MOLECULAR PHYLOGENY AND TAXONOMY OF PLANT PATHOGENIC GENERA *Diaporthe* (*Phomopsis*) AND *Colletotrichum*

DHANUSHKA UDAYANGA

DOCTOR OF PHILOSOPHY
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BIOTECHNOLOGY

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DISSERTATION COMMITTEE

.....CHAIRPERSON
(Dr. Siraprapa Brooks)

.....ADVISOR
(Assoc. Prof. Dr. Kevin D. Hyde)

.....CO-ADVISOR
(Asst. Prof. Dr. Ekachai Chukeatirote)

.....EXTERNAL EXAMINER
(Prof. Paul Taylor)

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Co-Advisor	Asst. Prof. Dr. Ekachai Chukeatirote

ABSTRACT

The genus *Diaporthe* (*Phomopsis*) is an important genus of phytopathogenic fungi with a worldwide distribution. Cryptic diversification, phenotypic plasticity and extensive host associations have complicated accurate species identification in the genus. In this study, the phylogenetic species recognition of *Diaporthe* is re-evaluated with worldwide collections with special reference to the significant phytopathogens on crops, ornamentals and forest trees and species complexes.

The Genealogical Concordance Phylogenetic Species Recognition (GCPSR) was applied to resolve the species limits with four gene phylogenetic analysis of ITS (nuclear ribosomal internal transcribed spacer), EF1-α (translation elongation factor 1-α), TUB (β tubulin) and CAL (calmodulin). The four gene combined phylogeny was used to define novel taxa from Thailand including *D. ptercarpicola*, *D. siamensis* and *D. thunbergii*. The species of *Diaporthe* associated with melanose and stem end rot of Citrus were re-defined, implementing the combined analysis of ITS, EF1-α, TUB, CAL and ACT (actin) genes and Genealogical Sorting Index methods.

Diaporthe citri, *D. cytosporella*, *D. foeniculina* and *D. rudis*, are epitypified with clarification of nomenclature and taxonomy.

The *Diaporthe sojae* species complex associated with soybean, cucurbits, and other herbaceous hosts were resolved based on five gene analysis including ITS, EF1-α, TUB, CAL and HIS (histone-3) genes. The seed decay pathogen *D. longicolla* was distinguished from the pod and stem blight pathogen *Diaporthe sojae*. The Lima bean pathogen *D. phaseolorum*, other herbaceous crop pathogens including *D. arctii*, *D. batatas* and *D. cucurbitae* were resolved with epitypes designated with the introduction of a new species *D. ueckerae* from cucurbits in USA.

The generic type species *Diaporthe eres* was redefined with the strict application of genealogical concordance and non-discordance criteria in conjunction with morphology with the epitypification of six phylogenetic species within the complex. Eight molecular markers including ACT, Apn2 (DNA-lyase), CAL, EF1-α, Fg1093 (ribosomal protein L37), HIS, ITS and TUB were used in the phylogenetic analyses and testing the informativeness. The caution is warranted using the ITS sequence data within the cryptic species complexes, and EF1-α is proved to be the best single marker to resolve the species. The utility of EF1-α, Apn2, HIS and ACT genes are found to be superior to the other genes used with reference to the phylogenetic informativeness. New primers were designed and made available for the amplification of Apn2, ACT and CAL genes within *Diaporthe*. Guidelines for the identification and description of new species are provided with the discussions on insights in to the evolution and the pattern of speciation within the genus.

A molecular phylogenetic analysis of anthracnose pathogens of tropical fruits was performed, with fresh collection of economic and wild fruits in northern Thailand. The species within *Colletotrichum gloesporioides* species complex were found to be the dominant among anthracnose pathogens. A six gene combined phylogeny of ITS, ACT, TUB, CAL, GPDH (glyceraldehyde-3-phosphate

dehydrogenase), GS (glutamine synthetase) was used to resolve the *C. gloesporioides* species complex with the description of the new species, *C. syzygicola*. In general, this thesis provides a comprehensive account in terms of utility of molecular data to resolve plant pathogenic species complexes in fungi. A number of economically important species of common plant pathogenic genera, *Diaporthe* and *Colletotrichum* were re-defined with accurate phylogenetic placement, morphology and insights in to the speciation and evolution.

Keywords: Canker / Diaporthales / DNA-lyase / Endophytes / Epitypification / Evolution / Gene discordance / Genealogical sorting index / Melanose / New primers / Phylogenetic informativeness / Soybean seed decay / Speciation / Species complex / Tropical fruit pathogens

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CHAPTER 1

INTRODUCTION

1.1 Plant pathogenic genus *Diaporthe* (*Phomopsis*)

Plant pathogenic genera represent a diverse and widely distributed assemblage within the Kingdom of Fungi causing a considerable impact on agricultural and forest ecosystems worldwide (Agrios, 2005). These pathogenic fungi can infect on a wide array of plants including field crops, ornamentals and forest trees. Pathogenic taxa often have complex and poorly understood life cycles, cryptic associations with plants and also lacking discriminatory micro and macro morphological characters among species. The identification of the causal agents of a plant disease is an essential step towards meaningful measures of control and disease surveillance (Rossman & Palm-Hernández, 2008).

Diaporthe Nitscke (synonym *Phomopsis* (Sacc.) Bubák) is an important phytopathogenic genus which needs an urgent taxonomic reappraisal (Farr, Castlebury & Rossman, 2002; Farr, Castlebury, Rossman & Putnam, 2002; Murali, Suryanarayanan & Geetha, 2006; Santos, Correia & Phillips, 2010). Until recently species in these genera were assumed to be host-specific, thus when a fungus was encountered on a host for which a name did not exist, a new species was described (Uecker, 1988; Crous, 2005; Cai et al., 2011). However, initiating with Rehner and Uecker (1994), in which DNA sequence data were first used in this genus, it is now known that most of the species of *Diaporthe* are not host-specific; i.e., one species may occur on many different host genera and one host may harbor several different species of *Diaporthe* (Zhang et al., 1997; Zhang, Hartman, Curio-Penny, Pedersen & Becker, 1999; Santos & Phillips, 2009; Santos et al., 2010; Diogo, Santos & Phillips, 2010). The genus belongs to class Sordariomycetes, order Diaporthales and the family

Diaporthaceae and the genus is typified by the species *Diaporthe eres* Nitscke (Nitscke, 1870). With the change to one scientific name for pleomorphic fungi (McNeill et al., 2012) based on relative priority, *Diaporthe* Nitschke (1870), represents the generic name that is older than the synonym *Phomopsis* (Sacc.) Bubak (1905). At present the Index Fungorum (www.indexfungorum.org/names/names.asp) lists 901 names of *Diaporthe* and 984 names of *Phomopsis* respectively. However, majority of these names have been applied based on host association and morphology, therefore most of them are obscured or uncertain due to synonyms. Therefore merging the species in two generic names is a significantly difficult and challenging task (Mostert, Crous & Petrini, 2000; Rossman, Farr & Castlebury, 2007). The definition of species limits within the genus *Diaporthe* has been on-going and species are presently being redefined based on a combination of morphological, cultural, phytopathological, mating type and multi-gene sequence data (Farr et al., 2002, Santos & Phillips, 2009; Santos et al., 2010).

1.1.1 Nomenclatural history

The precise naming of living organisms is crucial, since the name is the key to access all accumulated knowledge (Hawksworth & Rossman, 1997; Hawksworth, 2011). The occurrence of dual or multiple morphological forms of a fungal species (i.e. pleomorphism) and the dual nomenclature system has resulted in difficulties to develop a natural system of classification of fungi and therefore resulting a confusion in names (Shenoy et al., 2010). For these reasons a stable nomenclatural system with a single, clearly defined, systematically accurate names for genera and species are essential for all aspects of scientific studies.

The name *Phomopsis* in its first documented records was applied to anamorphs (asexual state) of nectriaceous fungi, with several changes over time in its nomenclatural status (Uecker, 1988). *Phomopsis* became more stable when Saccardo (1883) defined *Phomopsis* as a group of *Phoma* species that produced beta-conidia, but he did not transfer any species to *Phomopsis*. Later in the same volume of Sylloge Fungorum (Saccardo, 1884) treated *P. versoniana* and *P. brassicae* as species of *Zythia*. The present sense of the name *Phomopsis* (Sacc.) Bubak. (1905) resulted from the transfer of *Phoma lactucae* Sacc. to *Phomopsis*. Later, in the same year Saccardo

(1905) raised *Phomopsis* to generic rank and listed two species- *Phomopsis lamii* Sacc. and *P. pritchardiae* (Cooke & Harkn.) Sacc. Saccardo (1906) transferred three species of *Myxolibertella* to *Phomopsis*, while Höhnel and Litschauer (1906) agreed that *Phomopsis* and *Libertella* were the same and he used only *Phomopsis* in his writings.

Diaporthe is the teleomorphic (sexual state) name of *Phomopsis* with more than 900 names included in Index fungorum mostly independent of any anamorphic affinities. Since only 20% of anamorphic and teleomorphic connections are resolved for this genus, the need to link anamorphs with their teleomorphs using molecular data has been proposed (Sutton, 1980; Rehner & Uecker, 1994; Chi, Jiang & Xiang, 2007; Hyde, McKenzie & Ko Ko, 2011). Riedl and Wechtl (1981) formally proposed the conservation of the name *Phomopsis* as a valid generic name and this was accepted at the International Botanical Congress in 1987 and the need of lectotypification with *Phomopsis lactucae* (Sacc.) has been emphasised (Uecker, 1988). Wehmeyer (1933) in his comprehensive treatment of *Diaporthe* used morphology to differentiate the teleomorph and the asexual state was not considered. However, Chi et al. (2007) used *Phomopsis* as the preferred generic name in the Chinese compilation of over 200 species of *Phomopsis*. *Diaporthopsis* Fabre was described as a genus that is similar to *Diaporthe* but distinguished by non-septate ascospores. The type species of *Diaporthopsis*, *Diaporthe angelicae* (Berk.) Farr & Castl. was transferred to *Diaporthe* based on molecular and morphological data and therefore *Diaporthopsis* is now considered to be congeneric with *Diaporthe* (Castlebury, Farr, Rossman & Jaklitsch, 2003).

1.1.2 *Diaporthe* or *Phomopsis* - which name should be used?

There is a movement underway to provide pleomorphic fungal species with a single name instead of the conventional practice (until 2011) of providing a teleomorph and anamorph name for the different states (Shenoy, Jeewon & Hyde, 2007; Hawksworth, 2011; Hyde et al., 2011). The use of two names for a species is both confusing and unnecessary and has been the product of the dual nomenclature system (Shenoy et al., 2007). Several arguments have been made in the taxonomic history of *Diaporthe*/*Phomopsis* regarding the use of names of the teleomorph and

anamorph states (Chi et al., 2007; Santos et al., 2010). Since we are now able to link anamorph and teleomorph states through molecular sequence data regardless of whether the taxon in question expresses sexual or asexual structures the need for a dual nomenclature system is becoming redundant (Shenoy et al. 2007; 2010; Gehlot, Attitalla & Salleh, 2010; Hawksworth, 2011). However, in moving forward to use one name to represent the sexual and asexual states of a pleomorphic fungus many difficulties have to be overcome (Shenoy et al., 2010; Hyde et al., 2011).

In *Diaporthe*/*Phomopsis* we have the option of using the sexual name (*Diaporthe*), the older name (*Diaporthe*-1870 versus *Phomopsis*-1905), the name that is most often applied to important disease-causing organisms (i.e., *Phomopsis*), or maintaining the status quo as *Diaporthe* and *Phomopsis*. Santos and Phillips (2009) opted to use the older *Diaporthe* (1870) names, rather than the younger anamorphic genus, *Phomopsis* (1905), discouraging the introduction of separate anamorph names for new species of *Diaporthe* in their study. In this introductory chapter we generally use *Diaporthe* or *Phomopsis* species names more liberally as they have applied in literature referred, unless we clearly want to distinguish between two morphs.

The use of the dual nomenclature system in *Diaporthe*/*Phomopsis* can result in considerable confusion in taxonomy. Herein we detail several examples where confusion has been resulted by two names for identical taxa with advantages of using a single name. For instance, *Phomopsis vitimegaspora* Kuo & Leu associated with dead arm disease of grapevines in Taiwan was identified by Kuo and Liu (1998). The teleomorph was later recognised from Kyushu, Japan and designated the name *Diaporthe kyusuensis* Kajitani & Kanematsu with ITS sequence similarities (Kajitani & Kanematsu, 2000). Thus the same species has two completely unrelated species epithets. Two varieties of *Phomopsis* (*P. leptostromiformis* var. *leptostromiformis* (J.G. Kühn) Bubák, and *P. leptostromiformis* var. *occidentalis* Shivas) were identified as causing disease in *Lupinus* spp. *Diaporthe woodi* Punith. was later recognised as the teleomorphic state of *P. leptostromiformis* var. *occidentalis* (Punithalingam, 1974), while Williamson, Highet, Gams, Sivasithamparam and Cowling (1994) designated the name *Diaporthe toxica* P.M. Will., Highet, W. Gams & Sivasith. for the teleomorph of the toxicogenic variety of *P. leptostromiformis* var. *leptostromiformis*. In these, two examples more than one name represents a single

species (based on the dual nomenclature system). Now as it is easier to link names using molecular data, precise naming is needed in future understanding of taxa. The use of two names to represent species recorded from one host has introduced much confusion. For instance, *Phomopsis viticola* Sacc. and allied species of *Phomopsis* associated with grapes are have been reassessed in several previous studies (Merrin, Nair & Tarran, 1995; Phillips, 1999, 2000; Mostert, Crous, Kang & Phillips, 2001). *Phomopsis viticola* is, regarded as an anamorphic species as the sexual stage is not yet formed in recent studies, despite the amplification of both of mating type genes in different isolates (Santos et al., 2010).

Cryptosporella viticola Shear is now used as a synonym for *P. viticola*, which was previously thought to be the teleomorph. The names *Diaporthe austalafricana* Crous & Van Niekerk, *D. viticola* Nitschke and *D. perjuncta* Niessl have been given to the other taxa identified from grapevines. Eight species tentatively named as *Phomopsis* sp. 1 to 8, from grapevines were identified on basis of ITS and morphological data and not identified to species level due to the doubtful nature of host range or the frequency of occurrence (Niekerk et al., 2005). All records from grapes in this complex however, should belong to one genus (i.e., *Diaporthe*) although the existing nomenclatural system has made the situation confusing. The existence of homothallic and heterothallic taxa and compatible mating groups among species of this genus have been identified and confirmed by MAT gene-based rational selection and conventional mating experiments (Kanematsu, Adachi & Ito, 2007; Santos et al., 2010). Therefore, current knowledge supports the recognition of taxa within a biological and phylogenetic framework congruent with the linking of anamorphic and teleomorphic states. The significance of mating types of *Diaporthe* and other related concepts are discussed under the section of sexual state, mating types and molecular basis of mating experiments. Several important changes to the naming of fungi and needs to be further clarified. However, where anamorph and teleomorph names are involved, the oldest name will have priority unless a more commonly used name is conserved over the older name. Thus, *Diaporthe* is the oldest name and has priority over *Phomopsis* and *Diaporthe* should be used for all *Phomopsis* species although *Phomopsis* is generally the more commonly used name it could not be used unless it was conserved over *Diaporthe*. The utility of older name

Diaporthe is now widely accepted by the working community and included in the list of generic names of fungi for protection under the international code of Nomenclature for algae fungi and Plants (Kirk et al., 2013).

1.2 Life modes of *Diaporthe*

Species of *Diaporthe* have been reported as plant pathogens, endophytes, saprobes and even causing health problems in humans and other mammals (Sutton, Fothergill & Rinaldi, 1997; Farr, Castlebury & Pardo-Schultheiss., 1999; Garcia-Reyne et al., 2011). Several species isolated as pathogens of crops also have been isolated as endophytes from healthy tissues of the same or different hosts and also as saprobes from dead material (Promputtha et al., 2007). *Diaporthe helianthi* Munt.-Cvetk., a pathogen associated with the diseases of sunflower has been reported from pruning debris of *Vitis vinifera* in South Africa (Niekerk et al., 2005). In the same study, *Phomopsis amygdali* (Delacr.) Tuset & Portilla, a pathogen associated with shoot blight of almond and peach has been recorded from the asymptomatic nursery plant of *Vitis vinifera* in South Africa. In another case, *D. phaseolorum*, the causative agent of diseases of Lima bean and associated with soybean has been reported as endophytes in the estuarine mangrove plant *Kandelia candel* (Cheng et al., 2006).

1.2.1 Pathogens

Species of *Diaporthe* cause cankers, diebacks, root rots, fruit rots, leaf spots, blights, seed decay and wilts on a wide range of plants (Figure 1.1) including some economically important hosts worldwide (Uecker, 1988; Santos & Phillips, 2009, Niekerk et al., 2005). These species have been the subject of considerable phytopathogen research (Meyer, Zhang, Pedersen & Bradley, 2009; Li, Hartman & Boykin, 2010, Li, Wang, Huang & Shen, 2010). There has however, been no general review on this important pathogenic group so far concerning its taxonomy, phylogeny, nomenclature and evolution of species recognition. Most species of *Diaporthe* are thought to be hemibiotrophs in nature.

Notes. a. cane spot of grapevines (*Diaporthe ampelina*; b. leaf spots on grapevines (*D. ampelina*); c. stem canker of sunflower (*D. helianthi*); d. stem canker of peach (*D. amygdali*); e. stem blight of soybean (*D. sojae*); f. a soybean field infected with *Diaporthe*/*Phomopsis* complex.

Figure 1.1 Common diseases caused by several pathogenic species of *Diaporthe*.

Biotrophic fungi require living plants as a source of nutrients, while necrotrophic fungi kill their hosts and live off the dead tissue (Berger, Sinha & Roitsch, 2007). When the host is infected by a necrotrophic pathogen, the plant suffers severe effects, and the pathogen continues to survive on the host as a saprobe following tissue death (Van Kan, 2006), living on the nutrients from the tissue they have killed. *Diaporthe* pathogens are nectrotrophic at least for the latent phase of infection and are therefore called hemibiotrophs (Rosskopf, Charudattan, De Valerio & Stall 2000). Despite their significance as destructive plant pathogens, some species such as *Phomopsis leptostromiformis* which infects lupines (*Lupinus* spp.), also cause lupinosis, a type of mycotoxicosis in sheep which follows consumption of diseased plants (Van Warmelo & Marasas, 1972). The report of the occurrence of Human Phaeohyphomycotic Osteomyelitis (a subcutaneous infection of a finger of immunosuppressed female) by a species of *Diaporthe* resulted in the addition of the genus to the list of coelomycetous fungi capable of causing human diseases (Sutton, Timm, Morgan-Jones & Rinaldi, 1999). *Phomopsis longicolla* Hobbs was also reported from a human cutaneous infection in an immunosuppressed, renal transplant recipient from Guinea; the organism was previously known as a pathogen on seeds of soybean (Garcia-Reyne et al., 2011).

1.2.2 Endophytes

Species of *Diaporthe* are prevalent as endophytes of many hosts in both temperate and tropical regions and are especially common in the sapwood of angiosperms (Bussaban, Lumyong, Lumyong, McKenzie & Hyde, 2001; Tomita, 2003; Rossman et al., 2007; Murali et al., 2006; Suryanarayanan, Murali & Venkatesan, 2002; Botella & Diez, 2011; González & Tello, 2011). Endophytic species of *Diaporthe* were present in the sapwood of almost all angiosperm endophytes examined by Boddy and Griffith (1989). Promputtha, Hyde, McKenzie, Peberdy and Lumyong (2005) reported that, from a total of 31 morphospecies of sterile endophytes from *Magnolia liliflora* (Magnoliaceae) identified based on molecular phylogeny, 24 were *Diaporthe* species; this finding has been corroborated in several other recent studies with different hosts (Murali et al., 2006; Chaeprasert, Piapukiew, Whalley & Sihanonth, 2010; Rocha et al., 2011; Sun, Guo & Hyde, 2011).

The potential role of endophytes in protecting plants from fungal diseases such as Dutch elm disease has been explored (Brayford, 1990). An endophytic *Diaporthe* sp. from living bark of *Cavendishia pubescens* in Colombia produced paspalitrem A and paspalitrem C in batch fermentations. These compounds previously were known only from sclerotia of *Claviceps paspali* as tremorgenic mycotoxins causing neurological disorders of livestock (Bills, Giacobbe, Lee, Peláez & Tkacz, 1992). Thus the presence of endophytes in plant may be advantageous for the plants and may deter herbivory (Brayford, 1990; Hyde & Soytong, 2008; Weber, 2009; Vesterlund, Helander, Faeth, Hyvönen & Saikkonen, 2011).

1.2.3 Saprobes

There are abundant records species of *Diaporthe* as saprobes on decaying materials, as well as endophytes and pathogens later becoming early colonisers on wide range of dead plant substrata (Promputtha et al., 2007; Kodsueb, McKenzie, Lumyong & Hyde, 2008a; 2008b; Kumaresan & Suryanarayanan, 2002; Osono & Takeda, 2002; Yanna & Hyde, 2002; Hyde et al., 2007; Promputtha et al., 2010). Nine endophyte strains were isolated from leaves of *Magnolia liliflora* and three of them were *Diaporthe* (as *Phomopsis*) which are morphologically and phylogenetically similar to saprobes isolated from the early decay stage of leaves of the same host (Promputtha et al., 2010). Endophytic *Diaporthe* strains have also been shown to produce leaf degrading enzymes similar to those of saprobic strains which support the biochemical evidence that endophytes become saprobes at leaf senescence (Promputtha et al., 2010; Dai Chen, Tian & Shi, 2010; Meenavalli et al., 2011).

1.3 Diversity and species recognition

There has been considerable attention given to the need for revaluation of the taxonomy and phylogeny of *Diaporthe*; however it is currently well understood that the conventional taxonomic characters no longer resolve cryptic species (Brayford, 1990; Rehner & Uecker, 1994). Recent approaches have used nucleic acid sequence data to resolve species boundaries within the genus (Santos et al., 2010; Diogo et al., 2010). However, a polyphasic approach including morphology, molecular phylogeny,

pathogenicity and virulence of isolates biological species should be adopted in future studies (Santos & Phillips 2009; Diogo et al., 2010) as recommended for other genera such as *Colletotrichum*, *Fusarium* and *Pencillium* (Cai et al., 2009; Schroers Gräfenhan, Nirenberg & Seifert, 2011; Hawksworth, 2011). In general, species concepts/ species recognition criteria in fungi have evolved in sequential phases due to the complexity of identification of species (Shenoy et al., 2007); this includes the morphological, the ecological and physiological, the biological and the evolutionary and phylogenetic species recognition (Moncalvo, 2005). The species recognition criteria of *Diaporthe* have also been reviewed herein, based on similar phases that would facilitate to resolve the problems of this genus.

Based on the current knowledge of *Diaporthe*, it is challenging to identify a species isolated from a host for which a species has not been described previously. This is because many of known species have wide host range and there are few characters that can differentiate them (Uecker, 1988). Some species are thought to be host-specific; while others are able to infect a wide range of hosts and therefore caution is needed when concluding diversity in various hosts (Mostert et al., 2001; Crous, 2005; Schilder, Erincik, Castlebury, Rossman & Ellis, 2005; Santos & Phillips 2009; Diogo et al., 2010).

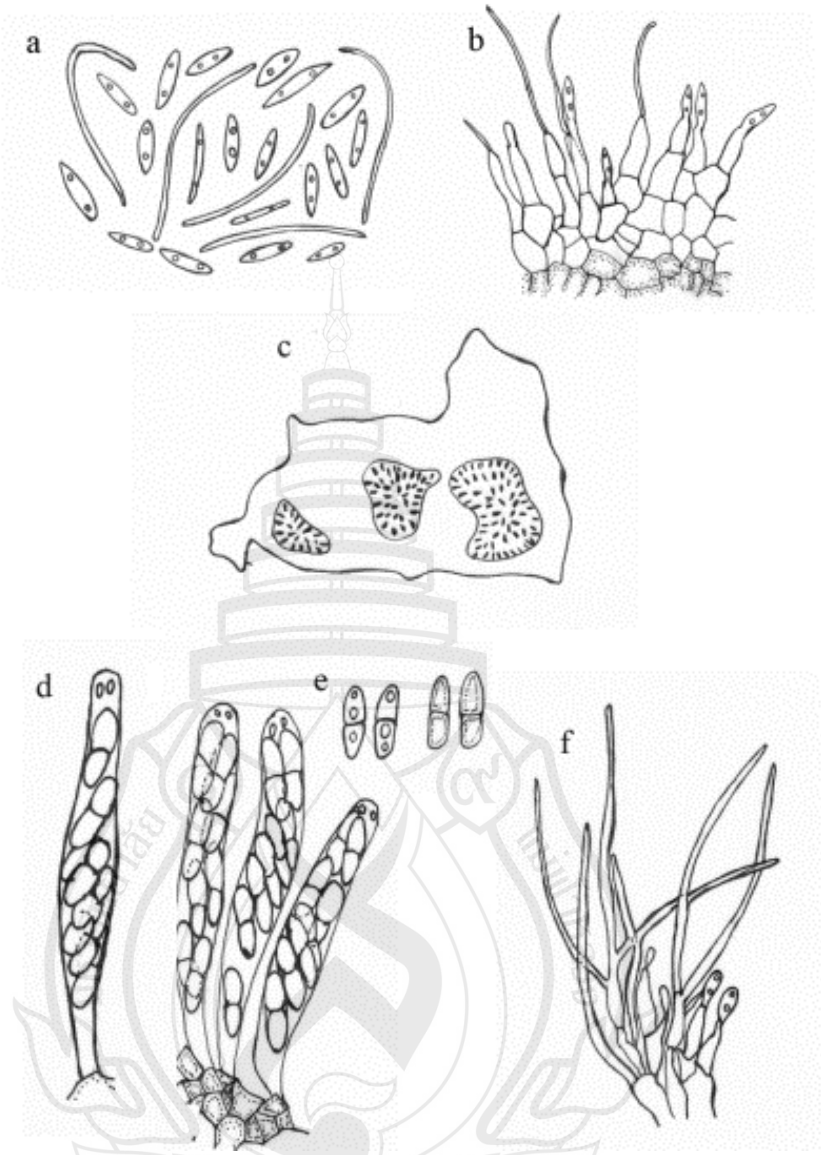
Diaporthe strains isolated from a single host may represent more than one taxon (Rehner & Uecker, 1994). There have been recent phylogenetic studies on several species complexes of *Diaporthe* associated with one particular host. Fifteen species of have been recorded from grapes (Vitaceae) (Crous, 2005; Niekerk et al., 2005), which is remarkable and more species could be found in future. Other examples include wild fennel (*Foeniculum vulgare*) which is host to several species of *Diaporthe* (Santos & Phillips, 2009), four to six species are known from soybean from different geographic locations (Nevena, Jelena & Franic-Mihajlovic, 1997; Zhang, Riccioni, Pedersen, Kollipara & Hartman, 1998; Mengistu, Castlebury, Smith, Rossman & Reddy, 2007) and five species from *Aspalathus linearis* in South Africa (Rensburg, Castlebury, Smith, Rossman & Reddy, 2006). There have been several unidentified species reported as endophytes in *Tectona grandis*, *Magnolia liliflora*, *Manglietia garrettii* and *Salix* sp. (Horn, Simmonds, Schwartz & Blaney 1996; Promputtha, Jeewon, Lumyong, McKenzie & Hyde 2005; Murali et al., 2006).

Species of *Diaporthe* associated with various hosts and multiple species complexes on single host need to be resolved with incorporation of DNA sequence data and morphology (Santos & Phillips, 2009). *Diaporthe* species associated with conifers, species from economic fruit trees and species associated with economic crops are awaiting a revaluation by precise identification of several different species records (Hahn, 1930; Kanematsu, Kobayashi, Kudo & Ohtsu, 1999). The tropical versus temperate endophytic assemblage, and species associated with members of families Cucurbitaceae, Euphobaceae, Fabaceae, Magnoliaceae and Rosaseae which are herbaceous and woody hosts in tropical and temperate regions needed a re-evaluation (Holliday, 1980; Chi et al., 2007; Murali et al., 2006).

1.3.1 Morpho-species recognition

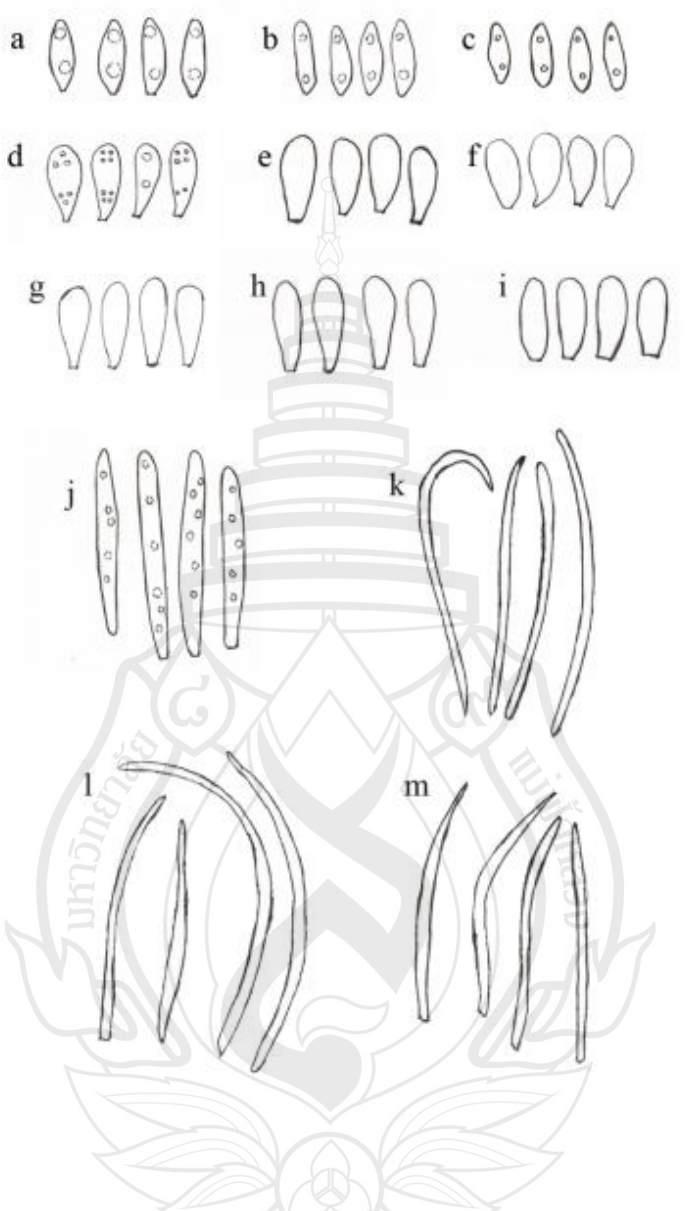
Morphology has been the basis of nearly all fungal taxonomic studies; therefore most previous compilations and monographs are based on morphological taxonomy (Hyde et al., 2010a; 2010b). Similarly, early species treatments of *Diaporthe* were based on morphology, culture characteristics and host association (Uecker, 1988; Brayford, 1990; Mostert et al., 2001; Chi et al., 2007). The asexual state is characterised by ostiolate, black conidiomata (Figure 1.2 c) containing elongate, cylindrical phialides (Figure 1.2 c) that may produce two types of hyaline, non septate conidia- namely alpha and beta (Rehner & Uecker, 1994). In some species, however, there are intermediates between these conidial types (Figure 1.3). The alpha conidia are aseptate, generally hyaline, fusiform and usually biguttulate, but sometimes lack of guttules or have more guttules (Figures. 1.2a, 1.3a–I). The beta conidia are also aseptate and hyaline, but are filiform, straight or more often hamate and lack guttules (Figures. 1.2a, 1.3) (Sutton, 1980).

Generally, conidiophores are hyaline, branched and occasionally short and 1–2 septate (Figure 1.2b). Frequently, they are multi-septate and filiform with enteroblastic, monophiladic conidiogenesis (Punithalingam, 1985; Crisescu, 2003). A third type of conidia called gamma conidia have been recorded (Rosskopf et al. 2000a; 2000b; Cristescu, 2007). These conidia are hyaline, multiguttulate, fusiform to subcylindrical with an acute or rounded apex, while the base is sometimes truncate (Figure 3J) (Punithalingam, 1974; Rodeva, Stoyanova & Pandeva 2009).



Notes. a. Alpha and beta Conidia (asexusl state); b. Conidiopores; c. Vertical section of stroma; d. Asci; e. Ascospores (sexual state); f. Conodiophores with paraphyses among conidiogenous cells (asexual states with paraphyses) (not in scale).

Figure 1.2 Asexual and sexual morphological characters of *Diaporthe.*



Notes. a-c. Biguttulate alpha conidia; d. Multi-guttulate alpha conidia; e-i. Eguttulate alpha conidia; j. Gamma conidia; k-m. Various types of beta conidia (not in scale)**.**

Figure 1.3 Comparison of conidial morphology of *Diaporthe*.

Those species described, having a third type of spores are *Phomopsis hordei* Punith. *P. oryzae* Punith., *P. phyllanthi* Punith., *P. amaranthicola* Rosskopf, Charud., Shabana & Benny., *P. capsici* (Magnaghi) Sacc., *P. elaeidis* Punith., *P. eugeniae* Punith., *P. viticola* Sacc. and *P. sedi* Punith.

The *Diaporthe* sexual state is characterised by ascomata which are usually immersed in the substrate, often erumpent through a pseudostroma mostly surrounding the ascomata and have more or less elongated perithecial necks (Figure 1.2d). The pseudostroma is distinct and often delimited with dark lines (Wehmeyer, 1933). Asci are unitunicate, clavate to clavate cylindrical, loosening from the ascogenous cells at an early stage and lying free in ascoma (Figure 1.2d). Ascospores are biseriate to uniseriate in the ascus, fusoid, ellipsoid to cylindrical, straight, inequilateral or curved, septate, hyaline and sometimes with appendages (Wehmeyer, 1933; Muntanola-Cvetković, ihaljčević & Petrov, 1981). Several different methods have been employed to induce anamorphic sporulation and teleomorphic structure formation of isolates in the absence in general methods (Onesirosan, 1978; Brayford, 1990; Kanematsu et al., 1999; Rawnsley, Wicks, Scott & Stummer, 2004; Luo, Xi, Jiang & Qi, 2004). However, because of the overlap in conidial size between species it is no longer possible to delimit species based on morphology alone (Van der Aa, Noordeloos & de Gruyter, 1990; Webber & Gibbs, 1984; Brayford, 1990; Rehner & Uecker, 1994). In addition, some of these characters vary with cultural conditions and media used, for example the zonation and pigmentation of aerial mycelium may be influenced by light (Brayford, 1990). Kanematsu, Minaka, Kobayashi, Kudo and Ohtsu (2000) identified two major morphologically distinct groups on the basis of colour of the colonies on PDA (Table 1.1).

They further recognised the same two basic types as Wand G types further on basis of virulence of *Diaporthe* (as *Phomopsis*) from peach, Japanese pear and apple in Japan where G type isolates are more virulent in inoculation in the field than that of W type (Kanematsu et al., 1999; 2007). Sutton (1980) used the term paraphyses for sterile hyphae in his descriptions for other genera of phialidic coelomycetes. A few species have been reported to have paraphyses (Rehner & Uecker, 1994). *Phomopsis javanica* (Uecker & Johnson, 1991) was distinguished from other taxa found on *Asparagus* such as *P. asparagi* (Sacc.) Grove, based on the occurrence of paraphyses.

Previous indications of such structures were also in *Phomopsis theae* Petch and *P. anacardii* Early & Punith (Punithalingam & Gibson, 1972).

Table 1.1 Designation of W and G types of cultures of *Diaporthe*

Type of colony	Surface view	Reverse view	Sporulation	Virulence
W type	White,aerial hyphae ,scattered relatively large stroma ,irregular pycnidial locules	Whitish and occasionally had pale pink ,brown and or grey zones	Both alpha and beta conidia on PDA	Less virulent
G type	a few aerial hyphae ,white to grey and formed abundant relatively small pycnidial stroma with irregular pycnidial locules	grey or brownish grey	Only alpha conidia on PDA	More virulent

Source Kanematsu et al. (2000)

A further occurrence of long paraphyses has been reported for *Phomopsis longiparaphysata* Uecker and Kuo, a taxon from grapes in Taiwan (Figure 1.2e) (Uecker & Kuo, 1992). Such a distinctive character is welcome in the study of a group noted for a dearth of such characters (Uecker & Johnson, 1991).

1.3.2 Pathogenicity and virulence

The capacity of a fungal species to cause a disease (i.e., pathogenicity) and the degree of pathogenicity (i.e., virulence) have been used to differentiate pathogenic species (Uddin & Stevenson, 1997; 1998a; 1998b; Schilder et al., 2005). The need for comparative studies of pathogenic *Diaporthe* species using morphology, and pathogenicity has also been emphasised by Kanematsu et al., (1999). Herein, we discuss several incidents of pathogenicity testing and cross inoculation experiments with arguments made for and against them. Pathogenicity testing of species of *Diaporthe* infecting grapes revealed that different isolates of *P. viticola* cause disease

symptoms, but differed in virulence, estimated on the size of lesions (Schilder et al., 2005). In the same study, specialisation of pathogens on specific plant tissues was observed and one distinct taxon was distinguished based on its severity of infection on grape fruits. Further characterisation revealed that, the isolate, which differed in virulence, resembles a species originating from another host in the vicinity of the vineyard. However the observations based on virulence and pathogenicity were mostly of a quantitative nature and thus it is difficult to assign any species on these observations alone (Schilder et al., 2005). Vidić (1991) studied the variability of virulence among isolates of *D. phaseolarum* var. *caulivora* on three varieties of soybean in Serbia but was unable to support or reject their separation into different physiological races based on severity of infection (Rehner & Uecker, 1994). Uddin and Stevenson (1998a; 1998b) reported pathogenic and molecular characterisation of three *Diaporthe* isolates from peach, plum and Asian pear. They observed that there was no significant difference between the length of cankers on peach shoots inoculated with plum and Asian pear isolates, and they were significantly smaller than those inoculated with peach isolate. All three isolates differed in morphology and ITS sequence data, although the phylogenetic affinity between the pear and plum isolates was closer than the peach isolate. Susceptibility of the apple, plum and pear to the pathogen causing shoot blight on peach was also confirmed, providing evidence of their capability to one particular host.

A species of *Diaporthe* occurring on grapes in Portugal was identified as *D. perjuncta*, which shows little resemblance to *P. viticola*, apart from its association with *Vitis*. Although several species of *Diaporthe* infect grapevines worldwide, it has been reported that Australian isolates of *Diaporthe australaficana* (formally *D. perjuncta*) do not cause *Phomopsis* cane and leaf spot disease in Australia (Rawnsley et al., 2004). Pathogenicity testing suggested that *D. australafricana* is less prone to be a pathogen and is more likely to be an endophyte in *Vitis*. However, *D. perjuncta* was recollected from *Ulmus glabra* in Germany, and distinguished from *D. viticola* by morphology and ITS based phylogeny and the taxon has been established (Niekerk et al., 2005). The wide host range of *Diaporthe* has great implications for the management of diseases caused by different species as alternative hosts might act as source of inocula which would be a challenge in management of disease and

quarantine. Therefore, the assessments of virulence, pathogenicity and the knowledge of disease cycles are equally important in future concerns in plant pathology and taxonomy. It is important to establish if a particular species is host specific or not and epitypification and performing Koch's postulates is important in describing new species, while pathogenicity alone could not contribute to the differentiation of taxa.

1.3.3 Chemotaxonomic markers, biochemistry and serology

In its broadest sense, chemotaxonomy is the use of chemical diversity as a taxonomic tool, which refers to the use of secondary metabolites in the classification of filamentous fungi (Frisvad et al., 2008). In this section we explore the use of chemotaxonomy as a tool to differentiate *Diaporthe* species. A profile of secondary metabolites consists of all the different compounds a fungus can produce on a given substratum and includes toxins, antibiotics and other different compounds. Chemotaxonomy is regularly used in polyphasic approaches to genera such as *Aspergillus* and *Penicillium* (Frisvad, Andersen & Thrane, 2008) and has been suggested for use in *Colletotrichum* (Abang et al., 2009; Cai et al., 2009). Although *Diaporthe* species have been extensively screened in bioassays for metabolite production (Isaka et al., 2001; Weber et al., 2005; Yang et al., 2010) the utilisation of chemotypes for species recognition has been limited.

Phomodiol, Phomopsolide B and Phomopsichalasin were recognised as potential chemotaxonomic markers in endophytic *Diaporthe* isolates from woody hosts (Horn Schwartz, Simmonds & Blaney, 1994). Two of these secondary metabolites were evaluated as potential chemotaxonomic markers for the endophytic isolates from Salix sp. (Willow) and several other hosts (Horn, Simmonds, Schwartz & Blaney, 1995). *Diaporthe* isolates from willows and non willow isolates were tested for the production of these two chemicals in both malt and millet media. Phomopsolide B was produced by all the isolates from willow and one isolate derived from different woody host. Phomodiol production however varied among all isolates (Horn et al., 1996). Shivas et al. (1991) demonstrated infraspecific variation in *Phomopsis leptostromiformis* from Western Australia using cultural and biochemical techniques. They recognised two different varieties of *Phomopsis leoptostromiformis* based on the observations from pectic estrase zymograms and quantities of

phomopsins A and C in assay conditions provided. Phomopsins were analysed in the extracts of culture by high-performance liquid chromatography (HPLC) (Shivas et al., 1991).

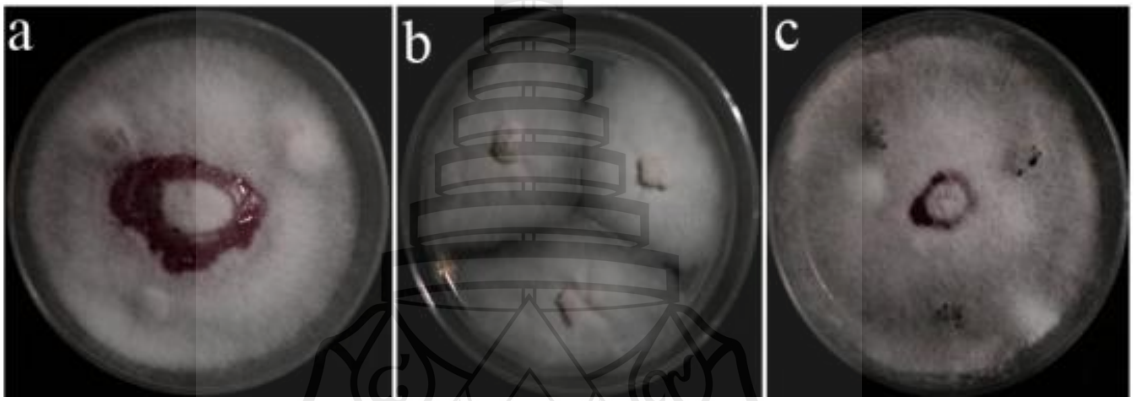
The antibodies derived from immunised rabbit serum for powdered mycelium of freeze dried *Diaporthe* was successfully used to detect the fungus infected to the soybean seed. Antiserum to freeze dried powdered mycelium of *Phomopsis longicolla* was used in an indirect ELISA (Enzyme-Linked Immunosorbent assay) and a modified immunoblot assay for seed born pathogen infection (Gleason, Ghabrial & Ferriss, 1987). Possible implications of detection of *P. longicolla* and its varieties using the monoclonal antibodies were also discussed in order to prevent the cross reactivity of antiserum in the above mentioned methodology (Gleason et al., 1987). Metabolites, mycotoxins and antibodies based diagnostic methods have prompted as alternative quick, specific and sensitive attempts in plant pathogen detection which surpass the traditional inconclusive methods (Ward, Foster, Fraaije & McCartney, 2004). The lack of utilisation of metabolite profiling and chemotaxonomic approaches in *Diaporthe* is not surprising due to rapid progress of molecular based identification.

1.3.4 Antagonism

The degree to which the growth of the fungal cultures is affected by the proximity of actinomycetes varied in quantitative expression, depending on the species interaction between two organisms (fungus and actinomycete) inhibiting the fungal growth (i.e. antagonism) has used in species recognition (Figure 1.4). Muntanola-Cvetković, Bojović-Cvetić , Franić-Mihajlović and Vukojević (1990), Muntanola-Cvetković, Mitić and Vukojević (1992) and Muntanola-Cvetković, Vukojević and Mihaljcêević (1996) reported on the repressive effect of some actinomycetes on the growth of *Diaporthe* isolates which could be relevant in distinguishing species. Fifty five asexual and sexual strains were analysed for antagonism by five selected Streptomyces species (Muntañola-Cvetkovic, ukojevic & Mihaljcevic, 2000).

The responses of the fungi varied, but two major groups could be distinguished. Group A encompassed isolates less affected by actinomycetes and Group B comprised those exhibiting high sensitivity in all experiments. Group A was

typically represented *by Diaporthe arctii*, *Phomopsis longicolla* and the *Phomopsis* type 1 culture from *Xanthium italicum*, whereas group B was typically represented by *Diaporthe helianthi* and *Phomopsis* type 1 culture from *X. italicum* and isolates from *Lactuca serriola*. The results obtained underscore the differences between *Diaporthe arctii* and *D. helianthi* and corroborate the value of the physiological aspects of congeneric isolates in considering taxonomic problems.



Notes. a. CBS 592.81 (ex-type of *D. helianthi*) Vs. CBS 100.56 (*Streptomyces diastaticus* subsp. *ardesiacus*) 2 weeks old coculture shows the increasing inhibition of the growth of *D. helianthi* by forming an inhibition area in the middle of the culture (positive results); b. CBS 592.81 (control) *D. helianthi* isolates without *Streptomyces* inoculation; c. CBS 117499 (ex-type of *D. cuppatea*) vs *Streptomyces diastaticus* subsp. *ardesiacus*: without considerable antagonistic reaction (negative results).

Figure 1.4 Repressive effects on *D. helianthi* by *Streptomyces diastaticus* subsp. *ardesiacus*.

However, the contributions based on these experiments are less and dependent therefore not recommended in initial stages of species identification (Muntañola-Cvetkovic et al., 2000).

1.3.5 DNA based alternative and comparative assays

DNA based comparative assays have proven to be useful in phylogenetic studies therefore utilised to evaluate the genetic diversity of *Diaporthe* especially when the DNA sequence facilities are not applied in large scale (Zhang et al., 1997, 1998; Chi et al., 2007; Santos & Phillips, 2009). These methods are in partial agreement with phenotypic and genotypic groups and some have been used in infraspecific taxonomy (Vergara, Capasso, Gobbi & Vannacci, 2005).

Restriction fragment length polymorphism (RFLP): RFLP has been used to distinguish between *Diaporthe* and other pathogens from soybean. PCR-RFLP patterns from ITS amplicons using 20 different restriction enzymes and was used in sequence analysis to distinguish *P. longicolla* and *D. phaseolorum* isolates from other soybean fungal pathogens (Zhang et al., 1997). The distinguishing patterns of RFLP from ITS amplicons were observed for *Diaporthe* isolates as compared to other associated pathogens using the same restriction enzymes. Zhang et al. (1998) used ITS based phylogeny and RFLP patterns of amplified products of ITS for the isolates derived from soybean as molecular markers in species detection. Restriction analysis of ITS by AluI, MseI, HhaI, RsaI, and ScrFI was used to detect subgroups of species of *P. longicolla* and *Diaporthe phaseolorum*. Extensive genetic variability was observed in *D. phaseolorum* isolates with the RFLP patterns. PCR-RFLP based analysis was undertaken to delineate the *Diaporthe* species from stone and pome fruits in south Africa with special reference to infection by a ds RNA mycovirus, *Diaporthe ambigua* RNAvirus (DaRV) (Preisig, Moleleki, Smit, B. Wingfield & M. Wingfield, 2000; Moleleki, Preisig, M. Wingfield, Crous & B. Wingfield, 2002). RFLP patterns and sequencing information were used to identify three different *Diaporthe* species namely *Diaporthe ambigua*, *D. perjuncta* and an unidentified *Diaporthe* sp. The species infected by the dsRNa virus was *D. perjuncta* and not *D. ambiguan* (Moleleki et al., 2002).

Random amplified polymorphic DNA (RAPD): Chen, Ntahimpera, Morgan and Michailides (2002a) and Chen, Jiang and Qi (2002b) evaluated the applications of RAPD and ITS sequence data on 34 *Phomopsis* isolates from China. This study revealed that RAPD data nearly coincides with morphological data and mostly with the ITS sequence data.

Microsatellite primed PCR (MSP-PCR): Microsatellite primed PCR profiles were generated for the isolates of *Diaporthe* (Santos & Phillips, 2009; Diogo et al., 2010). Representative isolates from meaningful groups at higher reproducibility levels were selected for phylogenetic analysis. This method is important to assess when large number of isolates are present from same or relative hosts and environment which avoids the repetitive sequencing of the same isolates and recognises the comparative genetic variability of isolates.

Species specific probes: Species specificity is an important criterion for DNA-based diagnosis. Melanson, Rawnsley and Scheper (2002) has used taxon-specific probes to detect *Phomopsis* sp. 1 and 2 from grapes. Their investigation used specific markers for *Phomopsis* infecting grapes to determine their origin in Australia. Zhang et al. (1997) used *Phomopsis* specific primers (PhomI and Phom II) for the amplification of 337 bp from the ITS region to distinguish isolates of *P. longicolla* and *D. phaseolorum* from other soybean pathogens. None of the amplified products was observed in the DNA of seven other soybean fungal pathogens or soybean plant genomic DNA. Species specific detection of *Diaporthe phaseolorum* and *Phomopsis longicolla* from soybean seeds was achieved using high throughput techniques (Zhang et al. 1999). They designed primer probe (Taq man) sets to detect *P. longicolla* in the seeds of soybean with high sensitivity at 0.15 ng (four copies) of plasmid DNA. Current advances in gene sequencing and analysis will result in many of the methods mentioned above being used less frequently as they no longer provide significant results as compared to modern techniques. However, alternative and comparative assays are still employed in selection of strains for sequencing, population studies, infraspecific taxonomy, evolutionary studies and studies of genetic diversity of fungi (Riccioni et al., 2008; Santos & Phillips, 2009; Diogo et al., 2010).

1.3.6 Phylogenetic species recognition

The current state of species of *Diaporthe*, effectively means that a particular isolate be identified to species level only if molecular techniques are employed (Crous, 2005). Classification of fungi based of DNA sequence data to infer evolutionary relationships has been widely recognised (Shenoy et al., 2007) and has successfully been used to differentiate species in several important pathogenic genera

including *Diaporthe* (Santos & Phillips, 2009; Diogo et al., 2010; Cai et al., 2011). Although the DNA-based methods provide convincing results, there are several challenges to establish a more precise phylogenetic framework for the genus.

1.3.6.1 ITS sequences in phylogenetic analyses: There have been several studies using ITS sequence data along with morphology to investigate the species of *Diaporthe*. Rehner and Uecker (1994) examined 43 North American and Caribbean strains of *Diaporthe* (as *Phomopsis*) from a diverse range of hosts by analysis of ITS1 and ITS2 sequence data. Three basic phylogenetic groups (A, B and C) were identified and defined on basis of geographic origin and the host association (Table 1.2). They defined ITS sequence based phylogenetic groups for the isolates obtained from Asia, Europe and North America. They also noted that variation in ITS sequence data may lead to the introduction of cryptic species of *Phomopsis* and therefore further refinement of available taxa was recommended. Groups of species were further interpreted with possible distinction on the basis of geographical, morphological and host affiliation. The disadvantage with this study, however, was that no extype strains were used and the isolates were randomly selected from various locations worldwide. Murali et al. (2006) studied the foliar endophyte assemblages from teak trees (*Tectona grandis*) in India using ITS sequence data from 11 different *Diaporthe* isolates (ten from teak and one from *Cassia fistula*). The data were analysed with more than 50 sequences downloaded from GenBank. The authors showed that the isolates fell into two strongly supported groups. The study did not describe any distinct species from teak, but supported earlier studies concluding that species from teak are not host-specific, and that the species concepts in *Diaporthe* need to be redefined. Santos and Phillips (2009) successfully used ITS sequence analysis combined with micromorphology to resolve the complex of *Diaporthe*/*Phomopsis* occurring in *Foeniculum vulgare* (wild fennel) in Portugal.

Table 1.2 Phylogenetic groups of *Diaporthe* isolates inferred based on ITS.

Group identity	Host range and specific characters	Geographic range / origin of isolates
Group A : Subclade A 1	Variety of host genera	Eastern and Midwestern USA
Group A : Subclade A 2	*Vaccinium* sp.	USA (MA and MI)
Group B	Woody and herbaceous plants produce paraphyses among their conidiogenous cells	Southern temperate to tropical regions
Group C	Primarily on herbaceous plant hosts, including agricultural field crops and some woody plants	Temperate to subtropical regions of USA

Source Rehner and Uecker (1994)

Four species were distinguished. *Diaporthe angelicae* (Berk.) D.F. Farr & Castl. was shown to be the most common pathogen of this host, *D. lusitanicae* Phillips & Santos was newly described, the teleomorph of *Phomopsis theicola* Curzi was revealed to be distinct from *Diaporthe theicola* Curzi and described as *D. neotheicola* Phillips & Santos.

1.3.6.2 Multi-locus phylogeny: Taylor et al. (2000), discussed operational species recognition criteria based on the ic principles of phylogenetic species, developed by Avise and Ball (1990). This is the widely accepted principle of modern phylogenetic approach to recognise fungal species based on concordance of multiple gene genealogies. The principles of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) is primarily applied for fungal species recognition, is based on the idea that recombination within a lineage will create conflict between gene trees, with the transition from conflict to congruence representing the species limit (Taylor et al., 2000). This approach has been utilised to delineate species in several fungal genera although the studies were lacking for *Diaporthe* until Udayanga, Liu, McEnzie, Chukeatirote and Hyde (2012) is published.

Rensburg et al. (2006) used ITS and partial elongation factor 1 α (EF1-α) sequence data, plus morphological and cultural observations to characterise species of

Diaporthe associated with dieback of Rooibos tea (*Aspalathus linearis*) in South Africa. The combined sequence data supported the differentiation of the same six species as identified by ITS phylogeny, but with a higher level of confidence. Farr et al., (2002a; 2002b) also discussed the importance of combining molecular and morphological characters in species identification. Ambiguities in the alignment of ITS sequence data across the genus *Diaporthe* were also noted (Farr et al., 2002a; 2002b). Large numbers of ambiguously aligned regions may obscure the true relationships among taxa and the parsimony analysis of ITS sequence data for this group indicates that there is a large amount of homoplasy across the entire genus (Farr et al., 2002a, b). Therefore the number of branching orders with fewest evolutionary events to explain contemporary sequences (i.e.; the number of parsimonious trees) might be higher than usual. Use of multiple sequence data using several combined genes in phylogenetic analyses would be needed as in *Colletotrichum* and other several complex genera (Vergara, Cristani, Regis & Vannacci, 2004; Cai et al., 2009; Crouch Clarke & Hillman, 2009; Prihastuti, Cai, Chen, McKenzie & Hyde, 2009; Aveskemp, Gruyter ,Woudenberg, Verkley & Crous 2010; Phoulivong et al., 2010), to better resolve species relationships.

ITS, EF-1α partial sequence data and MAT phylogenies of *Diaporthe* were compared without combining the genes in phylogeny to establish correlation between biological and phylogenetic species concepts (Santos et al., 2010). ITS sequence data was shown to be highly variable within a biological species of *Diaporthe*, while partial sequences from the translation elongation factor 1α were more reliable indicators of species limits.

Nevertheless, ITS sequence data can be used for reliable identification of phylogenetic relationships as long as they are interpreted with care. When compared to previously reported data for other genera such as *Colletotrichum* (Du, Schardl, Nuckles & Villancourt, 2005), the ITS region in *Phomopsis* appears to be evolving at much faster rates than EF1-α or even MAT genes (Santos et al., 2010). Therefore a slowly evolving gene region should be utilised in order to establish precise species limits. Santos et al. (2010) has suggested that the EF-1α derived sequences of *Diaporthe* are congruent with biological species clusters inferred by MAT phylogenies. Finding a relatively slowly evolving, single copy gene region with

minimum infraspecific variability is still a challenge for most fungal genera (Schmitt et al., 2009). Incongruence of single gene phylogenies is thought to be cause by various analytical and biological factors (Rokas, Williams, King & Carroll, 2003a). The impact of those errors would be eliminated by optimizing the conditions in change of the analytical criterion such as elimination of outgroup in analysis to a certain extent. Multi-gene gene phylogenies, would however, be more robust providing valuable information for selection of single genes to with less incongruence with true polygenetic relationships (Rokas, King, Finnerty & Carroll, 2003b).

There is an unprecedented need to use the multi-gene phylogenetic relationships in order to eliminate the incongruence that would result using single gene analysis and to establish meaningful evolutionary relationships.

1.4 Need for advancement in understanding *Diaporthe*

The need for of advancement in understanding of *Diaporthe* is driven because (1) many sequences deposited in GenBank are wrongly named because of lack of comparison with type derived sequences, (2) many GenBank sequences are named only to generic level, (3) there is a advancing trend of research of biological species concepts and infraspecific taxonomy, and (4) there is a lack of existing type derived cultures and sequences. This situation should be rectified in future studies of the genus. Isolates that represent type species are needed. Publication of new species should be amalgamated with type derived sequences and type derived cultures should be deposited in recognised culture collections (Shenoy et al., 2010; Abd-Elsalam et al., 2010; Hyde et al., 2010a, b). Species and infraspecific research should be expanded with incorporation of morphology and molecular data.

1.4.1 Ex-type ITS phylogenetic tree as a backbone for identification

Crouch et al. (2009) and Cai et al. (2009) revealed a high error rate and frequency of misidentification (86% and >86% respectively for *Colletotrichum graminicola* complex and *C. gloesporioides* complex), based on ITS sequence similarity comparison compared to type materials. It is therefore essential to use ex-type strains in molecular studies. Otherwise putatively named species from genera

with few distinguishing morphological characters used in phylogenetic studies will perpetuate the problem of wrongly named taxa in GenBank (Cai et al., 2011; Hyde et al., 2011). Even voucher or authentic strains should be treated with caution as there is no way of guaranteeing these are identical to the type of a species. ITS sequence data derived from ex-type isolates of *Diaporthe*/*Phomopsis* were located based on an extensive literature search until October in 2011 and downloaded from GenBank (Table 1.3). We also downloaded sequence data of authenticated or voucher cultures of *Diaporthe*/*Phomopsis*, accepting that these strains are less reliable than extype cultures and should be epitypified. All sequences were optimised manually to allow maximum alignment and maximum sequence similarity with gaps treated as missing data. The aligned dataset were analysed using PAUP* 4.0b10 (Swofford, 2002).

Ambiguously aligned regions of the dataset were excluded from all analyses. A heuristic search option with TBR branch swapping and 1,000 random sequence additions were used to infer trees. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Trees were figured in Treeview (Page, 1996). The first of 145 equally parsimonious trees obtained from the heuristic search is presented herein and provides a backbone of ex-type derived ITS sequences that can be used as a rough and quick identification guide for species of *Diaporthe* (Figure 1.5). The extype and voucher derived sequence data used here (46 taxa) are limited when compared to the large number of species names (981 names) listed for *Phomopsis* and its sexual *Diaporthe* (828 names) (Index Fungorum 2011). Not all described species of *Diaporthe*/*Phomopsis* have been sequenced; it would be an impossible task considering the short period we have used molecular data in fungal taxonomy and the history of species descriptions in the genus. The type derived ITS phylogenetic tree however, provides the basis for initial identification which can be improved and expanded on as more data become available.

Table 1.3 Ex-type and Ex-epitype cultures and sequences available at the beginning of this study.

Taxa	Culture collection	Status of culture	GenBank Accessions				References
			ITS	EF1-α	MAT1-1-1	MAT1-2-1	
P. amygdali	CBS 126679b	Ex-epitype	GQ281791	-	-	-	Diogo et al. (2010)
P. averrhoae	n.e.	Voucher	AY618930	-	-	-	Chang, Cheng , Xiang, Jiang and Qi (2004)
P. bougainvilleicola	n.e.	Voucher	AY601920	-	-	-	Chang et al. (2004)
P. camptothecae	n.e.	Voucher	AY622996	-	-	-	Chang et al. (2004)
P. chimoanthi	n.e	Voucher	AY622993	-	-	-	Chang et al. (2004)
P. castaneae-mollissimae	DNP128	Holotype	JF957786	-	-	-	This study.
P. cuppatea	CBS117499	Holotype	AY339322	AY339354	GQ250252	-	Rensburg et al. (2006) Santos et al. (2010)
P. columnaris	CBS109873	Holotype	AF439625	-	-	-	Farr et al. (2002b)
P. cotoneastri	CBS 439.82	Isotype	FJ889450	GQ250341	-	GQ250286	Santos et al. (2010)
P. dauci	CBS 315.49	Ex-epitype	FJ889451	GQ250348	-	GQ250289	Santos et al. 2010
P. emicis	BRIP 45089 a	Holotype	JF957784	-	-	-	This study.
P. eucommicola	n.e.	Voucher	AY578071	-	-	-	Chang et al. (2004)
P. eucommii	n.e.	Voucher	AY601921	-	-	-	Chang et al. (2004)
P. glabrae	n.e	Voucher	AY601918	-	-	-	Chang et al. (2004)
P. helianthi	CBS 592.81	Paratype	AY705842	GQ250308	GQ250234	-	Santos et al. (2010)
P. javanica	ATCC 24624	Holotype	-	-	-	-	-
P. lagerstromiae	n.e.	Voucher	AY622994				Chang, Xi, Xiang, Jiang and Chi (2005)
P. leptostromiformis var occidentalis	WAC 5364	Holotype	-	-	-	-	-
P. liquidambari	n.e.	Voucher	AY601919	-	-	-	Chang et al. (2004)
P. longicolla	ATCC 60325	Holotype	-	-	-	-	-
P. loropetali	n.e.	Voucher	AY601917	-	-	-	Chang et al. (2004)
P. magnoliae	n.e.	Holotype	AY622995	-	-	-	Chang et al. (2004)
P. mauritina	n.e	Holotype	EU012334	-	-	-	Yuan, Li and Wei (2008)
P. micheliae	n.e.	Holotype	AY620820	-	-	-	Chang et al. (2004)

Table 1.3 (continued)

Taxa	Culture collection	Status of culture	GenBank Accessions				References
			ITS	EF1-α	MAT1-1-1	MAT1-2-1	
P. saccharata	n.d.	Holotype	AF387817	-	-	-	Mostert et al. (2001)
P. phoenicicola	CBS 161.64	Holotype	FJ889452	GQ250349	-	GQ250290	Santos et al. (2010)
P. sclerotioides	CBS 296.67, ATCC 18585	Holotype	AF439626	GQ250350	GQ250253		Farr et al. (2002b) Santos et al. (2010)
P. theicola	CBS 187.27	Holotype	DQ286287	DQ286261	-	-	Rensburg et al. (2006)
P. tuberivora	CBS 268.32	Holotype	JF957785	-	-	-	This study
P. vaccinii	CBS 160.32	Holotype	AF317578	GQ250326	GQ250244	-	Santos et al. (2010)
P. viticola	CBS114016	Epitype	AF230751	GQ250351	GQ250254	-	Niekerk et al. (2005), Santos et al. (2010)
P. vitimegaspora	CCRC 33533 , ATCC 201952/ STE-U 2675	Holotype/ epitype	AF 230749	-	-	-	Niekerk et al. (2005)
Diaporthe alleghaniensis	ATCC 24097, CBS 495.72	Isotype	FJ889444	GQ250298	-	GQ250261	Santos et al. (2010)
D. ambigua	CBS 114015	Ex-epitype	AF230767	GQ250299	GQ250229	GQ250262	Mostert et al. (2002) Santos et al. (2010)
D. angelicae	CBS 111592	Holotype	AY196779	GQ250302	-	-	Castlebury et al. (2002) Santos et al. (2010)
D. aspalthi	CBS 117169/STE-U 5428	Holotype	DQ286275	DQ286249	-	GQ250267	Rensburg et al. (2006), Santos et al. (2010)
D. australafricana	CBS 113487	Holotype	AF230744	-	-	-	Niekerk et al. (2005)
D. crotalariae	CBS 162.33	Holotype	FJ889445	GQ250307	-	-	Santos et al. (2010)
D. hickoriae	CBS 145.26	Holotype	FJ889446	GQ250309	-	GQ250268	Santos et al. (2010)
D. lusitanicae	CBS 123213,CBS 123212	Holotype	GQ250190	GQ250311/ GQ250310	GQ250235	GQ250269	Santos et al. (2010)
D. melonis	CBS 507.78	Isotype	FJ889447	GQ250314	GQ250237	GQ250271	Santos et al. (2010)

Table 1.3 (continued)

Taxa	Culture collection	Status of culture	GenBank Accessions				References
			ITS	EF1-α	MAT1-1-1	MAT1-2-1	
D. neotheicola	CBS 123209,CBS 123208	Holotype	EU814480/ GQ250192	GQ250315/ GQ250316	GQ250238	GQ250272	Santos and Phillips (2009) Santos et al. (2010)
D. perjuncta	CBS109745	Ex-epitype	AY485785	GQ250323	GQ250242	-	Niekerk et al. (2005), Santos et al. (2010)
D. stewarti	CBS 193.36	Holotype	FJ889448	GQ250324	-	GQ250276	Santos et al. (2010)
D. strumella var. longispora	CBS 194.36	Holotype	FJ889449	GQ250325	GQ250243	-	Santos et al. (2010)
D. toxica	CBS 53493	Holotype	-	-	-	-	-
D. viburni	CBS 158.29	Holotype	-	-	-	-	-
D. viticola	STE-U 5683, CBS113201	Ex-epitype	AY485750	GQ250327	-	-	Niekerk et al. (2005) Santos et al. (2010)

Notes. n.e. culture not existing, n.d not deposited in public collections or available with author's collection, CBS. Centraalbureau voor Schimmelcultures, Netherlands, ATCC. American type culture collection, BRC. Biological resource center, Institute of Microbiology, Beijing, China, BRIP. Queensland Plant pathology herbarium/culture collection: Australia, WAC. Western Australia culture collection collection (as CCBD in publication), STE-U. Stellenbosch University culture collection, South Africa, CCRC. culture collection and research centre, Hsinchu, Taiwan.

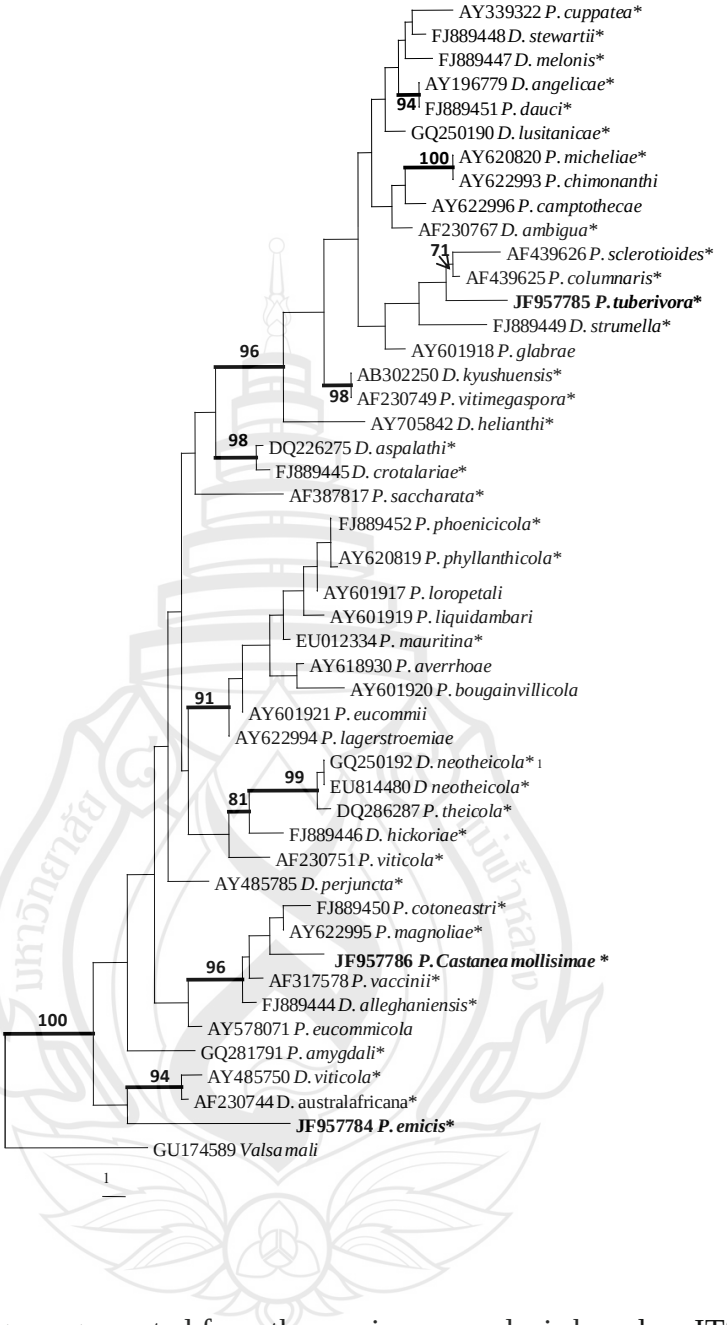


Figure 1.5 Phylogram generated from the parsimony analysis based on ITS sequence data derived from ex-type voucher specimens. Bootstrap support values >70% are shown below or above the branch and strict consensus branches are thickened. (*cultures derived from type specimens, newly generated sequences are in bold). The tree is rooted with *Valsa mali*.

1.4.2 Sexual state, mating types and molecular basis of mating experiments

A recent molecular based study on *Diaporthe* (Santos et al., 2010) focused mainly on the principles of biological species recognition with the rational selection of mating types by a genetic approach, therefore widening the understanding of biological species concept in this genus. Herein, we discuss the significance of incorporation of biological species concepts in future research on the genus. *Diaporthe* comprises homothallic, heterothallic and asexual members and therefore biological species recognition is important (Rossman et al., 2007; Kanematsu et al., 2007). In heterothallic (self-sterile) species, sexual development depends on mating between isolates of opposite mating types. Homothallic (self-fertile) species isolates produce the sexual stages without the need of a mating partner and therefore mating types cannot be defined in these organisms. Purely anamorphic organisms do not form any sexual stage although the mating type genes can be amplified (Santos et al., 2010). The identity of mating types of fungi is determined by the gene content at the mating type/mating type like (MAT or MTL) locus, which usually includes more than one open reading frames (ORFs) and encode for transcription factors that regulate the sexual identity (Butler, 2010). Mating types in the ascomycota are usually bipolar, which means that the mating types are determined by two possible DNA sequences at the mating type locus comprising unrelated and unique sequences even though they are in the same locus (Coppin, Debuchy, Arnaise & Picard, 1997). This lack of sequence similarity between the two alternate mating types is a characteristic property previously related to four model ascomycetes, i. e. *Neurospora crassa*, *Podospora anserine*, *Cochliobolus heterostrophus* and *Magnaporthe grisea* (Coppin et al., 1997). The term "idiomorph" have been used to denote unrelated sequences although present in the same homologous locus, rather than using the term alleles (Butler, 2010; Coppin et al., 1997). Kanematsu et al. (2007) revealed that the structure of MAT loci of *Diaporthe* Wand G types, is distinctive feature bearing homologous genes in opposite mating type loci. Other heterothallic filamentous ascomycetes have dissimilar structures in opposite mating type loci. Thus researchers working on *Diaporthe* tend to use "mating types" rather than "idiomorphs" (Santos et al., 2010).

There have been several attempts to apply the biological species concepts in *Diaporthe* using conventional means. Brayford (1990) identified two morphological

types of *Phomopsis* termed group one and two using isolates from *Ulmus* species from the British Isles and Italy; the groups also corresponded to two mating types. Cross mating experiments confirmed that group one consisted of two mating types and was thus self sterile, whereas group two was self fertile. No cross fertilisation was detected between the two groups. Linders, Enrico and Van Der Aa (1995) demonstrated that *D. adunca* (Roberge ex Desm.) Niessl was heterothallic with two mating types by cross fertilisation and development of the *Diaporthe* sexual stage the following spring. Kanematsu et al. (2000) employed morphology and molecular techniques to elucidate the diversity of *Diaporthe*/*Phomopsis* isolates from fruit trees. In the mating test experiments they recognised that the W-type isolates from fruit trees were heterothallic and inter-fertile even between isolates belonging to different monophyletic groups inferring the phylogeny of rDNA ITS comparison.

In the same experiment, the isolates of the G-type and *P. amygdali* collected in Japan were cross fertile. They have also shown the cross fertility between the isolates from different hosts in same morphological type by cross mating tests. As well as conventional mating experiments, molecular based methods have been utilised in mating type diagnosis. Kanematsu et al. (2007) stressed that it was important to use mating type genes in evolutionary relationships in *Diaporthe*. They also assumed that mating type genes would ultimately resolve most of the problems in species recognition. This study was based on previous data on the sexually incompatible groups of *Diaporhe/Phomopsis* from fruit trees isolated from Japan (Kanematsu et al. 2000). The hypothesis is that the reproductive isolation between *Diaporthe* W and G types might occur because of the differences of the mating type loci. Kanematsu et al. (2007) cloned and sequenced the mating type genes of both reproductively isolated groups, and found that the mating type loci are similar in structure in contrast to other filamentous fungi. Structure and expression analysis of mating type loci was reported related for *Diaporthe* W-type and G-type by PCR based methods. Sequence information was provided in GenBank with accession numbers for those mating type genes as *Diaporthe* W-type (MAT1-1: AB199324; MAT1-2:AB199325), *Diaporthe* G-type (MAT1-1: AB199326; MAT1-2: AB199327) (Kanematsu et al., 2007). These sequences were used to design suitable genus specific primers for mating type genes (Kanematsu et al., 2007; Santos et al., 2010). Santos et al. (2010) designed the primers

for the mating type diagnosis of *Diaporthe* using the alignments of the mating type genes of conserved regions of *Diaporthe* Wand G types (Kanematsu et al., 2007). These primers were successfully utilised for the amplification of part of the α1 box from MAT1-1-1 gene and part of the HMG (high mobility group) domain from MAT 1-2-1 gene. Mating experiments were conducted to verify the molecular diagnosis of mating types (Santos et al., 2010). The method of utilisation of MAT primers in the molecular diagnosis of homothallic and heterothallic members, and the selection of compatible mating pairs has drastically reduced the number of crossings in teleomorph induction in vitro (Santos et al., 2010). Homothallic species were used to induce the teleomorph in vitro whereas heterothallic species were tested in cross mating tests. The only requirement for successful mating was co inoculation with opposite mating types of the same species (Santos et al., 2010). MAT genes however, influence the determination of sex hence; they play a key role in population genetics and evolution of fungi and therefore provide meaningful justifications in evolutionary studies (Kronstad & Staben, 1997). Molecular phylogenetic approach in *Diaporthe* should therefore be meaningful with incorporation of ITS, MAT, EF1α and other reliable gene sequence based evidence to overcome different problems in taxonomic conclusions (Santos et al., 2010).

1.4.3 Infraspecific taxonomy

Infraspecific taxonomy considers taxa below the rank of species according to the International Code of Botanical Nomenclature-Vienna code, which includes subspecies, variety and forma (McNeill & Turland, 2005). Plant pathologists use the categories of forma specialis and pathotypes although they are not formal taxonomic ranks (Cannon, Bridge & Monte, 2000; Cai et al., 2009). Phenotypic and genotypic characters can be used in infraspecific taxonomy including pathogenicity, virulence, biochemistry, physiology and gene sequence data. Infraspecific variation of phylogenetically utilisable genes is a parameter for the selection of possible secondary barcoding regions for a particular genus of fungi (Herbert, Cywinska, Ball & Dewaard, 2003; Zhao, Luo & Zhuang, 2011). There have been several infraspecific taxonomic investigations on important plant pathogens of *Diaporthe* which cause significant losses to economically important crops. This includes pathogens of

sunflower (i.e. *Diaporthe helianthi*), the complex of *Diaporthe/Phomopsis* pathogens on soybean, and *P. viticola* and other species that causes the diseases of grapes (Merrin et al., 1995; Zhang et al., 1997; Rekab, Sorbo, Reggio, Zoina & Firrao, 2004; Viguié, Vear & de Labrouhe, 1999; Vergara et al., 2005). Whether infraspecific ranks should be used for species of *Diaporthe* is as yet undetermined and presently it would be wise to avoid such usage until molecular data can validate such ranking. Infraspecific studies on common pathogens are needed in future studies in order to recognise distinct biotypes.

1.4.4 Type cultures, epitypification and novel species

The study of type derived cultures and specimens are fundamental to future studies on the taxonomic studies. Some important type derived cultures has been lost due to poor storage facilities. For example, many of the type cultures for the species described from southern China (Chi et al., 2007) are no longer viable or are lost (Pers. comm. Prof. Zide Jiang). It is paramount that efforts are made to preserve these important cultures (Abd-Elsalam et al., 2010). If cultures are maintained in the regional collections with limited resources, they should also be deposited in international collections such as CBS and ATCC. It is necessary to distribute holotypes, isotypes and extype specimens and cultures in several herbaria and culture collections, and deposit the type derived sequences in public databases (Ozerskaya, Vasilenko, Verslyppe & Dawyndt, 2010; Abd-Elsalam et al., 2010). There is an urgent need for re-inventory of plant pathogens which as resulted from rapid progress in molecular identification identification of cryptic species, but this is hampered by the lack of type cultures (Cai et al., 2011; Hyde et al., 2010a; 2010b; 2011). DNA sequence data and living cultures significantly enhances the value of type material and the published species description and thus every effort should be made to generate and deposit such resources in public collections (Seifert & Rossman, 2010; Hyde et al., 2010a; 2010b). Changes in the botanical code may be needed to encourage this. In the parsimony analysis using 42 ITS sequences named as *Diaporthe helianthi* in GenBank numerous entries had considerable evolutionary divergence from the type derived sequence (Cai et al., 2011). This work shows the need for comparison with type material and type sequence data in phylogenetic studies of sexual Diaporthe in

order to avoid the possible misidentification. *Phomopsis amygdali*, the causal agent of twig canker and blight of almonds was recently epitypified in a survey of the pathogens in Portugal (Diogo et al., 2010). Although an epitype should be derived from the same locality and host as the type (Hyde & Zhang, 2008), the justification for epitypifying *P. amygdali* was based on morphological and ITS similarity of isolates from Italy, Portugal and Spain. The specimens had been described from *Prunus dulcis* (CBS-H 20420) and CBS 126679b was recognised as ex-epitype culture (Diogo et al., 2010). This is the only recent case of epitypification of a species of *Diaporthe/Phomopsis* that is an important phytopathogen. There is an unprecedented need for mycologists to return to the field, recollect species re-typify taxa with living cultures and fully characterise the taxa which has a large number of species names mostly without DNA sequence data or type cultures (Hyde et al., 2010a; 2010b).

1.4.5 Potential resource for future research initiatives

Fungal genomics and proteomics, genetic transformations, gene knockout strategies and different molecular biological applications have revolutionised the studies of fungal biology in recent decades (Lorang et al., 2001; Birren, Fink & Lander., 2002; An, Wang, Liu & Bennett, 2010; González-Fernández et al., 2010; Kano, Kurita, Kanematsu & Morinaga, 2011). The use of *Diaporthe* species for various applications including biocontrol, adaptive responses of endophytes and hosts, studies on host pathogen interactions, model systems for studying fungal pathogenicity, mycotoxins and fungal metabolite research have been significant other than its ubiquity as a pathogen (Anco, Kim, Mitchell, Madden & Ellis, 2009; Nevena et al., 1997; Hyde et al., 2011; Dai et al., 2010).

There have been initiatives of using species of *Diaporthe* as a tool of studying fungal pathogenesis also involved in genetic transformation (Guido et al., 2003; Anco et al., 2009). In one study, *Phomopsis viticola* was transformed by GFP (green fluorescent protein) using protoplast mediated transformation and penetration and invasion of the host by the fungus studied by fluorescent microscopy. Transformations yielded mitotically stable strains without any change in virulence on grape internodes and leaves in comparison to the wild type. The transformed *P.*

viticola strains were considered to be a critical tool for elucidating fungal penetration of host plants, invasive growth and the nature of its host association and to explore the unknown physiological function of beta conidia. The study speculated on the potential use of *Diaporthe/Phomopsis* as a model organism to study the molecular mechanisms related to pathogenesis (Anco et al., 2009).

The endophytic *Phomopsis* strain B3 isolated from *Bischofia polycarpa* (Chinese bishopwood) is thought to have a symbiotic relationship with rice, peking spurge (*Euphorbia* pekinensis) and peanut, stimulating growth and acting as a pathogenicide (Dai, Yu, Zhao, Jiang & Yang, 2006; Yuan, Dai, Li, Tian & Wang, 2007). The fungus can colonise rice plants from inoculated mycelium available in the soil (Dai et al., 2010). The possible mechanisms of plant colonisation by this strain were examined. The ability to produce laccase enzymes and form cavities in the surface of straw was observed by enzyme assays and microscopy. It was suggested that the endophyte can produce enzymes with entry points at the surface of plant; in *Phomopsis* strain B3 the dominating enzyme was laccase (Dai et al., 2010). This initial study holds promise for future studies on horizontal transmission of endophytes into living plants which may be important in development of pathogen resistant crops. Phylogenetic and evolutionary genomic research has been a focal interest since this will resolve a wide range of biological problems (An et al., 2010). Rapid advancement in the genomics of plant pathogenic fungi will speed up understanding of plant pathogens in many areas including host range and specificity, pathogenicity factors, epidemiology, fungicide resistance, control and evolution (An et al., 2010). Despite the importance as a pathogen on crops and the fascinating biology of the genus, species of *Diaporthe* have not yet been widely used in fungal genomics and proteomics research. *Diaporthe* is potentially important as a model genus for the studies of biology, pathology, reproduction, genetics and evolution of ubiquitous pathogens and therefore should be used as a resource organism for future research.

In conclusion of the overview, species recognition criteria within *Diaporthe* have evolved from morphological species to phylogenetic and biological species incorporating molecular data. The rapid advancement of understanding of molecular phylogeny to resolve the species questions in fungi has been utilised to understand the confusion in the taxonomy of *Diaporthe*. An overview of the current knowledge of

species of *Diaporthe* provides a foundation for future taxonomic and phylogenetic studies. The best practices for the resolution of taxonomy in the genus are epitypification of existing names and linking the species to reliable sequence data, which could be achieved by a collaborative effort among interested groups. Fresh collections are needed for most of the significant pathogens which need to redefine with the clarification of nomenclature and taxonomy.

1.5 Applications

Phytopathogenic genera of fungi also have gained the attention in the discovery of novel biochemically and physiologically active compounds and their direct use in agricultural biotechnology and medicine (Dai, Yuan, Yang, Shi & Li, 2008; Kathiravan & Raman, 2010; Xu, Ebada & Proksch 2010; Senthil Kumaran, Jung & Kim, 2011). The ubiquity, diversity and biology of the species of *Diaporthe* encourage the need for evaluation of potential applications of these fungi. A key argument in favor of studying taxonomy and conserving biodiversity is that as yet undiscovered biodiversity will yield products of important and beneficial use for humans. However, any link between undiscovered biodiversity and useful products is however, largely conjectural (Smith et al., 2008).

1.5.1 Biocontrol agents

Biological control of weeds by plant pathogens has gained acceptance as a practical, safe, environmentally beneficial, weed management method applicable to agroecosystems (Charudattan, 2000). There has been remarkable attention directed towards bioherbicides or mycoherbicides (i.e. inundative use of fungal pathogens) in advancing biocontrol strategies (Mortensen, 1997; Charudattan, 2000; Trujillo, 2005). Some species of *Diaporthe* have been reported as potential mycoherbicides to control invasive and destructive weeds due to their hemibiotrophic to necrotrophic life mode, extensive sporulation and persistence in the environment (Rosskopf, Charudattan, Shabana & Benny, 2000; Rosskopf et. al., 2000b). The toxins and enzymes involved in physiological and biochemical functions of hemibiotrophs and necrotrophs are important targets for the studies in biocontrol and molecular plant pathology and

instrumental to design rational strategies for disease control (Van Kan, 2006). Knowledge of the pathogen life cycle also drives the effective control of plant diseases (González-Fernández, Prats & Jorrín-Novo, 2010).

Table 1.4 *Diaporthe*/*Phomopsis* species as biocontrol agents

Pathogen (name as in publication)	**Host /Target**	**Reference(s)**
Phomopsis sp.	*Carthamus lanatus* (Safron Thistle)	Ash et al. (2010)
P. emicis Shivas	*Emex australis*	Shivas and Scott (1993)
P. convolvulus Ormeno	*Convolvulus arvensis*	Ormeno-Nunez (1988); Morin, Watson and Reeleder (1989)
P. amaranthicola Rosskopf, Charud., Shabana & Benny	*Amaranthus* sp.	Ortiz-Ribbing and Williams (2006)
P. cirsii Grove	*Cirsium arvense*	Leth, Netland and Andreasen (2008)

A greater use of mycoherbicides is important with the movement towards organic farming and the restricted use of herbicides (Ash, 2010; Bailey, Boyetchko & Langle, 2010). Examples of potentially available *Diaporthe* in biocontrol of weeds are listed in Table 1.4. Research on biological control of weeds should target the most urgent and problematic weeds where management by conventional methods is not working and biocontrol agents also provide significant benefits for users (Auld & Morin, 1995; Greaves, Holloway & Auld, 1998; Charudattan, 2001). Therefore the discoveries on bioherbicidal fungal strains should follow the urgent needs and pathogens on invasive plants should be reassessed and reported as potential biocontrol agents (Charudattan, 2000; Ortiz-Ribbing & Williams, 2006). The wide host range of species of *Diaporthe*,

host specificity of some species and mechanisms infection, pathogen persistence in the environment has been proven an utilisable tool in integrated weed management systems (Ortiz-Ribbing & Williams, 2006).

1.5.2 Secondary metabolites

The discoveries of biologically active fungal metabolites including new antibiotics, chemothereputic agents, and agrochemicals have been the focus of the scientific community worldwide. These fungal metabolites are generally recognised as highly effective, possess low toxicity, and have a minor environmental impact (Pearce, 1997; Strobel & Daisy, 2003; Smith et al., 2008; Xu et al., 2010). *Pestalotiopsis*, another coelomycetous genus, has been shown to be highly creative with more than 130 novel potentially medicinal metabolites discovered (Aly, Debbab, Kjer & Proksch 2010; Xu et al., 2010; Liu, 2011). *Diaporthe* is a similarly creative genus with several important discoveries including exclusive and structurally significant, physiologically active fungal metabolites (Table 1.5).

Fungal endophytes have received increasing attention by natural product chemists due to their diverse and structurally unprecedented compounds which make them interesting candidates for drug discovery (Strobel & Daisy 2003; Zhigiang, 2005; Huang, Cai, Hyde, Cork & Sun, 2008; Mitchell, Strobel, Moore, Robison & Sears, 2010; Liu, 2011). Endophytic *Diaporthe* strains have gained attention in most cases involving metabolite research. Because of the practical difficulty in identification at the species level, most of these metabolite producing strains are only recognised at generic level. The utility of some of the novel metabolites in functional in vitro systems are still unknown (Li, Wang, Huang & Shen, 2010).

Table 1.5 Secondary metabolites from species of *Diaporthe*/*Phomopsis*

Source isolate	Host	Metabolites / enzymes	Known applications of metabolites	Reference(s)
Endophytic *Phomopsis* sp. BCC 1323	*Tectona grandis*	Phomopxanthone A,B	*In vitro* antimalarial ,antitubercular activities, cytotoxicity	Isaka et al. (2001)
Endophytic *Phomopsis* sp. BCC 9789	*Musa acuminata*	Six new oblongolides	Cytotoxicity	Bunyapaiboonsri, Yoiprommarat, Srikitikulchai, Srichomthong and Lumong (2009)
Endophytic *Phomopsis* sp.	*Taxus cuspidate*	Taxol	Anticancer activity	Senthil Kumaran et al. (2009)
Endophytic *Phomopsis* sp. B3	*Bischofia Polycarpa*	Laccase enzymes	Biological Oxidation/microbial industry	Dai et al. (2010)
Phomopsis cassiae	*Cassia spectabilis*	ethyl 2,4-dihydroxy-5,6-dimethylbenzoate, phomopsilactone	Antifungal activity, cytotoxity against human cervical tumor cell line	Silva et al. (2005)
Phomopsis oblonga	*Ulmus* sp.	Several novel compounds	Insecticidal activity	Claydon , Grove and Pople (1985)
Phomopsis leptostromiformis	*Lupinus* sp.	Phomopsin	Antimitotic activity (inhibition of microtubule assembly)	Yin, Hlsayoshi, Yoshiyuki and Shigeo (1992) Shivas, Allen and Williamson (1991)
Endophytic *Phomopsis* sp. (#zsu-H76)	*Excoecaria agallocha*	Phomopsis-H76 A ,B ,C (novel)	In vitro antibacterial activity and cytotoxicity	Yang et al. (2010)
Phomopsis sp. A123	*Kandelia candel*	Five novel nonenolides, phomonol, phomotone, phomophene.	Not detected	Li et al. (2010)
Endophytic *Phomopsis* sp. Lz42	*Maytenus hookeri*	A new sesquiterpenoid , sterol and 5 known compounds	Not detected	Yuan et al. (2009)

Table 1.5 (continued)

Source isolate	Host	Metabolites / enzymes	Known applications of metabolites	Reference(s)
Phomopsis sp.	*Erythrina crista-galli*	mellein, nectriapyrone, 4-hydroxymellein, scytalone, tyrosol, clavatol, mevinic acid, mevalonolactone, Phomol	Antimicrobial activity ,antinfammatory activity	Redko et al. (2007) Weber et al. (2004)
Phomopsis sp. KS-37-2	Stem of cherry tree	benzophomopsin A (I)	Not detected	Shino et al. (2009)
Endophytic *Phomopsis* sp.	living bark of *Cavendishia pubescens*	paspalitrems A 40 , C 41	Tremorgenic activity	Bills et al. (1992)
Endophytic *Phomopsis* sp.	twigs of *Salix gracilostyla var. melanostachys*	Phomopsichalasin	Antibacterial and antifungal activity	Tan and Zou (2001)
Endophytic *Phomopsis* sp.	*Azadirachtae indica*	Five ten-membered Lactones	Antifungal activity against plant pathogens	Wu et al. (2008)
Phomopsis longicolla (endophytic)	*Dicerandra frutescens* : stem segment	Dicerandrol A,B,C	Antibiotic and cytotoxic activity	Wagenaar and Calrdy (2001)
Endophytic *Phomopsis* sp.	*Hydnocarpus anthelminthicus*	Mycoepoxydiene derivatives	Cytotoxicity	Prachya et al. (2007)
Phomopsis archeri (endophytic)	cortex stem of *Vanilla albidia*	Three new sesquiterpenes	Cytotoxicity against cancer cell lines , Antimalarial activity	Hemtasin et al. (2011)
Endophytic *Phomopsis* sp.	*Allamanda cathartica*	Terpenes	Antibacterial activity	Nithya and Muthumary (2011)

1.6 *Colletotrichum*: the need for accurate identification

In addition to the phytopathogenic genus *Diaporthe*, this thesis includes one chpater on the genus *Colletotrichum* related to the tropical fruit patogens. These two genera are more common pathogens encounter in both temperate and tropical trees causing diseases on crops, ornamentals and forest trees. Both of the genera belongs to Class Sordariomycetes, although the genus *Diaporthe* is in the order Diaporthales, family Diaporthaceae where the genus *Colletotrihum* classified in the order Glomerellales, and family Glomerellaceae. These two genera need phylogenenetic re-aseessments in order to define the species limits and the studies concerning importnt pathogens are ongoing.

The genus *Colletotrichum* corda (sexual state *Glomerella* Spauld. & H. Schrenk) cause diseases on a wide range of hosts including grasses, legumes, vegetables, perennial crops and fruits (Hyde et al., 2009a; 2009b; Cai et al., 2009). Species of *Colletotrichum* were listed in eighth position of important plant pathogens in a recent survey among plant pathologists (Dean et al., 2012). Accurate species identification of *Colletotrichum* is critical to understand the epidemiology and to develop effective control of diseases. Identification of C*olletotrichum* species based on morphology has always been problematic, because there are few reliable characters which are plastic, dependent upon methods and experimental conditions (Cai et al., 2009). Rapid progress in molecular phylogenetic methods is now making it possible to recognise stable and well-resolved species limits within *Colletotrichum* (Weir, Johnston & Damm, 2012; Damm, Cannon, Woudenberg & Crous, 2012a). An important step in providing a stable taxonomy for the genus is to epitypify existing names, and in so doing link them to genetically defined clades.

Colletotrichum has long served as a model system for hemibiotrophic pathogens, having a short biotrophic stage, followed by a switch to tissue ramification and necrotrophic development (Dean et al., 2011). In addition to economically important crops, some species of *Colletotrichum* infect a variety of wild and weedy plants (Crouch et al., 2009). Most *Colletotrichum* species cause diseases known as anthracnose, characterised by sunken necrotic lesion on leaves (also known as leaf

spot), stems or fruits, reducing the commercial value of the fresh produce. Moreover, since several *Colletotrichum* species cause latent infections, particularly of fruit crops, they are one of the major causes of post harvest diseases in tropical countries (Phoulivong, 2011). General morphological characters of *Colletotrichum* are useful in order to indentify the strains at generic level. Some of these include : on the plant, the conidiomata are typically acervular, with a basal tissue composed of brown or sub hyaline pseudo parenchyma, with fairly thick-walled cells, often vertically arranged, bearing conidiophores which sometimes are branched near the base. The conidiogenous cells are cylindrical, hyaline, phialidic, often with a distinct apical thickening and collarette. The conidia are one-celled, hyaline, straight or curved, cylindrical and germinate with brown appressoria. The conidiophores are usually intermixed with dark brown, thick-walled, apically attenuated setae. In pure culture, the conidiomata are often reduced, basal tissues and setae can be absent or conidiogenous cells can be formed directly on the mycelium (Cai et al., 2009, Than et al., 2008a; 2008b).

1.6.1 Current status of species recognition

The current status of species recognition in *Colletotrichum* has been revised in several comprehensive contemporary reviews for the genus (Hyde et al., 2009, Cai et al., 2009; Cannon, Damm, Johnston & Weir, 2012). Molecular phylogenetic studies have revolutionised the understanding of species diversity and evolutionary relationships of this ubiquitous genus. The current outline of the genus comprised of nine major species complexes and several intermediate lineages. These major species aggregates are informally classified as clades of acutatum, boninense, dematium, destructivum, gloeosporioides, gramonicola, orbiculare, spaethianum and truncatum (Cannon et al., 2012). Many of these species complexes were previously been thought to be single taxon, molecular phylogeny has later revealed to be species complexes. The species within the major aggregates share high degree of phenotypic, genotypic, biological and ecological similarities. Therefore, the studies focus on these separate clades and exploring the gene regions informative for each of clusters has been a focal interest of the taxonomic community at present.

For instance, *Colletotrichum gloesporioides* species complex was revised by Weir et al. (2012), which is recognised as a highly diverse clade. This species complex is now comprised on at least 28 distinct species, however more to be resolved. With the availability of well defined ex-type cultures and multiple DNA sequence data contribute to the understanding of evolutionary relationships within the clade. Many of the tropical fruit pathogens are aggregate with the species in this clade including *Colletotrichum gloesporioides, C. musae*, *C. kahawae* and *C. siamense*. Although these major species complexes are comprehensively studied, large part of the described species within the genus is yet to be resolved. These species need to be recollected and epitypified with the placement within the known phylogeny. Linking to the old species names for the newly collected taxa can be difficult and time consuming endeavor, however once the precise identification is done it provide promising implications in taxonomy. Describing novel taxa from largely untouched niches and hosts also reveals new lineages contributing undiscovered biodiversity.

1.6.2 *Colletotrichum* species associated with tropical fruits

Anthracnose and rots of tropical fruits caused by *Colletotrichum* species are major pre and post harvest diseases which seriously constrain the production, marketing and export of fruits. Therefore it is important in phytosanitation and quarantine purposes (Sreenivasaprasad, Brown & Mills 1992, Alahakoon & Brown 1994; Hindorf, 2000; Hyde et al., 2009a; Johnston & Jones, 1997). The impact of fruit spoilage ranges from a minor reduction of market quality and total loss of fresh produce (Bailey & Jeger, 1992). The fruit rots are mainly caused by *Colletotrichum gloeosporioides*, and to a lesser extent by *C. acutatum*. However, these two taxa are thought to be species complexes and accurate information concerning the causal species within these complexes is lacking (Hyde et al., 2009a, Cai et al., 2009). The '*gloeosporioides*' complex arose because of the artificially enlarged spore range (especially spore length) placed on *C. gloeosporioides* (Arx, 1957a; 1957b) to overcome the instability of spore morphology under different conditions or from different hosts. Although *C. gloeosporioides*, and to a lesser extent *C. acutatum*, has previously been shown to be the causal agent of tropical fruit rots, Phoulivong et al. (2010) discovered that none of the 25 strains isolated from Laos and Thai fruits were

either of these species. Previous understanding that anthracnose of most tropical fruits is caused by *C. gloeosporioides* or *C. acutatum* should therefore be reconsidered. The re–evaluation of the *Colletotrichum* species from which causes the tropical post post-harvest fruit diseases is needed as this would resolve more genetically unidentified species causing severe losses on fruits in tropics.

1.7 Research Objectives

The objectives of this study are,

1.7.1 To re-evaluate the phylogenetic species recognition in *Diaporthe* based on multiple molecular markers and application of GCPSR to introduce novel taxa.

1.7.2 To re-define the species limits of globally important phytopathogenic species and species complexes of *Diaporthe* based on molecular phylogenetic assessments and designation of epitypes for the species associated with *Citrus*, soybean and other herbaceous crops and the generic type species *D. eres*.

1.7.3 To re-evalaute the species diversity and phylogenetic relationsips of *Colletotrichum* species associated with tropical fruits emphasis on *C. gloeosporioides* species complex.

CHAPTER 2

A MULTILOCUS PHYLOGENETIC EVALUATION OF *Diaporthe* (*Phomopsis*)

2.1 Introduction

The genus *Diaporthe* Nitschke, includes phytopathologically important taxa with wide host ranges and geographic distributions (Uecker, 1988; Crous & Groenewald, 2005; Rossman et al., 2007). *Diaporthe* species have also been reported as endophytes in healthy leaves and stems, saprobes on decaying wood and leaf litter, and even parasites in humans and other mammals (Van Warmelo et al., 1970; Sutton et al., 1999; Garcia-Reyne et al., 2011; Iriart et al., 2011; Botella & Diez, 2011; Sun et al., 2011; Rocha et al., 2011). The host specificity and geographic distributions of most phyopathogenic species of *Diaporthe* are unknown, hindering the international exchange of agricultural commodities (Udayanga et al., 2011; Cowley, Ash, Harper & Luckett, 2012; Sun et al., 2012). Studies on phytopathogenic *Diaporthe* species are therefore particularly important to plant pathologists working on wide range of crop diseases (e.g. grapes, sunflower, soybean and various diseases associated with fruit and ornamental trees).

Molecular phylogenetic analyses have made it possible to reliably connect sexual and asexual morphs of the species of pleomorphic genus *Diaporthe*. Being the older name, *Diaporthe* has priority over *Phomopsis* and should be the generic name for these taxa in future studies (Santos et al., 2010; Santos, Vrandečić, Ćosić, Duvnjak & Phillips 2011; McNeill, Turland, Monro & Lepschi., 2011; Crous et al., 2011; Hawksworth, 2011; Wingfield et al., 2012). In exceptional cases, where the name *Phomopsis* is used in this study, it is used explicitly to identify the two morphs and to

distinguish between existing names that are not yet been formally transferred to *Diaporthe*. Species recognition criteria in *Diaporthe* have historically been based on morphology, culture characteristics and host affiliation (Wehmeyer, 1933; Van der Aa et al., 1990; Rehner & Uecker, 1994; Mostert et al., 2001; Niekerk et al., 2005; Santos & Phillips, 2009). The current status of taxonomic knowledge of *Diaporthe* effectively means that strains can be identified to species level only if molecular techniques are employed (Castlebury et al., 2003; Castlebury, 2005; Crous, 2005; Crous & Groenewald, 2005; Santos et al., 2010). Nuclear ribosomal internal transcribed spacer (ITS), partial sequences of translation elongation factor 1-α (EF 1-α) and mating type genes (MAT) have commonly been used in contemporary molecular phylogenetic studies of the genus (Niekerk et al., 2005; Rensburg et al., 2006; Santos et al., 2010; 2011; Udayanga et al., 2011; Sun et al., 2012). In the current study, we infer the first multi-locus phylogeny of *Diaporthe* using combined sequences of ITS, EF1-α, TUB and CAL genes. Establishing a well-resolved phylogenetic basis for the genus is important not only for validating diagnostic methods and resolving cryptic species (Udayanga et al., 2011), but also for interpreting the evolutionary history of various genetic traits of interest, such as pathogenicity (De Guido et al., 2003; Kanematsu et al., 2007; Garcia-Guzman & Morales, 2007; Catalano, Rekab, Firrao, Vannacci & Vergara, 2012), host diversity, geographic distribution (Rehner & Uecker, 1994) and mating types (Santos et al., 2010).

Genealogical Concordance Phylogenetic Species Recognition (GCPSR), which uses the concordance of multiple gene genealogies in various combinations, has been provided a better approach for the species delimitation as compared to species concepts based on morphology and reproductive behavior (Avise & Ball, 1990; Templeton, 1989; Hudson & Coyne, 2002; Taylor et al., 2000). Protein-coding genes are widely used in fungal phylogenetics both for higher level phylogenetic placements and species level diagnostics with the addition of new molecular markers to the fungal taxonomists' toolbox (Einax & Voigt 2003; Hofstetter, Miadlikowska, Kauff & Lutzoni, 2007; Schmitt et al., 2009; Walker, Castlebury, Rossman & White, 2012a). Multi-locus phylogenetic analyses have become a routine procedure to identify novel fungal species, especially in those genera that lack distinctive

morphological characters, and to resolve species complexes where conventional taxonomy has resulted in confusion (Rokas, Williams, King & Carroll, 2003a; Rokas, King, Finnerty & Carroll, 2003b; Lumbsch et al., 2005; James et al., 2006; Alves, Correia & Phillips, 2006; Schoch et al., 2006; Cai et al., 2011a; 2011b; Manamgoda, Cai, Bahkali, Chukeatirote & Hyde, 2011).

The objectives of this study are (1) to compare the effectiveness of individual and combined gene analyses to resolve species boundaries and relationships within *Diaporthe* and (2) to provide a backbone phylogenetic tree for future studies in *Diaporthe* based on available ex-type cultures using a multi-gene analysis and (3) to introduce new species combinations for the well resolved species in *Diaporthe* based on multi-locus phylogeny, and observations of ex-type cultures and specimens.

2.2 Materials and methods

2.2.1 Collection and isolation

Plant pathogenic and endophytic strains of *Diaporthe* were collected in field surveys in different locations from various hosts in Chiang Rai and Chiang Mai Provinces in northern Thailand (Table 2.1). Specimens with disease symptoms were observed using a stereo microscope and sporulating fruiting bodies were used for single spore isolation by a modified spore suspension method as described for different fungal groups (Choi, Hyde & Ho, 1999; Chomnunti et al., 2011). The sporulating pycnidia or ascomata were excised using a sterile needle, crushed with a few drops of sterile distilled water and spore suspension was then transferred to water agar (WA) plates. The inoculated WA plates were incubated for 24 h and germinating single spores were then transferred to malt extract agar (MEA) plates and incubated at 25 °C in the dark. Endophytic fungi from leaves were isolated using the protocol outlined by Murali et al. (2006). All fresh cultures were deposited in Mae Fah Luang University Culture Collection (MFLUCC) and herbarium material in MFLU. Duplicate cultures are deposited in BCC and CBS, the latter under Material Transfer Agreement (MTA: C27/2011). Details of nomenclatural novelties and new combinations were added to MycoBank (Crous, Gams, Stalpers, Robert & Stegehuis,

2004). Ex-type and ex-epitype cultures were obtained from CBS (Utrecht, Netherlands), BRIP (Queensland, Australia), and directly from authors of recently described new species of *Diaporthe* (Table 2.1).

2.2.2 DNA extraction, gene amplification and sequencing

Isolates were grown on potato-dextrose agar (PDA) overlaid with sterilised cellophane for 5 days at 25 ℃ (Murali et al., 2006) and total genomic DNA was extracted from 0.05 to 0.10 g of axenic mycelium scraped from the edge of the growing culture (Wu, Wang, Huang & Qu, 2001). Mycelia were ground with half volume of PVP (polyvinylpyrrolidone), sterile quartz sand and 200 µl of 2 % CTAB buffer using a sterilised glass pestle in micro centrifuge tubes. Then, 400 µl of CTAB was added and incubated in 65 ℃ for about 40 min and centrifuged at 12,000 rpm for 10 min. The supernatant was subjected to phenol/chloroform extraction followed by precipitation of DNA from the aqueous phase with ice-cold iso-propanol at 4 ℃ for 1 h. Precipitated DNA was recovered by centrifugation of 12,000 rpm for 10 min and washed with 70 % ethanol, air dried, dissolved in 50 µl of TE buffer and stored at -20 ℃ until use for amplification reactions.

Six loci were sequenced including ITS (White et al., 1990), EF1-α, CAL (Carbone & Kohn, 1999), TUB (Glass & Donaldson, 1995), MAT 1-1-1 and MAT 1-1-2 (Santos et al., 2010). The primers, references and PCR protocols are summarised in Table 2.2. The 50 µl reaction volume (1×PCR buffer, 0.2 mM dNTP, 0.4 µM of each primer; 1.5 mM $MgCl_2$, 2 % formamide, 1 % DMSO (variable), 0.8 units Taq polymerase and 10 ng template DNA), was used for each of the reaction with the adjustments of components when needed. The PCR products, spanning approximately 300–500 bp (ITS, EF 1-α, TUB, CAL) were visualised on 1 % agarose gels stained with Goldview (Geneshun Biotech, China) with D2000 DNA ladder (Realtimes Biotech, Beijing, China). For the MAT 1-1-1 and MAT 1-2-1 genes (200 and 300 bp, respectively), the amplicons were subjected to simultaneous electrophoresis in 1.5 % agarose gels with a 100 bp DNA ladder (Realtimes Biotech, Beijing, China) in 100 V for 45 min and visualised in GelDoc image system with modifications as described in Santos et al. (2010). All the PCR products were then purified according to the company protocols and DNA sequencing was performed using the above-mentioned

primers in an Applied Biosystem 3730 DNA analyser at the Sinogenomax Company, Beijing, China.

2.2.3 Sequence alignment and phylogenetic analyses

Sequence homologies for the assembled consensus sequences were analysed using the BLAST search in the National Center for Biotechnology Information (NCBI) and for the rough identification of fresh isolates used in the analyses. Sequences of the available ex-type cultures were obtained from GenBank (Table 2.1) listed in Udayanga, Liu, McKenzie, Chukeatirote and Hyde (2012). The consensus sequences for each gene were initially aligned by Clustal-W (Thompson, Higgins & Gibson, 1994) as implemented in Bioedit (Hall, 1999) and improved in MAFFTv6 (Katoh, Misawa, Kuma & Miyata, 2002; Katoh & Toh, 2008), online sequence alignment editor under the default settings and optimised manually when needed. Ambiguously aligned regions were excluded from all the analyses and confirmed that there is not conflict in datasets. A partition homogeneity test (PHT)/ Incongruency Length Difference Test (ILD) (Farris, Kallersjo, Kluge & Bult, 1994) was applied as implemented in PAUPv4.0b10 (Swofford, 2002) to evaluate the feasibility of combining datasets. PAUPv4.0b10 was used to conduct the parsimony analysis to obtain the phylogenetic trees. Trees were inferred using the heuristic search option with 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony (Tree Length [TL], Consistency Index [CI], Retention Index [RI], Relative Consistency Index [RC] and Homoplasy Index [HI] were calculated for trees generated under different optimality criteria. Kishino-Hasegawa test (KHT) (Kishino & Hasegawa, 1989) was performed in order to determine whether trees were significantly different. Trees were figured in Treeview (Page, 1996). In total, five data matrices were analysed and compared: based on ITS sequences (alignment I),

Table 2.1 Isolates and GenBank accessions for the gene sequences in this study.

Species	Collection Code	Host	Country of Origin	Collector	GenBank accessions				Detection of Mating type	
					ITS	EF1-α	TUB	CAL	MAT 1-1-1	MAT 1-2-1
D. cotoneastri	CBS 439.82	*Cotoneaster* sp.	UK, Scotland	H Butin	FJ889450	GQ250341	JX275437	JX197429	-	+
D. castaneae-mollissimae	DNP 128	*Castanea mollissima*	China	SX Jiang	JF957786	JX275401	JX275438	JX197430	+	+
	DNP 129	*Castanea mollissima*	China	SX Jiang	JQ619886	JX275402	JX275439	JX197431	+	+
D. vaccinii	CBS 160.32	*Oxycoccus macrocarpus*	USA	HF Bain	AF317578	GQ250326	JX275436	n.d.	+	-
D. vticola	CBS 113201	*Vitis vinifera*	Portugal	AJL Phillips	AY485750	GQ250327	JX275454	JX197445	-	-
	DNP 086-g1	*Vitis vinifera*	Italy	XZ Liu	JQ619896	JX275412	JX275455	JX197446	-	-
	DNP 086-g2	*Vitis vinifera*	Italy	XZ Liu	JQ619896	JX275413	JX275456	JX197447	-	-
D. australafricana	CBS 113487	*Vitis vinifera*	South Africa	L Mostert	AF230744	n.d.	JX275457	JX197448	-	-
D. thunbergii	MFLUCC 10-0576a	*Thunbergia laurifolia*	Thailand	DS Manamgoda	JQ619893	JX275409	JX275449	JX197440	-	+
	MFLUCC 10-0576b	*Thunbergia laurifolia*	Thailand	SC Karunarathna	JQ619894	JX275410	JX275450	JX197441	-	+
	MFLUCC 10-0576c	*Thunbergia laurifolia*	Thailand	D Udayanga	JQ619895	JX275411	JX275451	JX197442	-	+
D. amygdali	CBS 126679	*Prunus dulcis*	Portugal	E Diogo	GQ281791	JX275400	JX275435	JX197428	-	+
D. neoviticola	CBS114016	*Vitis vinifera*	France	P Larignon	AF230751	GQ250351	JX275452	JX197443	+	-
D. perjuncta	CBS 109745	*Ulmus glabra*	Austria	W Jaklitsch	AY485785	GQ250323	JX275453	JX197444	+	-
P. emicis	BRIP 45089a	*Emex australis*	Auatralia	RG Shivas	JF957784	JX275414	JX275458	JX197449	-	+
	BRIP 45089b	*Emex australis*	Auatralia	RG Shivas	JQ619898	JX275415	JX275459	JX197450	-	+
D. phoenicicola	CBS 161.64	*Areca catechu*	India	HC Sivastava	FJ889452	GQ250349	JX275440	JX197432	-	+
Diaporthe sp.	MFLUCC 10-0609	*Mangifera* sp.	Thailand	SC Karunarathna	JQ619892	JX275408	JX275446	JX197437	-	+
	MFLUCC 10-0587	*Tectona grandis*	Thailand	D Udayanga	JQ619890	JX275406	JX275444	JX197436	-	+

Table 2.1 (continued)

Species	Collection Code	Host	Country of Origin	Collector	GenBank accessions				Detection of Mating type	
					ITS	EF1-α	TUB	CAL	MAT 1-1-1	MAT 1-2-1
Diaporthe sp	MFLUCC 10-0590	*Cassia spectabilis*	Thailand	D Udayanga	JQ619891	JX275407	JX275445	n.d.	-	+
D. pterocarpicola	MFLUCC 10-0580a	*Pterocarpus indicus*	Thailand	D Udayanga	JQ619887	JX275403	JX275441	JX197433	-	+
	MFLUCC 10-0580b	*Pterocarpus indicus*	Thailand	NF Wulandari	JQ619888	JX275404	JX275442	JX197434	-	+
Diaporthe sp.	MFLUCC 10-0583	*Tectona grandis*	Thailand	D Udayanga	JQ619889	JX275405	JX275443	JX197435	-	+
D. aspalathi	CBS 117169	*Aspalathus linearis*	South Africa	JCJ van Rensberg	DQ286275	DQ286249	JX275447	JX197438	-	+
D. crotalariae	CBS 162.33	*Crotalaria spectabilis*	unknown	GF Weber	FJ889445	GQ250307	JX275448	JX197439	-	-
D. pterocarpi	MFLUCC 10-0571	*Pterocarous indicus*	Thailand	D Udayanga	JQ619899	JX275416	JX275460	JX197451	-	+
	MFLUCC 10-0575	*Pterocarous indicus*	Thailand	NF Wulandari	JQ619901	JX275418	JX275462	JX197453	-	+
	MFLUCC 10-0588	*Magnolia* sp.	Thailand	D Udayanga	JQ619900	JX275417	JX275461	JX197452	-	+
*D. **neo**theicola*	CBS187.27	*Camellia sinensis*	Italy	M Curzi	DQ286287	DQ286261	JX275463	n.d.	-	-
	CBS 123208	*Foeniculum vulgare*	Portugal	AJL Phillips	EU814480	GQ250315	JX275464	n.d.	+	+
D. phaseolorum	MFLUCC 10-0608	*Hylocerus undatus*	Thailand	D Udayanga	JQ619875	JX275389	JX275424	JX197418	-	+
	MFLUCC 10-0603	*Hylocerus undatus*	Thailand	D Udayanga	JQ619876	JX275390	JX275425	JX197419	-	+
D. melonis	CBS 507.78	*Cucumis melo*	USA	L Berha	FJ889447	GQ250314	JX275423	JX197417	+	+

Table 2.1 (continued)

Species	Collection Code	Host	Country of Origin	Collector	GenBank accessions				Detection of Mating type	
					ITS	EF1-*α*	TUB	CAL	MAT 1-1-1	MAT 1-2-1
D. ambigua	CBS 114015	*Pyrus communis*	South Africa	S Denman	AF230767	GQ250299	JX275434	JX197427	+	+
D. helianthi	CBS 592.81	*Helianthus annuus*	Serbia	M Muntanola Cvetkovic	AY705842	GQ250308	JX275465	JX197454	+	-
D. angelicae	CBS 111592	*Heracleum sphondylium*	Austria	W Jaklitsch	AY196779	GQ250302	n.d.	n.d.	-	-
D. stewartii	CBS 193.36	*Cosmos bipinnatus*	unknown	AL Harrison	FJ889448	GQ250324	JX275421	JX197415	-	+
D. cuppatea	CBS 117499	*Aspalathus linearis*	South Africa	JCJ van Rensberg	AY339322	AY339354	JX275420	JX197414	+	-
D.lusitanicae	CBS 123212	*Foeniculum vulgarae*	Portugal	JM Santos	GQ250190	GQ250311	JX275422	JX197416	+	-
D. sclerotioides	CBS 296.67	*Cucumis sativus*	Netherlands	HA Van der Kesteren	AF439626	GQ250350	JX275426	JX197420	+	-
D. strumella	CBS 194.36	*Ribes* sp.	Canada	LE Wehmeyer	FJ889449	GQ250325	JX275427	n.d.	+	-
Diaporthe sp. 2	MFLUCC 10-0601	*Coffea arabica*	Thailand	D Udayanga	JQ619902	JX275419	JX275466	JX197455	-	+
	MFLUCC 10-0584	*Tectona grandis*	Thailand	D Udayanga	JQ619884	JX275398	n.d.	n.d.	-	+
	MFLUCC 10-0582	*Aeschynanthus radicans*	Thailand	SC Karunarathna	JQ619885	JX275399	JX275433	JX197426	-	+
	MFLUCC 10-0570	Dead wood-unknown	Thailand	D Udayanga	JQ619877	JX275391	JX275428	JX197421	-	+
	DEN 009	*Tectona grandis*	Thailand	D Udayanga	JQ619882	JX275397	JX275432	JX197425	-	+
D. siamensis	MFLUCC 10-0573a	*Dasymaschalon* sp.	Thailand	D Udayanga	JQ619879	JX275393	JX275429	JX197423	-	+
	MFLUCC 10-0573b	*Dasymaschalon* sp.	Thailand	NF Wualandari	JQ619880	JX275395	JX275430	JX197423	-	+

Table 2.1 (continued)

Species	Collection Code	Host	Country of Origin	Collector	GenBank accessions				Detection of Mating type	
					ITS	EF1-α	TUB	CAL	MAT 1-1-1	MAT 1-2-1
D. siamensis	MFLUCC 10-0573c	*Dasymaschalon* sp.	Thailand	D Udayanga	JQ619881	JX275396	JX275431	JX197424	-	+
Diaporthe sp. 3	MFLUCC 10-0589	*Magnolia* sp.	Thailand	D Udayanga	JQ619878	JX275392	n.d.	n.d.	-	+
	MFLUCC 10-0581	*Rhapis* sp.	Thailand	D Udayanga	JQ619883	JX275394	n.d.	n.d.	-	+

Notes. MFLUCC: Mae Fah Luang University Culture Collection CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands BRIP: Australian plant pathogen culture collection, Queensland DNP/DEN: First author's personal collection (deposited in MFLUCC), (+) : Mating type gene present, (-): mating type genes absent, n.d. : not determined.

Table 2.2 Genes used in the study with PCR primers, references and protocols

Gene/loci	ITS	CAL	EF1-α	TUB	MAT 1-1-1	MAT 1-2-1
PCR primers (for/rev)	ITS 1 / ITS 4	CAL228F /CAL737R	EF1-728F / EF1-986R	Bt2a / Bt2b	MAT1-1-1-FW/MAT1-1-1RV	MAT1-2-1FW/MAT1-2-1RV
References for primers used	White, Bruns , Lee & Taylor (1990)	Carbone and Kohn, (1999)	Carbone and Kohn, (1999)	Glass and Donaldson, (1995)	Santos et al., (2010)	Santos et al., (2010)
PCR: thermal cycles: ** (Annealing temp. in bold)	(95 °C : 30 s, **55 °C**:50 s, 72 °C:1 min) x40 cycles Same conditions are applicable to both markers		(95 °C: 30 s**, 58 °C**:50 s, 72 °C:1 min) x 40 cycles Same conditions are applicable to both markers		(94 °C : 30 s, **50 °C** 30 s, 72 °C:1 min) x x40 cycles	(94 °C ; 30 s, **56 °C** : 30 s, 72 °C:1 min) x40 cycles

Note. All the PCR thermal cycles include an initiation step of 95 °C, 5 min, and final elongation step of 72 °C, 10 min.

EF 1-α sequences (alignment II), TUB sequences (alignment III), CAL sequences (alignment IV), combined ITS, TUB, EF 1-α, CAL genes (alignment V). For the alignment V, Bayesian analysis was performed as described in Phillips, Crous and Alve (2007), setting burn-in at 2000 generations. The amplification reactions for MAT 1-1-1 and MAT 1-2-1 genes were performed, but the sequences were not used in phylogenetic analysis. The results of the detection of mating type genes via simultaneous electrophoresis as described in Santos et al. (2010) are presented in Table 2.1.

2.3 Results

2.3.1 PCR optimisation and amplification of selected loci

One hundred and fifty new sequences were generated in this study (Table 2.1) from 23 ex-type cultures and fresh collections of *Diaporthe* from northern Thailand and elsewhere. Other sequences were downloaded from GenBank and used in the phylogenetic analysis. PCR optimisation and phylogenetic performance of selected loci PCR conditions at optimum annealing temperature and reagent concentrations were optimised to develop effective amplification of ITS, EF **1-α**, TUB and CAL loci. The optimum annealing temperatures were recognised to amplify ITS, CAL (55 °C) and EF **1-α**, TUB (58 °C). This procedure reduces the time required for working with multiple strains of *Diaporthe* to generate the multiple DNA sequences. Two PCR thermal cycling conditions can be used for the amplification of four loci in separate systems. Phylogenetic performance of each locus was compared with the multi-locus phylogeny based on alignment properties of data matrices and selected characters in parsimony analysis (Table 2.3).

2.3.2 ITS phylogenetic analysis in species delimitation

The ITS phylogenetic tree (Figure 2.1) consists of sequences derived from fresh isolates and ex-type, ex-epitype and authentic sequences as indicated in the ITS backbone phylogenetic tree (Udayanga et al., 2011).

Table 2.3 Comparison of PCR success and alignment properties

Genes/loci	ITS	EF1-α	TUB*	CAL*	combined 4 genes
PCR success	100 %	99 %	95 %	95 %	-
Alignment strategy (MAFFT v6)	FFT-NS-I	FFT-NS-I + manual	FFT-NS-I	FFT-NS-I	-
Characters included (with gaps)	522	484	479	555	2040
Characters excluded in parsimony analysis	45	45	-	54	144
Invariable characters	309	80	226	211	826
Parsimony informative characters (%)	95 (18 %)	254 (52 %)	167 (35 %)	245 (44 %)	761 (37 %)
Uninformative polymorphic characters	73	67	86	43	269
Number of branches >70% bootstrap in parsimony analysis	15	24	25	23	32

The ITS data matrix contains 52 taxa including the outgroup and averaging 522 characters (including gaps); 45 characters are excluded in the parsimony analysis. The resulting statistics for the parsimony analysis revealed that 309 characters are constant, 95 characters are parsimony informative and 73 variable characters are parsimony-uninformative. The parsimony analysis yielded 50 equally parsimonious trees and the first tree (Figure 2. 1) was recognised as the best tree and presented here as the basic identification guide to the isolates used in this study (TL=368, CI=0.563, RI=0.797, RC=0.448, HI=0.438).

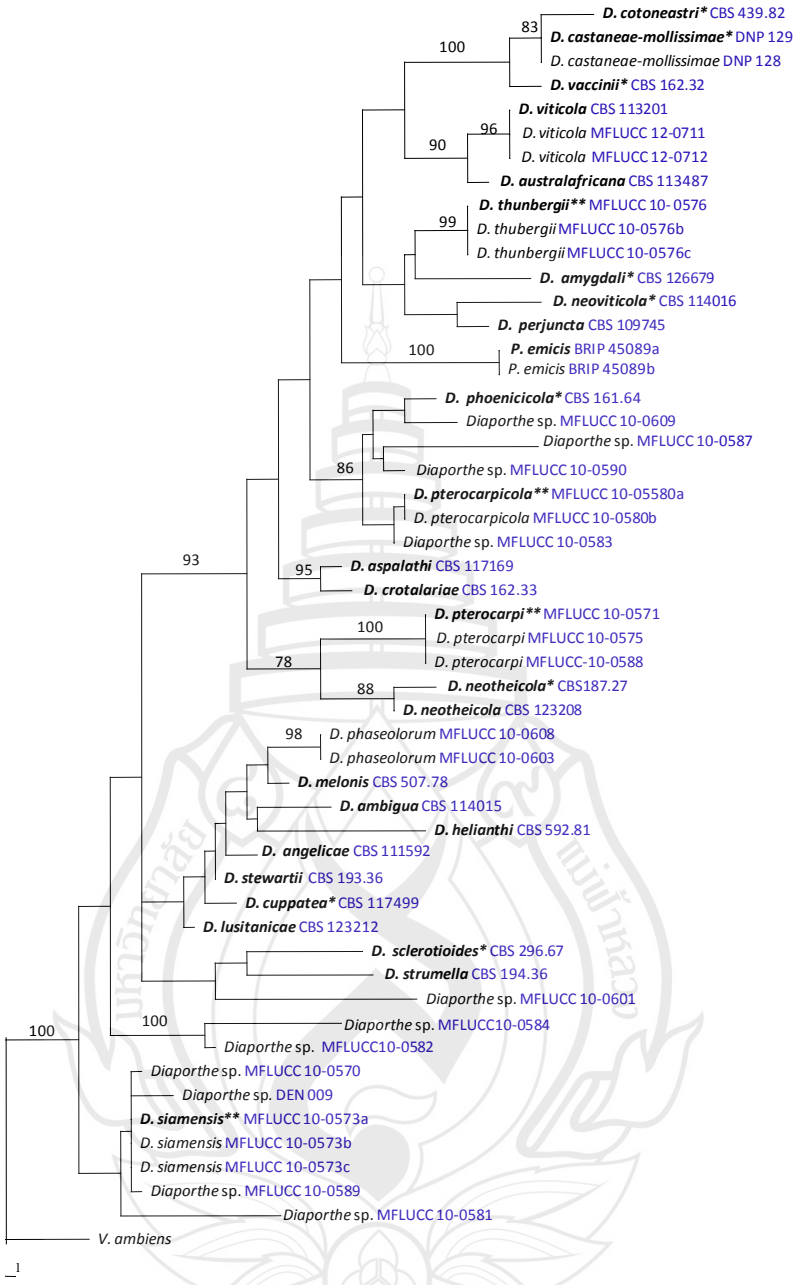


Figure 2.1 The phylogram inferred from a parsimony analysis of ITS sequences from ex-type, ex-epitype (in bold), and fresh isolates collected in Thailand. MP bootstrap values >70 % are shown above or below the branches. The tree is rooted with *Valsa ambiens*.

The phylogenetic tree (Figure 2.1) includes ex-type cultures from a wide range of hosts and various geographic locations while the fresh isolates were chiefly from northern Thailand. Although the terminal nodes differentiate each taxon included in the analysis, the bootstrap support values were inconclusive in most cases and unable to distinguish cryptic taxa.

2.3.3 EF 1-α phylogenetic analysis in species delimitation

The EF 1-α data matrix contains 52 taxa including the outgroup and averaged 484 characters (including gaps) 45 characters were excluded in the parsimony analysis. The statistics for the parsimony analysis revealed that 80 characters are constant, 254 characters are parsimony informative, while 67 variable characters are parsimony uninformative. The parsimony analysis of the alignment yielded six equally parsimonious trees and the first tree (Figure 2.2) was recognised as best tree and presented here as the basic identification guide to the isolates used in this study (TL=1378, CI=0.464, RI=0.745, RC=0.346, HI=0.536). The amplified segment contains part of EF 1-α gene spanning an entire intron (with more variable characters) and partial sequences of the flanking exons. Some isolates identified to be the same species based on EF 1-α phylogeny (with 100 % bootstrap similarity), were further tested in ITS, TUB, CAL and MAT 1-2-1 phylogenetic trees (data not shown). We observed that the groups of isolates identified as siblings in terminal clades in the EF 1-α phylogeny show phylogenetic variability when employing different protein coding genes used in this study. Therefore we recognised that there may be distinct phylogenetic species that are obscured in the analysis of the EF 1-α gene.

2.3.4 Combined analysis of ITS, EF1-α, CAL and TUB genes

The combined gene data matrix contains 52 taxa including the outgroup and an average of 2040 characters; 184 characters were excluded in parsimony analysis. The statistics for the parsimony analysis revealed that 826 characters are constant, 761 characters are parsimony informative, while 269 variable characters are parsimony-uninformative.

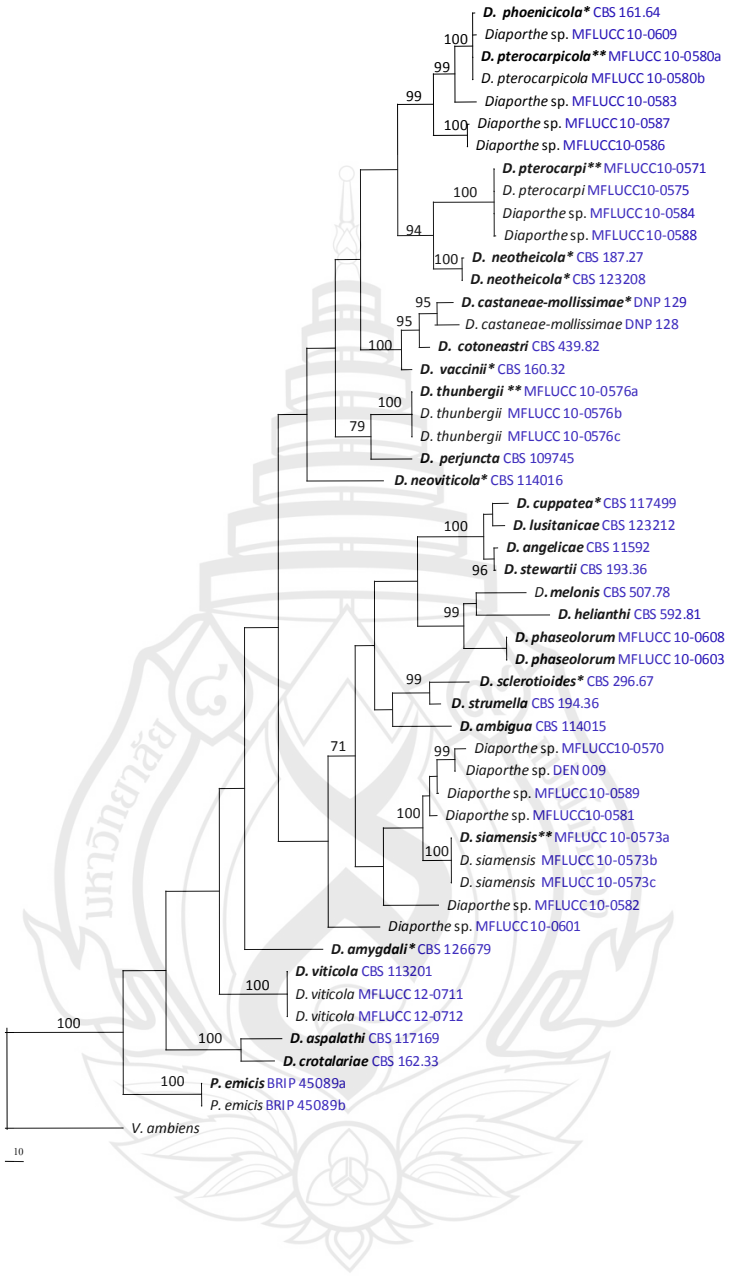


Figure 2.2 The phylogram inferred from a parsimony analysis of EF1- α from ex-type, ex-epitype (in bold), and fresh isolates collected in Thailand. MP bootstrap values >70 % are shown above or below the branches. The tree is rooted with *Valsa ambiens*.

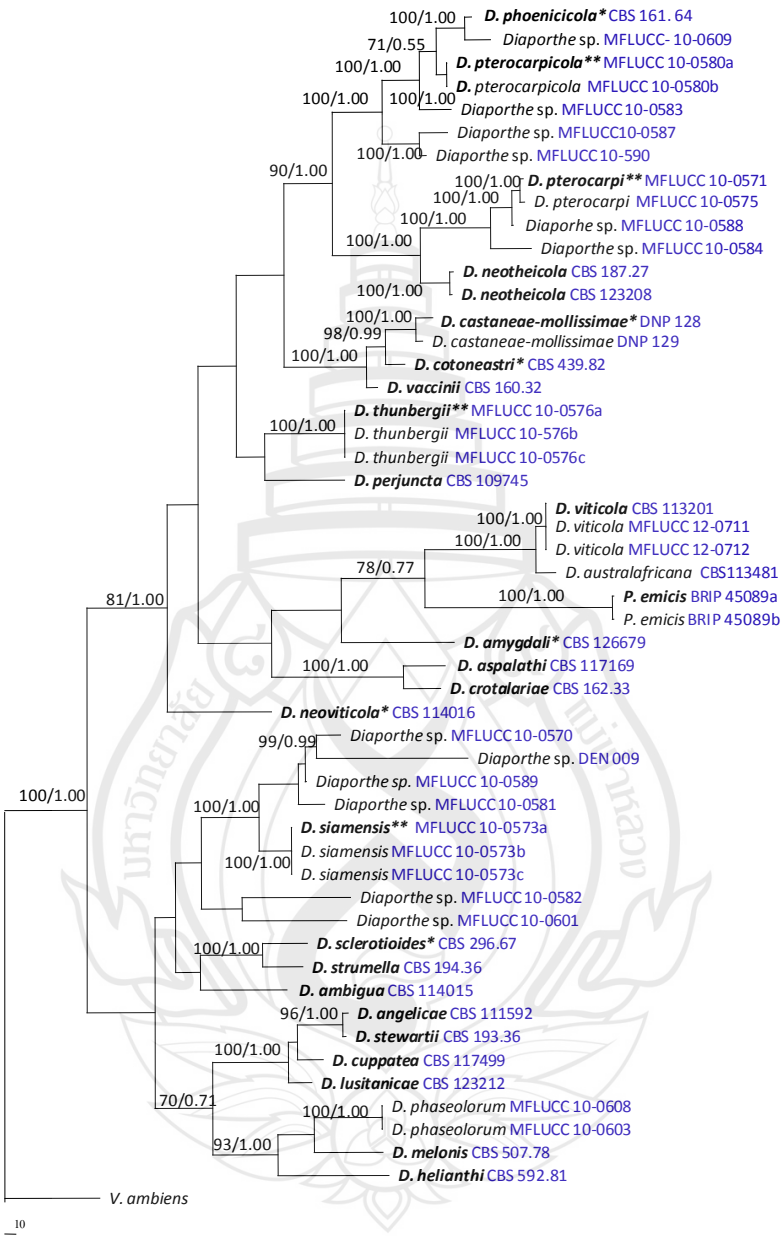


Figure 2.3 The phylogram inferred from a parsimony analysis of combined EF1- α. ITS, TUB and CAL genes from ex-type and ex-epitype (in bold), fresh isolates collected in Thailand. MP bootstrap values >70 % are shown above or below the branches. The tree is rooted with *Valsa ambiens*.

The parsimony analysis of the alignment yielded four equally parsimonious trees and the first tree (Figure 2.3) was recognised as the best tree and presented here as the basic identification guide to the isolates used in this study (TL=3460, CI=0.492, RI= 0.747, RC=0.368, HI= 0.508).

Based on the combined phylogenetic tree, we recognised that most of the ex-type derived taxa are placed in terminal clades and highly supported, without conflict between well-recognised taxa. However the branch lengths of several sub-clades are shorter, indicating the speciation occurred in short time frames. The terminal branch support values and additional internal branch support values (>70 %) has been increased by combining the four genes compared to ITS and EF 1-α phylogenetic trees.

2.3.5 Taxonomy

In this section we transfer nine species of *Phomopsis* to *Diaporthe* based on multi-locus DNA sequence data, annotated with the details of ex-type and ex-epitype isolates and specimens. Brief notes are given where the novel combination is not straightforward.

Diaporthe amygdali (Delacr.) Udayanga, Crous & K.D. Hyde, comb. nov.

MycoBank 800722

Basionym. *Fusicoccum amygdali* Delacr., Bull. Soc. mycol. Fr. 21: 280 (1905)

≡ *Phomopsis amygdali* (Delacr.) J.J. Tuset & M.T. Portilla, Can. J. Bot. 67(5): 1280 (1989)

Type Specimen examined. PORTUGAL:Trás-os-Montes, Mirandela, on twigs of *Prunus dulcis*, Sept. 2005, E. Diogo (CBS-H 20420, epitype), ex-epitype culture, CBS 126679.

Diaporthe castaneae-mollisimae (S.X, Jiang & H.B. Ma) Udayanga, Crous & K.D. Hyde, comb. nov.

MycoBank 800702

Basionym. *Phomopsis castaneae-mollisimae* S.X. Jiang & H.B. Ma, Mycosystema 29: 467 (2010)

Type specimen examined. CHINA: Shangdong Province, on leaves of *Castanea mollissima*, April 2006, S.X. Jiang (CLS-0612, holotype), ex-type culture, DNP 128.

Diaporthe cotoneastri (Punith.) Udayanga, Crous & K.D. Hyde, comb. nov. MycoBank 800697

Basionym. *Phomopsis cotoneastri* Punith., Trans. Br. mycol. Soc. 60 (1): 157 (1973)

Type specimen examined. SCOTLAND: Ayr, on *Cotoneaster* sp., May 1982, H. Butin (CBS-H 7633: isotype), ex-isotype culture, CBS 439.82.

Diaporthe cuppatea (E. Jansen, Lampr. & Crous) Udayanga, Crous & K.D. Hyde, comb. nov. MycoBank 800698

Basionym. *Phomopsis cuppatea* E. Jansen, Lampr. & Crous, Stud. Mycol. 55: 72 (2006)

Type specimen examined: SOUTH AFRICA, Western Cape Province, on *Aspalathus linearis*, 2006, J. Janse van Rensburg (CBS H-19687, holotype), ex-type culture, CBS 117499.

Diaporthe neotheicola A.J.L. Phillips & J.M. Santos, Fungal Diversity 34: 120 (2009)

= *Phomopsis theicola* Curzi, Atti Ist. bot. R. Univ. Pavia, 3 Sér. 3: 65 (1927)

Type specimens examined: PORTUGAL Évora, on *Foeniculum vulgare*, Nov. 2007, A.J.L. Phillips (CBS-H 20131, holotype), ex-type culture: CBS 123208.

Notes: The sexual morph of *Phomopsis theicola* was originally proposed as a distinct taxon from *Diaporthe theicola* Curzi, and was described as *Diaporthe neotheicola* in Santos and Phillips (2009). The multi-locus phylogeny (Figure 2.3), however, reveals that the ex-type isolates of *P. theicola* and *D. neotheicola* represent the same taxon as also stated by Santos and Phillips (2009). Because the name *Diaporthe theicola* is already occupied, we opt to use the name *D. neotheicola*. Further study and epitypification of *D. theicola* Curzi is needed. This taxon is revisited broadly in chapter 4 in this thesis.

Diaporthe phoenicicola (Traverso & Spessa) Udayanga, Crous & K.D. Hyde, comb. nov. MycoBank 800699

Basionym. *Phomopsis phoenicicola* Traverso & Spessa, Bolm Soc. broteriana, Coimbra, sér. 1 25: 177 (1910)

= *Subramanella arecae* H.C. Srivastava., Zakia & Govindar., Mycologia 54(1): 7 (1962)

Specimen examined: INDIA, on fruit of *Areca catechu*, Feb. 1964, H.C. Srivastava (CBS H-7808, isotype), ex-isotype culture, CBS 161.64.

Notes: The sequences available as *P. phoenicicola* are derived from an isotype of *Subramanella arecae* which is a later synonym. An ex-type culture of *P. phoenicicola* does not exist, and therefore the ex-isotype of *Subramanella arecae* will serve as the representative strain for this taxon until revisited. However, identification of this isolate as *Phomopsis phoenicicola* (Santos & Phillips, 2010) is doubtful, therefore not confirmed in this study.

Diaporthe sclerotioides (Kesteren) Udayanga, Crous & K.D. Hyde, comb. nov.

MycoBank 800700

Basionym. *Phomopsis sclerotioides* Kesteren, Neth. Jl Pl. Path. 73: 115 (1967)

Specimen examined: NETHERLANDS, Maarssen, on roots of *Cucumis sativus*, June 1967, H.A. Van der Kesteren (IMI 151828, PD 68/690, holotype), ex-type culture, CBS 296.67.

2.4 Discussion

2.4.1 ITS as a phylogenetic marker in *Diaporthe*

We evaluated the phylogenetic species recognition in *Diaporthe* based on the four utilisable loci individually and in combination to establish robust concept to circumscribe species in the genus. The ITS phylogenetic tree generated here is based on the phylogenetic backbone tree presented in Udayanga et al. (2011) as a rough and quick identification guide for fresh isolates of *Diaporthe* species. Analyses of ITS coupled with morphology, pathogenicity or EF 1-α sequence data have been used in successful taxonomic revisions in contemporary molecular phylogenetic studies (Farr et al., 2002a; Rensburg et al., 2006; Santos & Phillips, 2009; Diogo et al., 2010; Santos et al., 2011; Thompson, Tan, Neate, Aitken & Shivas, 2011). ITS sequences provide persuasive evidence for species delineation with a few distantly related taxa analysed (e.g.; species associated with the diseases of soybean, and sunflower; Jurković, Vrandečić, Ćosić, Riccioni & Duvnjak, 2007; Thompson et al., 2011; Santos et al., 2011), but confusion occurs when large numbers of species from a wide

range of host species are analysed. Typically, branches in phylogenetic trees are bifurcate. Any node that has only two intermediate decedents is said to be resolved. The internal nodes are polytomous (more than two descendents i.e., sister taxa), where relationships are unclear. This can be seen in ITS phylograms of *Diaporthe* when large numbers of taxa are incorporated. A large amount of homoplasy (similarity of sequences among species of different ancestry) across the genus may have resulted in a large number of most parsimonious trees using ITS sequence data (Farr et al., 2002a). These problems can be eliminated in the combined gene analysis we have used in this study. Morphological and culture-based studies have revealed that there may be different species that are not well resolved using only ITS sequence data in preliminary analyses (Farr et al., 2002a; 2002b; Rensburg et al., 2006; Thompson et al., 2011). Different gene genealogies are therefore needed to resolve the cryptic species of *Diaporthe* by either individual or combined gene analysis. In the barcoding initiatives of a wide range of fungi, ITS sequence data can reliably identify 73 % of taxa studied across kingdom Fungi (Schoch et al., 2012). The ITS region has also been used to develop molecular markers, speciesspecific probes and other alternative and comparative assays in the detection of pathogens of *Diaporthe* which is significantly important in rough and quick identification for plant pathogens (Zhang et al., 1997, 1998; Moleleki et al., 2002).

2.4.2 Multiple protein coding genes as phylogenetic markers

An approximately 350 base pair region of the translation elongation factor-1 alpha gene (EF 1-α) has been used, to better resolve the species in *Diaporthe* in several consecutive studies (Castlebury, Farr & Rossman, 2001; Castlebury, 2005; Rensburg et al., 2006; Santos et al., 2010). The species resolved by the EF 1-α gene is congruent with the taxa identified by MAT gene genealogies. We compared individual gene analysis of ITS and EF 1-α, TUB, CAL and combined analysis. Species resolved in the terminal nodes of EF 1-α and combined gene analyses were congruent with a few exceptions. For instance, *Diaporthe pterocarpicola* is not clearly differentiated as a distinct species using EF 1-α analysis. However this was resolved when we combined the ITS, EF 1-α, TUB, CAL genes, where the phylogenetic species, *D. pterocarpicola* is distinguished from *D. phoenicicola*

(Udayanga et al., 2012). The TUB sequences yielded concordant support for the species recognised in EF 1-α phylogenies with higher branch support values in terminal clades (tree not shown). Therefore, the TUB gene could also be used as a phylogenetic marker for *Diaporthe* to assess diversity and to delineate taxonomic units where the confusion occurs in single gene analysis with ITS and EF 1-α gene sequences. When compared to ITS and EF 1-α sequence datasets, the TUB data matrix contains fewer ambiguously aligned regions and less homoplasy across the genus, and should be considered as secondary phylogenetic marker for the genus. The CAL sequences yielded less resolution for some of the cryptic species, although the overall data matrix contains a higher percentage of variable characters. This would be, due to ambiguous alignment in the second intron of the CAL data matrix, thus technically eliminated in all the analyses. For instance *D. viticola* and *D. australafricana* could not be reliably distinguished when employing only CAL genes in the phylogenetic analysis. However, *D. viticola* and *D. australafricana*, two species associated with grapevines in Europe, Africa and Australia are distinct taxa and represent the ex-type sequence data incorporated in the analysis. Therefore care should be taken when interpreting the circumscription of some of the cryptic species of *Diaporthe* based on CAL gene genealogies. However, the CAL gene sequences are also able to resolve the species similarly congruent with EF, and TUB, and are thus recommended in the combined gene analysis. This study confirms that all four gene regions used in this study are useful markers to assess diversity and identify species boundaries and relationships of *Diaporthe*. However, the combined gene analysis provides robust support to delineate cryptic species at the terminal nodes, and to recognise sub-clades of closely related taxa across the genus.

2.4.3 Genealogical concordance phylogenetic species recognition

The multi-locus phylogenetic tree presented here has basically resolved the problems of phylogenetic species in *Diaporthe*. However, we have observed a high correlation in some of the monophyletic sub-clades (closely related taxa), which should be revised in future studies.

The adoption of GCPSR has profound implications for accurate species recognition and resolution of complexes of cryptic taxa (Taylor et al., 2000; Cai et al.,

2011a; 2011b). Cryptic species of common plant pathogenic genera (e.g. *Armillaria*: Coetzee et al., 2005; *Fusarium*: Aoki, O'Donell & Scandiani, 2005, Summerell, Laurence, Liew & Leslie, 2010; Summerell & Leslie 2011; *Calonectria*: Lombard, Crous, B. Wingfield, M. Wingfield, 2010; *Plagiostoma*: Mejia, Castlebury, Rossman, Sogonov & White, 2011; *Phyllosticta*: Glienke et al., 2011; Wikee et al., 2011; Wang et al., 2011; *Cercospora*: Groenewald, Groenewald & Crous, 2005; Crous et al., 2006; *Colletotrichum*: Crouch et al., 2009; Phoulivong et al., 2010; Damm et al., 2012a), and *Diaporthe* (present study) have been resolved using a combined gene analysis. Therefore, GCPSR has become a tool to unravel the cryptic species complexes especially, where there is a dearth of distinguishing morphological characters (Hyde et al., 2010a; Shivas & Cai, 2011). The multi-locus phylogeny of *Diaporthe* would serve as a fundamental tool for consolidating the evolutionary biology, host diversity, biogeographic structure and ecology of this non-modal organism. Therefore future studies are needed based on collections from various geographical locations worldwide as shown in *Diaporthe* and other taxa in *Diaporthales* (Rehner & Uecker, 1994; Zhang et al., 1998; Zhang, 2002; Walker, Castlebury, Rossman, Mejía & White, 2012b; Mejia et al. 2011a; 2011b).

2.5 Conclusion

In the present study, phylogenetic species recognition in *Diaporthe* is re-evaluated using a multi-locus phylogeny based on a combined data matrix of rDNA ITS, and partial sequences from the translation elongation factor 1-α (EF 1-α), β tubulin (TUB) and calmodulin (CAL) molecular markers. DNA sequences of available ex-type cultures have been included, providing a multi-locus backbone tree for future studies on *Diaporthe*. The backbone phylogenetic tree comprising type-derived sequences presented here provides an additional resource for accurate species identification, resolve cryptic species complexes of *Diaporthe* in future studies.

The multi-locus phylogeny and observations of ex-type cultures and specimens has made it possible to reliably connect the sexual and asexual morphs of *Diaporthe* with required changes in nomenclature concerning unified nomenclature

system with one name for a species instead of dual nomenclature for sexual and asexual morphs. More ex-type sequences are needed to analyse increasing its validity, thus aiding identification of species and avoiding misapplication of names.

CHAPTER 3

MULTI-LOCUS PHYLOGENY REVEALS NEW SPECIES OF *Diaporthe* FROM THAILAND

3.1 Introduction

Diaporthe Nitschke comprises important phytopathogens, often with wide host ranges and geographic distributions (Uecker, 1988; Rossman et al., 2007; Crous, 2005; Udayanga et al., 2011; Cowley et al., 2012). Species recognition criteria in *Diaporthe* have historically been based on morphology, cultural characteristics and host affiliation (Rehner & Uecker, 1994; Mostert et al., 2001; Niekerk et al., 2005; Murali et al., 2006; Santos & Phillips, 2009). The current state of taxonomic knowledge of *Diaporthe* effectively means that strains can be identified to species level only if molecular techniques are employed (Santos et al., 2010). Udayanga et al. (2012) provided the first multi-locus phylogenetic tree of *Diaporthe* using combined sequences of ITS, and partial sequences of EF1-α, TUB and CAL genes. In this study, we analyse the sequence data from fresh collections from Thailand. Three new species are described with full descriptions and illustration with the introduction of new combination and epitype designated for *Phomopsis pterocarpi* which is synonymised under *D. pterocarpi*.

3.2 Materials and methods

3.2.1 Fungal isolates and morphological characters

Fungal isolates were obtained as described in section 2.2.1 and the host, collection details and genbank accessions used in this study are listed in Table 2.1. Morphological descriptions are based on sporulating pycnidia on WA+alfalfa stem,

and for the sterile cultures as observed on the original host. Wherever possible, 20-30 measurements (× 40 and × 100 magnifications) were made of structures mounted in 5% KOH, using a compound light microscope (Nikon Elipse 80i). The extremes of measurements are given in parentheses. Three duplicate cultures of each isolate were used for determining colony characters on potato-dextrose agar (PDA, Difco) at 25 ºC in the dark following the methods of Brayford (1990). Colony diameters on PDA and MEA were recorded at intervals of 24 hours for 7 days and used to calculate the growth rate of four replicates per isolate. After 7 days, colony size and colour of the colonies (Rayner, 1970) and zonation were recorded.

3.2.2 Molecular methods and phylogenetic analysis

DNA extraction, gene amplification, sequencing, sequence alignment are as described in section 2.2.2**.** Combined sequences of ITS, and partial sequences of EF1-α, TUB and CAL genes were used in phylogenetic analysis as described in chapter 2.2.3. The sub-set of gene sequences used and the collection details of the isolates used in this study were also included in Table 2.1. We analysed the newly generated sequences with all available type derived sequences listed in Udayanga et al. 2011; 2012. A sub-set of taxa were selected to represent the combined phylogenetic tree presented here (Figure 3. 1).

3.3 Results

3.3.1 Molecular phylogenetic analysis

The combined gene data matrix contains 31 taxa including the outgroup taxon; 126 characters were excluded in parsimony analysis. The statistics for the parsimony analysis revealed that from the remaining 1824 characters 996 characters are constant, 539 characters are parsimony informative while 289 variable characters are parsimony-uninformative. The parsimony analysis of alignment yielded one equally parsimonious trees and which was recognised as the best tree and presented here (Figure 3.1, TL=1721, CI=0.668, RI=0.822, RC=0.549, HI=0.332). The tree generated from Bayesian analysis was identical to the tree generated from parsimony analysis and the Bayesian posterior probabilities were summarised on the same tree.

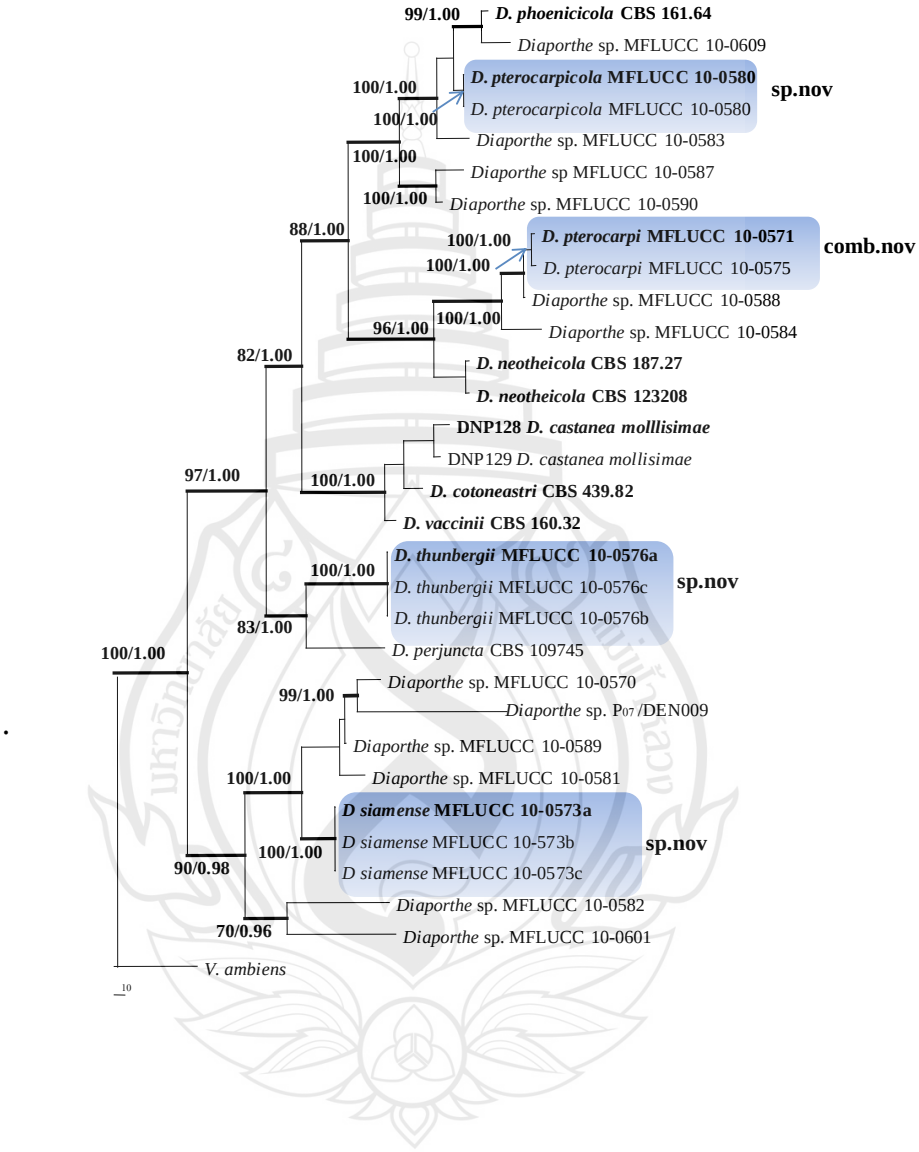


Figure 3.1 The phylogram generated from the parsimony analysis of combined ITS, EF1-α, CAL and TUB loci of *Diaporthe* Ex-type cultures are in bold. The bootstrap support values >70% from 1000 replicates are shown below or above the thickened branches followed by Bayesian posterior probabilities. Novel species and combinations are highlighted. The tree is rooted with *Valsa ambiens***.**

Based on the combined phylogenetic tree, we recognised that all the ex-type derived taxa are placed in terminal clades with higher bootstrap values without any conflict between phylogenetic species delimitation. Three new species were recognised based on phylogenetic inferences coupled with morphological features and are described below. The phylogenetic placement of the *D. pterocarpi* is determined within *Diaporthe* based on the fresh collection. According to the combined phylogenetic tree inferred, *D. pterocarpi* is closely related to *D. neotheicola*. *Diaporthe pterocarpicola* is highly similar to *D. phoeniciola*, referring to the phylogenetic placement. *Diaporthe thunbergii* is closely cluster with *D. perjucta*. A few isolates closely cluster with *D. simense* are not unequivocally identified, not introduced as new species in this study.

3.3.2 Taxonomy

In this section we provide full descriptions and illustrations of three novel species of *Diaporthe* from northern Thailand and a modern description for the new combination *D. pterocarpi* based on the fresh collections. Taxonomic notes are provided with the comparison of the morphology of taxa described and the information retrieved from the standard megablast search for the ITS barcodes.

Diaporthe siamensis Udayanga, X. Z. Liu and K.D. Hyde, sp. nov. Figure 3.2

MycoBank: MB 800826.

Etymology: *Siam*, former name for Thailand where fungus was first collected.

Pycnidia associated with necrotic leaf tissue; stem, up to 70 µm diam, 50 µm high, erumpent, with slightly elongated black necks, mostly submerged in tissue; walls consisting of 2–4 layers of medium dark brown *textura angularis*; yellowish white, spiral conidial cirri exuding from ostioles; walls consisting of 2–4 layers of medium dark brown textura angularis. Conidiophores hyaline, 1–2 septate, branched, densely aggregated, cylindrical-filiform, straight to sinuous, 7–18 × 1.0–1.5 µm. Conidiogenous cells phialidic, cylindrical, terminal and lateral, with slight taper towards apex, 0.5–1 µm diam, with visible periclinal thickening; collarette not seen. Paraphyses present, hyaline, sub-cylindrical, septate, extending above conidiophores, straight, flexuous, branched, up to 20 µm long, 1.0–1.5 µm wide at base. , Alpha conidia aseptate, hyaline, smooth, mono or biguttulate, ellipsoidal–fusiform, base

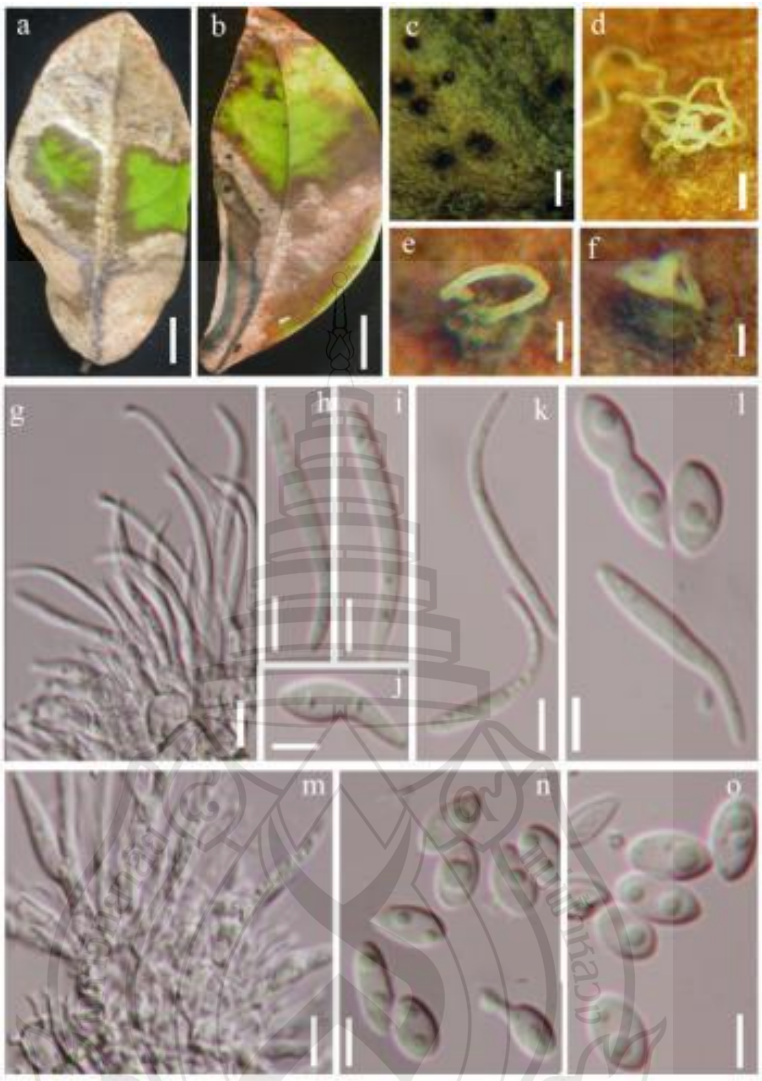
subtruncate, (3.5–)4–5(–6) × (2–)2.5(–3) µm. Gamma conidia aseptate, hyaline, smooth, fusiformfusoid, eguttulate or biguttulate, apex acutely rounded, base subtruncate, 12–13 × 1.5–2 µm. Beta conidia present on the host, conidia aseptate, hyaline, hamate or curved, apex acutely rounded, base truncate, (14–)15–18(–19) × (1.5–)2 µm.

Cultural characteristics: Colonies on PDA and MEA in the dark, 25 °C, after 2 wk, with moderate growth rate (7.8 mm/day), colonies on PDA with white to cream, cottony, smooth, margin lobate, reverse of the culture greenish yellow at the centre due to pigment formation.

Material examined: THAILAND, Chiang Rai Province, Thasud, Muang District, Mae Fah Luang University Park, N 18^{O} 05' 59.1", E 102^{O} 40' 02.9", on leaves of *Dasymaschalon* sp. (Annonaceae), 11 March 2010, D. Udayanga, DPH 004 (MFLU 12-0121 holotype); ex-type cultures MFLUCC 10-0573a,10-573b,10-57c = CBS 135770.

Notes: Three *Diaporthe/Phomopsis* species are known from Annonaceae hosts, but none of them are from *Dasymaschalon*. *Diaporthe siamensis* has paraphyses intermixed among conidiophores and intermediate gamma conidia, which also distinguish it from other species reported from Annonaceae. *Diaporthe annonae* Spegazzini (1906) associated with *Annona cherimolia* in Argentina was described with only a sexual morph (type LPS 2468). However, *D. siamensis* did not produce a teleomorph in culture or on the host. *Phomopsis annonacearum* Bond.-Mont. described from a green-house in Russia on living leaves of *Annona cherimolia*, and *A. squamosa* bears conidiomata (120–140 µm), conidiophores (12–15× 2.5–3 µm) and alpha conidia (5–8 × 2–2.5 µm), while *P. annonae* Urries, described from Spain associated with *A. cherimolia* has conidiomata (300–1000 µm), conidiophores (8–14 × 2 µm) alpha conidia (6.5–9.5 × 1.5–2.5 µm) and beta conidia (20–30 ×1–1.5 µm).

Based on a Megablast search of NCBI's GenBank nucleotide database, the closest matches using the ITS sequence of *D. siamensis* are an endophytic, undescribed *Phomopsis* sp. 122AC/L from a fruit tree in Malaysia (GU066685;



Notes. a–b. Necrotic leaves of *Dasymaschalon* sp.; c. pycnidia on infected areas; d–f. conidia cirri exuding from ostioles; g. conidiophores and beta conidia; h,i,k. beta conidia; j. gamma conidia; l. alpha and beta conidia; m. Paraphyses; n–o. alpha conidia; Bars. a–b= 2 cm, c–f= 1 mm g–o= 5 µm.

Figure 3.2 Morphology of *Diaporthe siamensis*.

Identities = 497/501 (99%), Gaps = 2/501 (0%), and an endophytic *Phomopsis* sp. 45GP/T from *Garcinia parvifolia* from Malaysia (GQ352480; Identities = 485/487 (99%), Gaps = 0/487 (0%)). The association with *Phomopsis/Diaporthe* was confirmed by the EF, CAL and TUB gene sequences. MAT1-2-1 gene is detected in PCR amplification of all three isolates used. A sexual morph was not formed in culture suggesting the fungus is heterothallic or asexual.

Diaporthe thunbergii Udayanga, X.Z. Liu and K.D. Hyde, sp. nov. Figure 3.3

MycoBank: MB800959

Etymology: Named after the host from which it was isolated, *Thunbergia laurifolia* (Acanthaceae).

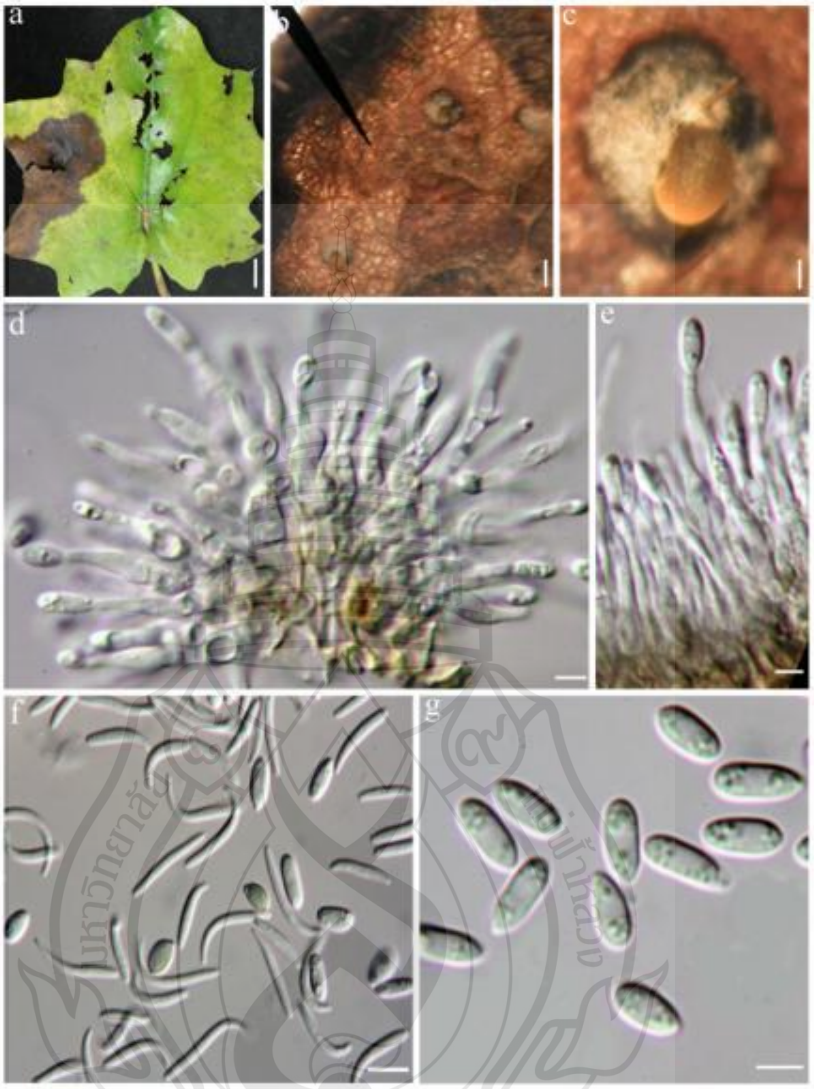
Pycnidia associated with necrotic leaf tissue, globose, up to 90–100 μm diam, erumpent, with slightly elongated black necks, mostly submerged in the host tissue; yellowish translucent conidial droplets exuded from the ostioles; walls consisting of 5–6 layers of medium brown textura globosa-angularis. Conidiophores hyaline, 1–3-septate, unbranched, densely aggregated, subcylindrical, straight to sinuous, 5–20 × 0.8–2.8 μm. Conidiogenous cells phialidic, sub-cylindrical, terminal, slightly tapering towards the apex, 0.5–1 μm long, with visible periclinal thickening; collarette not seen. Paraphyses hyaline, smooth, cylindrical, septate, extending above conidiophores, straight, flexuous, unbranched, or branched below, up to 10–20 μm long, 2–2.5 μm wide at base. Alpha conidia aseptate, hyaline, smooth, multi-guttulate, ovoid-, base sub-truncate, (5–)6–7(–7.5) × (1.5–)2–2.5(–2.6) μm. Beta conidia present on the host, aseptate, hyaline, smooth, straight to hammate, base truncate (14–)15–17(–18) × (1–)1.7 μm. Gamma conidia aseptate, hyaline, smooth, fusiform, multi-guttulate, apex acutely rounded, base subtruncate, 8–11 × 1–2 μm.

Cultural characteristics: colonies on PDA in the dark, 25 °C, after 1 wk, fast growing (11.4 mm/day), on MEA with moderate growth rate (7.1 mm/day), colonies on PDA with abundant dirty white, fluffy aerial mycelium, concentric zonation, margin fimbriate, and in reverse the centre of the culture is greenish yellow colour development due to pigment formation.

Materials examined: THAILAND, Chiang Mai Province, Doi Suthep-Pui National Park, Medicinal Garden, 18°48.62′N 98°54.60′E, on leaves of *Thunbergia*

laurifolia, 7 April 2010, D. Udayanga (DPH 008/MFLU 12-0117, holotype); ex-type cultures MFLUCC 10-0576a.10-576b.10-0576c = CBS 135769.

Notes: *Diaporthe*/*Phomopsis* species have not been previously reported from *Thunbergia* spp. (Acanthaceae). The present species has distinct morphological characters including multi-guttulate, small, bacillus-like alpha conidia on the host and in forming gamma conidia. The conidiophores are also long and tightly aggregated. *Diaporthe thunbergii* sp. nov., was compared with herbarium specimens of two morphologically similar species, *Phomopsis ampelopsidis* Petr., and *P. elaeidis* Punith. and with the ITS sequence analysis of available *Diaporthe*/*Phomopsis* type sequences. The type specimen of *P. ampelopsidis* on stems of *Ampelosis quinquefolia* (PR 7579) was examined. The pycnidia, found on dead stems measured 1000 × 500 µm diam. Alpha conidia were biguttulate and 6–11 × 2–3 µm), but beta and gamma conidia could not be found, nor were they recorded in the original description. *Phomopsis elaeidis* on *Elaeidis guineensis* (K: IMI 172622), had pycnidia up to 2000 µm in diam., conidiophores 8–12 × 3 µm, and beta conidia 18–24 × 0.5–1 µm) . Most anamorphic *Diaporthe* species have overlapping conidial dimensions, therefore, other distinct morphological characters such as paraphyses, conidiophore aggregation, guttulation and presence of gamma conidia, were compared. The new species does not morphologically resemble any previously described species examined here. However, it has a 99 % similarity in standard BLAST search to an unidentified endophyte from *Magnolia liliflora* from the vicinity of the type location in a different study. These endophytes, (MS24 and MS28) from *Magnolia liliflora* (Magnoliaceae) from Doi-Suthep Pui National Park, Thailand (DQ485957; Identities = 488/490 (99%), Gaps = 1/490 (0%)) and DQ485961: Identities = 485/488 (99%), Gaps = 1/488 (0%), respectively). MAT 1-2-1 gene is detected in PCR amplification of all three isolates used. The sexual morph did not form in culture and one mating type was detected in all isolates used, therefore, *D. thunbergii* is presumed to be heterothallic or asexual.



Notes. a. necrotic leaf of *Thunbergia laurifolia* with irregular, brown leaf spot; b–c. conidial droplets exuding from ostioles; d–e. Conidiophores; f–g. alpha, beta and gamma conidia. Bars. a. 2 cm, b. 200 µm c. 20 µm d,e. 5 µm f, g. 8 µm.

Figure 3.3 Morphology of *Diaporthe thunbergii*.

Diaporthe pterocarpicola Udayanga, X.Z. Liu and K.D. Hyde, sp. nov. Figure 3.4

MycoBank: MB 801053

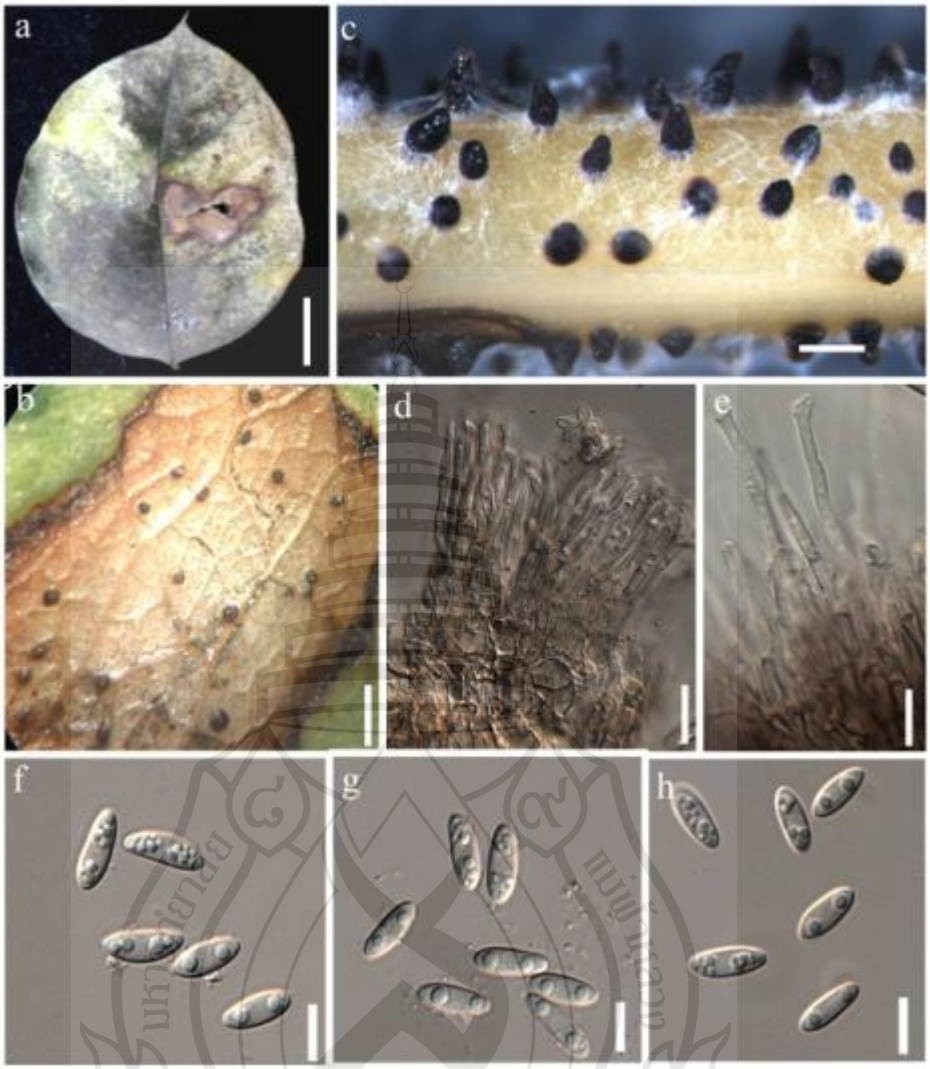
Etymology: Named after the host from which it was isolated, *Pterocarpus indicus* (Fabaceae).

Pycnidia associated with infected leaf tissue; hemi-spherical, up to 120 µm diam, 75 µm high, immersed, with slightly elongated black necks, mostly submerged into tissue; yellowish translucent conidial droplets emerging from ostioles; walls consisting of 3–4 layers of medium dark brown textura angularis. Conidiophores hyaline, multi-guttulate, 1–2-septate, unbranched, densely aggregated, subcylindrical to cylindrical, wide at the base, straight to sinuous, 7–18 µm x × 1.5–3.5 µm, 2.5–3.5 µm wide at the base. Conidiogenous cells phialidic, cylindrical, terminal, slightly tapering towards apex, 1–2 µm diam, with visible periclinal thickening; collarette not clearly observed. Paraphyses occasionally present, hyaline, smooth, cylindrical, septate, extending above the conidiophores, straight, flexuous, unbranched, up to 25 µm long, 1.5–2 µm wide at base. Alpha conidia unicellular, hyaline, multi-guttulate, ellipsoid or clavate, and base subtruncate, (5–)6–7(–8) (2–)2.5(–3.5) µm. Beta or gamma conidia not observed on host or on pine needles in culture.

Cultural characteristics: Colonies on PDA and MEA in the dark, 25 ºC, after 1 wk fast growing (12.1 mm/day), on PDA with abundant white fluffy aerial mycelium, with concentric zonation, margin fimbriate, reverse of the culture slightly greenish yellow with pigment formation.

Material examined: THAILAND, Chiang Rai Province, Thasud, Muang District, Arboratum Chiang Rai, N 18° 05’ 59.1”, E 102° 40’ 02.9”, on leaves of *Pterocarpus indicus*, 14 May 2010, D. Udayanga DPH 013 (MFLU 12-0128, Holotype, ex-type cultures MFLUCC 10-0580a, 10-0580b = CBS 135432).

Notes: *Phomopsis pterocarpi* is known from *Pterocarpus erinaceus*. A modern description for this species is provided (as *Diaporthe pterocarpi*) based on a study of type material (on leaves of *Pterocapus erinaceus* PDD 14878) and a new collection from Thailand. *Diaporthe pterocarpi* has 2-3 guttulate alpha conidia measuring 6–9 ×2.5–3 µm, which is different to *D. pterocarpicola*, which has multi-guttulate conidia and guttulate conidiophores.



Notes. a. Infected leaf of *Pterocarpus indicus*; b. visible pycnidia on leaf spot; c. Pycnidia on alfalfa stem; d,e. Conidiophores; h. alpha conidia. Bars. a.1 cm, b. 500 µm, c. 200 µm d,e. 10 µm, f–h. 6 µm.

Figure 3.4 Morphology of *Diaporthe pterocarpicola*.

Based on a Megablast search of NCBI's GenBank nucleotide database, the closest sequence to *D. pterocarpicola* is that of *Phomopsis* sp. 40GP/S an endophyte from *Garcinia parvifolia* from Malaysia (GQ352478; Identities= 485/486 (99%),

Gaps = 0/486 (0%) and endophytic fungus *Phomopsis* sp. from *Garcinia* sp. from Malaysia 89CN/F. (GU066658; Identities = 494/499 (99%), Gaps = 4/499 (1%). MAT1-2-1 gene is detected in PCR amplification of all three isolates used. The sexual morph did not form in culture and one mating type was detected in all isolates used. Therefore, this species is also heterothallic or asexual.

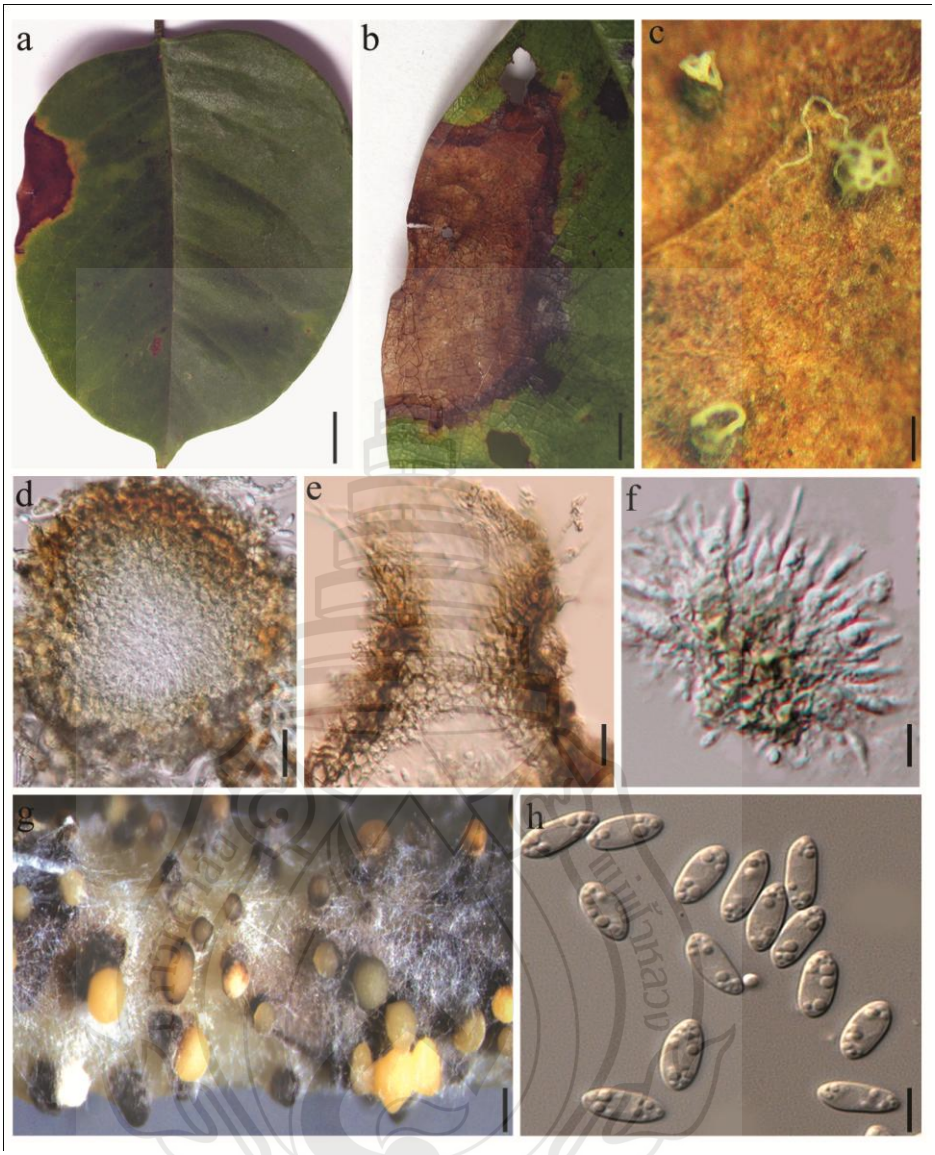
Diaporthe pterocarpi (Hughes) Udayanga, X.Z. Liu & K.D. Hyde, comb. nov. Figure 3.5

Basionym. *Phomopsis pterocarpi* S. Hughes, Mycol. Pap. 50:54 (1953).

MycoBank: MB 801055

Pycnidia associated with infected leaf tissue; globose 100-120 µm diam, later conical, up to 100 µm diam, 65-100 high, somewhat erumpent, with a slightly elongated black neck, mostly submerged in tissue; dirty white, spiral conidial masses (cirri) extruding from ostioles; walls consisting of 3–4 layers of medium brown textura globosa-angularis. Conidiophores hyaline, smooth, aseptate, unbranched, ampulliform, straight to sinuous, 10–15 × 1–2 µm. Conidiogenous cells phialidic, cylindrical, terminal, with slight taper towards apex, 0.5–1 µm diam; collarettes not seenobserved., Paraphyses absent. Alpha conidia aseptate, hyaline, smooth, fusiform, biguttulate, rarely 3-guttulate and base subtruncate, (5–)6–7(–9) × (2–)2.5(–3) µm. Gamma and beta conidia not observed on the host or in culture.

Cultural characteristics: Colonies on PDA and MEA in the dark, 25 ºC, after 1 wk slow growing (4.2 mm/day)., on PDA with white, fluffy aerial mycelium, underside greenish yellow pigmentation developing in the centre.



Notes. a. Infected leaf of *Pterocarpus indicus*; b. necrotic lesion on the leaf; c. Cirri exuding from ostioles; d. section of pycnidia in early stage (immature); e. section through matured pynidia with ostiole; f. Conidiophores; g. sporulation on alfalfa stem on WA; h. alpha conidia. Bars. a. 1cm, b. 0.5 cm, c. 200 µm, d. 20 µm, e. 50 µm, f. 5 µm, g. 6 µm.

Figure 3.5 Morphology of *Diaporthe pterocarpi*.

Material examined: TOGOLAND (Togo, West Africa), Kete Krachi, on leaves of *Pterocarpus erinaceus* (PDD 14878, isotype), THAILAND, Chiang Rai Province, Mae Fah Luang University Garden, leaves of *Pterocarpus indicus*, 12 April 2010, D. Udayanga DPH 002 (MFLU 12-0120, Epitype designated here, ex-epitype culture MFLUCC 10-0572, 10-0575 = CBS 137021).

Known host and distribution: *Aloe vera*, *Jatropha curcas*, *Ougeinia dalbergioides*, *Pterocarpus santalinoides*, *P. angolensis*, *P. erinaceus*, *P. indicus* from Ghana, Hong Kong, India, Sierra Leone, Thailand, Togo, Zambia.

Notes: Based on a megablast search of NCBI's GenBank nucleotide database for ITS sequence, the closest for *D. pterocarpi* are an endophytic *Phomopsis* sp. CML 1936 from *Phoradendron perrottetii* from Brazil (JN153068; Identities = 473/478 (99%), Gaps = 0/478 (0%)) and an endophyte P1808A from unknown tropical plant from Peru (EU977317; Identities = 479/490 (98%), Gaps = 8/490 (2%)). MAT1-2-1 gene is detected in PCR amplification of all three isolates used. The sexual morph did not form in culture and one mating type was detected in all isolates used, therefore, the taxon is heterothallic or asexual.

3.4 Discussion

Udayanga et al. (2012) showed that all four gene regions (ITS, EF, TUB, CAL) used in this study are useful markers to infer phylogenetic relationships and identify species of *Diaporthe*. The combined analysis of these four loci gives more robust support to define the species limits at the tips of branches in phylogeny. Recognition of new species, mainly from woody plants as pathogens and endophytes based on molecular data, suggests that many more species of *Diaporthe* remain to be discovered. A similar situation is occurring in other important plant pathogenic genera such as *Bipolaris*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Phyllosticta* and *Pestalotiopsis* (Manamgoda et al., 2011; Summerell et al., 2010, Summerell & Leslie, 2011; Wikee et al., 2011a; 2011b; Maharachchikumbura, Guo, Chukeatirote, Bahkali & Hyde, 2011).

The species described here were discovered during study of a wide range of hosts including mostly tropical woody and herbaceous plants in wild and ornamental plants in common in the tropics. The new species introduced herein, were originally identified as pathogens or secondary invaders on particular hosts, but may exhibit wide host ranges, geographic distribution and switching of life modes (e.g. to endophytes or saprobes, Promputtha et al., 2007, Dai et al., 2010). Therefore, the host diversity and geographic distributions should be re-assessed and updated in future studies with the availability of more isolates and better defined taxa. With a large number of unidentified endophytic *Diaporthe/ Phomopsis* sequences in GenBank (Murali et al., 2006, Abreu et al., 2012), there is a need to identify more species with wide range of hosts sharing variable ecological niches. Based on the current knowledge of *Diaporthe* it is challenging to identify a strain isolated from a host for which a species has not previously been described. This is because both host-specific as well as non-host specific generalist species reported from one host could represent more than one taxa (Udayanga et al., 2011; Santos et al., 2011, Thompson et al., 2011, Crous et al., 2011, 2012). However, the multi-locus backbone phylogenetic tree in Udayanga et al. (2012) provides an alternative approach to identify and describe different species occurring on one particular host as well as cryptic taxa found on wide range of hosts.

3.6 Conclusion

In the present study multi-locus phylogeny based on combined sequences of ITS, and partial sequences from the EF1-α, TUB and CAL genes, revealed three new species from fresh collections made in northern Thailand. The new species *Diaporthe siamensis*, *D. thunbergii* and *D. pterocarpicola* are introduced in this paper with full descriptions and comparison with similar taxa. *Phomopsis pterocarpi* which is epitypified and synonymised under *Diaporthe pterocarpi* was described based on a collection from northern Thailand.

CHAPTER 4

SPECIES LIMITS IN *Diaporthe* I: MOLECULAR RE-ASSESSMENT OF CITRUS PATHOGENS

4.1 Introduction

The genus *Diaporthe* is an economically important group of plant pathogenic fungi causing diseases on a wide range of crops, ornamentals and forest trees (Farr et al., 2002a; 2002b; Crous, 2005; Udayanga et al., 2011). Accurate species identification is vital for controlling the diseases caused by these fungi as well as for implementing quarantine regulations (Rossman & Palm-Hernández, 2008; Cai et al., 2011; Shivas & Cai, 2012). Until recently, species of *Diaporthe* have been defined based on morphology and host association. However, patterns of host association and speciation have yet to be fully understood within *Diaporthe*. Multiple species of *Diaporthe* can often be found on a single host and a single species of *Diaporthe* can be associated with many different hosts (Crous, 2005; Niekerk et al., 2005; Santos & Phillips, 2009; Diogo et al., 2010; Gomes et al., 2013). Using molecular data, much progress has been made towards identifying and characterising emerging pathogens, prevalent endophytes and saprobes in the genus *Diaporthe* (Santos & Phillips 2009; Diogo et al., 2010; Luongo, Santori, Riccioni, & Belisario, 2011; Udayanga et al., 2012a; Udayanga, Liu, Mckenzie, Chukeatirote & Hyde, 2012b; Thomidis, Exadaktylou & Chen, 2013).

Modern systematic accounts of *Diaporthe* have used DNA sequence data as the most accurate means to circumscribe species within this genus (Rehner & Uecker, 1994; Castlebury et al., 2003; Rensburg et al., 2006). Markers used in contemporary phylogenetic revisions include the complete nuclear ribosomal internal transcribed spacer regions (ITS) and more recently partial sequences of actin (ACT), beta-tubulin

(TUB), calmodulin (CAL), histone H3 (HIS), mating type genes (MAT 1-1-1 and MAT 1-2-1) and translation elongation factor 1-alpha (EF1-α) (Niekerk et al., 2005; Diogo et al., 2010; Santos et al., 2010; Udayanga et al., 2012a; 2012b; Gomes et al., 2013). Multi-gene phylogenetic species delineation has become the most effective tool for taxonomic studies of fungi compared to traditional mating experiments and morphology (Taylor et al., 2000; Dettman et al., 2003). Although the ITS region is often useful for identification of *Diaporthe* species, multi-gene phylogenetic analyses are required for accurate reconstruction of species boundaries and relationships (Udayanga et al., 2012a; Gomes et al., 2013). Intraspecific variation observed in ITS sequences in several species of *Diaporthe* can cause confusion in species recognition when used alone (Farr et al., 2002a; 2002b; Santos et al., 2010).

Diaporthe citri is a pathogen that causes melanose and stem end rot disease of *Citrus* spp. throughout the world (Whiteside & Timmer, 2000a; Mondal, Vincent, Reis & Timmer, 2007). Melanose disease can affect young leaves and fruits of different species and varieties of *Citrus* causing black blemishes on fruit rind and small, black, raised lesions often surrounded by yellow necrotic halos (Timmer & Kucharek, 2001). Symptoms of the disease may vary with host variety, geographic location, seasonal occurrence, ecophysiological factors and severity of infection (Timmer & Fucik, 1976; Whiteside, 1977; Kucharek, Whiteside & Brown, 1983). The range of symptoms varies from small spots, scab lesions and mudcake to star melanose on different tissues of *Citrus* spp. (Timmer, 2000; Whiteside & Timmer, 2000a; Agostini, Bushong, Bhatia & Timmer, 2003). Perithecia and pycnidia are only produced on dead and dying twigs and on fruit affected by stem end rot. Because perithecia are rarely formed, conidia produced by pycnidia are the prime source of inoculum (Bach & Wolf, 1928; Kuhara, 1999).

Although the biology and epidemiology of melanose are well studied, the phylogenetic relationships of the causal organisms, genetic variability and population structure have not been investigated (Burnett, 1962; Moherek, 1970; Mondal, Agostini, Zhang & Timmer, 2004; Mondal et al., 2007). *Diaporthe* pathogens of *Citrus* are usually identified as *Diaporthe citri* in taxonomic and plant pathological studies and regional checklists (Timmer & Kucharek, 2001; Udayanga et al., 2011). In addition to *Diaporthe citri*, several other species of *Diaporthe* have been reported

from *Citrus*, often as *Phomopsis.* These include *Diaporthe citrincola* described from the Philippines, *Phomopsis californica* from California, *P. caribaea* from Cuba and *P. cytosporella* (as *Phoma cytosporella*) from Italy, which have all previously been considered synonyms of *Diaporthe citri* (Rehm, 1914; Fawcett, 1922; 1936; Horne, 1922). Yamato (1976) recognised four unidentified morphological species on *Citrus* spp. in Japan. *Diaporthe citri* was also considered a synonym of *D. medusaea* by Wehmeyer (1933) who also listed *D. citrincola*, *P. citri* and *P. californica* as host or ecological forms of *D. medusaea*. Others followed this synonymy including Punithalingam and Holiday (1973) and Whiteside and Timmer (2000a). The name *D. medusaea* is used in several articles and checklists for the fungus causing melanose and stem end rot, therefore, the true host range and geographic distribution of *D. citri* are difficult to determine (Kobayashi, 1970**;** Pantidou, 1973; French, 1987)**.**

Given the vague species concept of *D. citri* and its broad application, a modern taxonomic and phylogenetic reappraisal of *D. citri* and other *Diaporthe* species on *Citrus* is necessary. In this study, we analyse DNA sequence data from recent collections of *Diaporthe* isolated from *Citrus* and other hosts in Asia, Europe and the United States to accurately identify the taxa associated with *Citrus*. The objectives of this study are: 1) to define the species of *Diaporthe* on *Citrus* worldwide based on phylogenetic analysis of multi-gene sequence data, the genealogical sorting index and morphological characters; 2) to resolve taxonomic and nomenclatural uncertainty by providing modern descriptions for *Diaporthe citri* and designating epitypes for *D. cytosporella*, *D. foeniculina* and *D. rudis* and their synonyms; 3) to evaluate their host range and geographic distribution; and 4) to assess the utility of individual genes for accurate circumscription of these species.

4.2 Materials and methods

4.2.1 Isolates and morphology

Strains of *Diaporthe* from *Citrus* hosts were obtained from China, Korea, New Zealand, Spain and the United States (California, Florida and Texas). These strains were isolated from specimens with typical symptoms of *Citrus* melanose and stem

end rot as well as saprobes on twigs and branches. Isolates from other hosts were obtained from culture collections including CBS (Netherlands), Fawcett Laboratory, University of California, Riverside (CA, USA), ICMP (New Zealand), MFLUCC (Thailand) and the SMML, USDA-ARS (MD, USA) and various contributors listed in Table 4.1. Morphological descriptions were based on sporulating pycnidia from inoculated alfalfa stems placed on 1.5 % water agar (WA) for living cultures as well as type and other specimens. Digital images of fruiting bodies were captured using a Discovery v20 stereomicroscope and AxioCam digital camera (Carl Zeiss Microscopy, Thornwood, NY, USA) imaging system. Whenever possible, 20–30 measurements were made of the structures mounted in 5 % KOH using a Carl Zeiss Axioplan2 compound light microscope using the 40× or 100× objectives. The extreme measurements are given in parentheses with mean and standard deviation. Three sets of duplicate cultures of each isolate were used for determining colony characters on potato-dextrose agar (PDA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 25 ºC in the dark following the methods of Brayford (1990). Colony diameters on PDA were recorded at intervals of 24 h for 1 wk and used to calculate the growth rate of eight replicates per isolate. After 1 wk, colony size and colour of the colonies (Rayner, 1970) and zonation were recorded.

4.2.2 DNA extraction, PCR and sequencing

Mycelial scrapings (50–60 mg) from the leading edge of cultures on PDA, incubated for 4–5 d at 25 ˚C were harvested and lysed in tubes containing 500 µm garnet media and a 6 mm zirconium bead (OPS Diagnostics, Lebanon, NJ, USA) with the Fast Prep FP120 (Fischer Scientific Inc, Waltham, MA, USA) for 20 s. Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. The ACT, CAL, EF1-α, ITS and TUB gene regions were amplified following the conditions outlined in Table 4.2.

Table 4.1 Isolates used and genes sequenced in the study.

Species	Isolate No.	Host	Origin	Collector/contributor	GenBank accession no.				
					ITS	EF 1-α	TUB	ACT	CAL
D. alleghaniensis	CBS 495.72*	*Betula alleghaniensis*	Canada: Ontario	R. Arnold	FJ889444	GQ250298	KC843228	JQ807299	KC343249
D. australafricana	CBS 113487*	*Vitis vinifera*	South Africa	L. Mostert	KC343039	KC843099	JX275457	KC843265	JX197448
	CBS 111886	*Vitis vinifera*	Australia	R.W.A. Schepers	KC343038	KC343764	KC344006	-	KC343280
	AR5209 = CBS 135771	*Persea americana*	USA: California	Akif Eskalen	KF199875	KF199877	KF199879	KF199883	KF199881
	AR5210 = CBS 135772	*Persea americana*	USA: California	Akif Eskalen	KF199876	KF199878	KF199880	KF199884	KF199882
D. canthii	CBS 132533*	*Canthium inerme*	South Africa	P.W. Crous	JX069864	KC843120	KC843230	KC843291	KC843174
D. citri	AR3405* = CBS 135422	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843311	KC843071	KC843187	KC843234	KC843157
	AR3404	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843316	KC843076	KC843192	KC843239	KC843162
	AR3406	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843320	KC843080	KC843196	KC843243	KC843166
	AR4469 = CBS 135423	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843321	KC843081	KC843197	KC843244	KC843167
	AR4470	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843318	KC843078	KC843194	KC843241	KC843164
	AR4471	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843317	KC843077	KC843193	KC843240	KC843163
	FAU583 = CBS 135424	*Citrus paradisi*	USA: Florida	F.A. Uecker	KC843327	KC843087	KC843203	KC843250	KC843173
	AR3403	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843310	KC843070	KC843186	KC843233	KC843156
	AR4473	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843319	KC843079	KC843195	KC843242	KC843165
	AR3407	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843313	KC843073	KC843189	KC843236	KC843159
	AR4472	*Citrus* sp.	USA: Florida	L.W. Timmer	KC843312	KC843072	KC843188	KC843235	KC843158
	AR4364 = CBS 135425	*Citrus* unshiu cv. juwadeun	Korea: Odeung-dong	S.K. Hong	KC843326	KC843086	KC843202	KC843249	KC843172

Table 4.1 (continued)

Species	Isolate No.	Host	Origin	Collector/contributor	GenBank accession no.				
					ITS	EF 1-α	TUB	ACT	CAL
D. citri	AR4370 = CBS 135426	*Citrus* unshiu cv. juwadeun	Korea: Odeung-dong	S.K. Hong	KC843324	KC843084	KC843200	KC843247	KC843170
	AR4350	*Citrus* unshiu cv. juwadeun	Korea: Odeung-dong	S.K. Hong	KC843325	KC843085	KC843201	KC843248	KC843171
	CT003 = CBS 135767	*Citrus reticulata*	China	D. Udayanga	KC843322	KC843082	KC843198	KC843245	KC843168
	DA103 = CBS 135427	*Citrus reticulata*	China	D. Udayanga	KC843323	KC843083	KC843199	KC843246	KC843169
	ICMP 10355	*Citrus reticulata*	New Zealand: Kerikeri	G.J. Samuels	KC843314	KC843074	KC843190	KC843237	KC843160
	ICMP 6981	*Citrus* sp.	USA: Texas	G.J. Samuels	KC843315	KC843075	KC843191	KC843238	KC843161
D. cotoneastri	CBS 439.82*	*Cotoneaster* sp.	UK: Scotland	H. Butin	FJ889450	GQ250341	JX275437	KC843231	JX197429
	DP0667 = CBS 135428	*Juglans cinerea*	USA: North Carolina	S. Anagnostakis	KC843328	KC843121	KC843229	KC843232	KC843155
D. cynaroidis	CBS 122676*	*Protea cynaroidis*	South Africa	P.W. Crous	EU552122	EU552093	KC344026	-	KC343300
D. cytosporella	FAU461 =CBS 137020*	*Citrus limon*	Spain	M. Palm	KC843307	KC843116	KC843221	KC843285	KC843141
	AR5148	*Citrus sinensis*	USA: California	A. Eskalen	KC843308	KC843117	KC843222	KC843286	KC843142
	AR5149*	*Citrus sinensis*	USA: California	A. Eskalen	KC843309	KC843118	KC843222	KC843287	KC843143
D. foeniculina	FAU460	*Citrus limon*	Spain	M. Palm	KC843304	KC843113	KC843218	KC843282	KC843138
	MEP1289-1	*Citrus limon*	Spain	D. Grenier	KC843305	KC843114	KC843219	KC843283	KC843139
	FAU462 = CBS 135429	*Citrus limon*		M. Palm	KC843292	KC843101	KC843206	KC843270	KC843126

Table 4.1 (continued)

Species	Isolate No.	Host	Origin	Collector/contributor	GenBank accession no.				
					ITS	EF 1-α	TUB	ACT	CAL
D. foeniculina	ICMP 6986	*Citrus limon*	New Zealand: Hope	-	KC145897	KC145989	-	-	-
	ICMP 6970	*Acacia* sp.	New Zealand: Auckland	G.J. Samuels	KC145896	KC145984	-	-	-
	ICMP 12285	*Juglans regia*	New Zealand	K. Knight	KC145853	KC145937	-	-	-
	ICMP 6987	*Malus domestica*	New Zealand: Nelson	G.J. Samuels	KC145894	KC145990	-	-	-
	ICMP 17058	*Paraserianthes lophantha*	New Zealand: Auckland	C.F. Hill	KC145842	KC145977	-	-	-
	ICMP 11892	*Fuchsia excorticata*	New Zealand: Taupo	J.M. Young	KC145898	KC145931	-	-	-
	DP0454	*Ribes nigrum*	New Zealand: Nelson	C.F. Hill	KC843297	KC843106	KC843211	KC843275	KC843131
	AR3607= STE-U2654	*Vitis vinifera*	South Africa	L. Mostert	AF230743	JQ807419	KC843204	JQ807344	KC843123
	DP0392 = CBS 111554	*Foeniculum vulgare*	Portugal: Marcos	A.J.L. Phillips	KC843296	KC843105	KC843210	KC843274	KC843130
	DP0391 = CBS 111553*	*Foeniculum vulgare*	Portugal: Marcos	A.J.L. Phillips	KC843295	KC843104	KC843209	KC843273	KC843129
	AR5151	*Citrus latifolia*	USA: California	A. Eskalen	KC843303	KC843112	KC843217	KC843281	KC843137
	AR5142 = CBS 135430	*Citrus limon*	USA: California	A. Eskalen	KC843301	KC843110	KC843215	KC843279	KC843135
	AR5145	*Citrus limon*	USA: California	A. Eskalen	KC843306	KC843115	KC843220	KC843284	KC843140
	AR5147	*Citrus limon*	USA: California	A. Eskalen	KC843299	KC843108	KC843213	KC843277	KC843133
	AR5144	*Citrus limon*	USA: California	A. Eskalen	KC843302	KC843111	KC843216	KC843280	KC843136

Table 4.1 (continued)

Species	Isolate No.	Host	Origin	Collector/contributor	GenBank accession no.				
					ITS	EF 1-α	TUB	ACT	CAL
	AR5143	*Citrus limon*	USA: California	A. Eskalen	KC843294	KC843103	KC843208	KC843294	KC843128
	AR5146	*Citrus limon*	USA: California	A. Eskalen	KC843298	KC843107	KC843212	KC843298	KC843132
	AR5150 = CBS 135431	*Citrus latifolia*	USA: California	A. Eskalen	KC843293	KC843102	KC843207	KC843293	KC843127
	AR5152	*Citrus latifolia*	USA: California	A. Eskalen	KC843300	KC843109	KC843214	KC843300	KC843134
D. foeniculina (syn. *P. theicola*)	CBS187.27*	*Camellia sinesis*	Italy	M. Curzi	DQ286287	DQ286261	JX275463	JQ807298	KC843122
D. foeniculina (syn. *D. neotheicola*)	CBS 123208*	*Foeniculum valgare*	Portugal	A.J.L. Phillips	EU814480	GQ250315	JX275464	KC843269	KC843125
D. foeniculina (syn. *D. rhusicola*)	CBS 129528*	*Rhus pendulina*	South Africa	P.W. Crous	JF951146	KC843100	KC843205	KC843268	KC843124
Diaporthe helianthi	CBS 592.81	*Helianthus annuus*	Serbia	M Muntanola - Cvetkovic	AY705842	GQ250308	JX275465	KF199885	JX197454
D. pterocarpi	MFLUCC10-588	*Magnolia sp.*	Thailand: Chiang Rai	D. Udayanga	JQ619900	JX275417	JX275461	KC843289	JX197452
	MFLUCC10-575	*Pterocarpus indicus*	Thailand: Chiang Rai	N.F. Wulandari	JQ619901	JX275418	JX275462	KC843288	JX197453
	MFLUCC10-571* = CBS 135768	*Pterocarpus indicus*	Thailand: Chiang Rai	D. Udayanga	JQ619899	JX275416	JX275460	KC843290	JX197451
D. pterocarpicola	MFLUCC10-580a* = CBS 135432	*Pterocarpus indicus*	Thailand: Chiang Rai	D. Udayanga	JQ619887	JX275403	JX275441	KF214779	JX197433

Table 4.1 (continued)

Species	Isolate No.	Host	Origin	Collector/contributor	GenBank accession no.				
					ITS	EF 1-α	TUB	ACT	CAL
D. pterocarpicola	MFLUCC10-580b	*Pterocarpus indicus*	Thailand: Chiang Rai	N.F. Wulandari	JQ619889	JX275405	JX275443	KF214780	JX197435
D. rudis	AR3654 = CBS 135433	*Rosa canina*	Austria	W. Jaklitsch	KC843338	KC843097	KC843184	KC843262	KC843153
	DP0423	*Pyrus sp.*	New Zealand	W. Kandula	KC843335	KC843094	KC843181	KC843258	KC843150
	DP0350 = CBS 135434	*Castanea sp.*	New Zealand: Churchill	H. Smith	KC843338	KC843098	KC843185	KC843264	KC843154
	ICMP 12522	*Ileostylis micranthus*	New Zealand: Central Otago	P.R. Johnston	KC145906	KC145940	-	-	-
	ICMP 15267	*Alnus* sp.	New Zealand: Mid Canterbury	K. Eade	KC145839	KC145998	-	-	-
	ICMP 16419	*Castanea sativa*	New Zealand: Mid Canterbury	H.C. Smith	KC145904	KC145976	-	-	-
	ICMP 7025	*Vaccinium corymbosum*	New Zealand: Waikato	P.R. Johnston	KC145885	KC145995	-	-	-
	AR3422 = CBS 109292*	*Laburnum anagyroides*	Austria	W. Jaklitsch	KC843331	KC843090	KC843177	KC843254	KC843146
	AR3646	*Epilobium angustifolium*	Canada: British Columbia	M. Barr	KC843330	KC843089	KC843176	KC843253	KC843145
	AR3478 = CBS 109768	*Epilobium angustifolium*	Canada: British Columbia	M. Barr	KC843329	KC843088	KC843175	KC843252	KC843144

Table 4.1 (continued)

Species	Isolate No.	Host	Origin	Collector/contributor	GenBank accession no.				
					ITS	EF 1-α	TUB	ACT	CAL
	DA243 = CBS 135435	*Brugmansia* sp.	Germany	R. Schumacher	KC843332	KC843091	KC843178	KC843255	KC843147
	DA244	*Brugmansia* sp.	Germany	R. Schumacher	KC843334	KC843093	KC843180	KC843257	KC843149
	ER285A = CBS 135437	*Acer opalus*	Italy	E. Camporesi	KC843336	KC843095	KC843182	KC843259	KC843151
	ER286C	*Acer opalus*	Italy	E. Camporesi	KC843337	KC843096	KC843183	KC843260	KC843152
	ER286D	*Acer opalus*	Italy	E. Camporesi	KC843333	KC843092	KC843179	KC843256	KC843148
	DPG01	*Vitis viniferra*	Italy	X.Z. Liu	JQ619896	JX275412	JX27545	KC843261	JX197446
	DPG02	*Vitis viniferra*	Italy	X.Z. Liu	JQ619897	JX275413	JX275456	KC843263	JX197447
D. rudis (syn. *D. viticola*)	CBS 113201*	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY485750	GQ250327	JX275454	KC843251	JX197445
D. thunbergii	MFLUCC10-576a* = CBS 135769	*Thunbergia laurifolia*	Thailand: Chiang Mai	D.S. Manamgoda	JQ619893	JX275409	JX275449	KF199886	JX197440
	MFLUCC10-576b	*Thunbergia laurifolia*	Thailand: Chiang Mai	S.C. Karunaratne	JQ619894	JX275410	JX275450	KF199887	JX197441
	MFLUCC10-576c	*Thunbergia laurifolia*	Thailand: Chiang Mai	D. Udayanga	JQ619895	JX275411	JX275451	KF199888	JX197442
D. vaccinii	CBS 160.32*	*Vaccinium macrocarpon* (as *Oxycoccus macrocarpos*)	USA: Massachusetts	C. Shear	AF317578	GQ250326	JX275436	JQ807297	KC343470
	FAU446 =CBS122113	*Vaccinium macrocarpon*	USA: Massachusetts	F. Caruso	U11317,U11367	JQ807398	KC843224	JQ807322	KC849455
	DF5032 = CBS 135436	*Vaccinium corymbosum*	USA: North Carolina	D. Farr	AF317570	JQ807380	KC843225	JQ807303	KC849456
	FAU633	*Vaccinium* sp.	USA: Michigan	-	U11360,U11414	JQ807413	KC843226	JQ807338	KC849457

Table 4.1 (continued)

Species	Isolate No.	Host	Origin	Collector/contributor	GenBank accession no.				
					ITS	EF 1-α	TUB	ACT	CAL
D. vaccinii	FAU468	*Vaccinium macrocarpon*	USA: New Jersey	-	U113327,U11377	JQ807399	KC843227	JQ807323	KC849458

Notes. AR, D, DA, FAU,ER, DLR, DF, DP, DPG: isolates in SMML culture collection, USDA-ARS, Beltsville, MD, USA; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; ICMP: International Collection of Microorganisms from Plants, Landcare Research, New Zealand; MFLUCC: Mae Fah Luang University Culture Collection; ICMP: Landcare Research, New Zealand; MFLUCC: Mae Fah Luang University Culture Collection; *= ex-type cultures.

Table 4.2 New and available primers, PCR protocols and references

Locus	Primers	Optimised PCR protocols	Approximate sizes of the PCR amplicons obtained	References for primers[a] & protocols[b]
ACT	ACT-512F: ATGTGCAAGGCCGGTTTCGC ACT-783R (5' TACGAGTCCTTCTGGCCCAT-3') ACT878R (new): ATCTTCTCC ATGTCGTCCCAG	(95 °C : 30 s, 55 °C:50 s,72 °C:1 min) ×39 cycles for ACT512F/ACT783R (95 °C : 30 s, 58 °C:50 s, 72 °C:1 min) ×39 cycles for ACT512F/ACT878R	280bp (ACT512F/ACT783R) 380bp (ACT512F/ACT878R)	Carbone & Kohn (1999)[a] This study[a,b]
TUB	Bt-2a: GGTAACCAAATCGGT GCTGCTTTC BT-2b: ACCCTCAGTGTAGTG ACCCTTGGC	(95 °C : 30 s, 58 °C:50 s, 72 °C:1 min) ×39 cycles	500bp	Glass & Donaldson (1995)[a] Udayanga et al., (2012a)[b]
CAL	CAL-228F: GAGTTCAAGGAGGCCTTCTCCC CAL-737R: CATCTTCTGGCCAT CATGG CL1F: GARTWCAAGGAGGCCTTCTC CL2A : TTTTTGCATCATGAGTTGGAC CAL563F (new): GACAAATCA CCACCAARGAGC	(95 °C : 30 s, 55 °C:50 s,72 °C:1 min) ×39 cycles for CAL228F/CAL737R (95 °C : 30 s, 52 °C:50 s,72 °C:1 min) ×39 cycles for CL1/CL2A (95 °C : 30 s, 51 °C:50 s,72 °C:1 min) ×39 cycles for CAL563F/CL2A	500bp (CAL228F.CAL737R) 800bp (CL1/CL2A) 570bp (CAL563F/CL2A)	Carbone & Kohn (1999)[a] O'Donnell et al., 2000[a]; Udayanga et al., (2012a)[b] This study[a,b]
EF1-α	EF1-728F: CATCGAGAAGTTCGAGAAGG EF1-986R: TACTTGAAGGAACCC TTACC	(95 °C : 30 s, 58 °C:50 s, 72 °C:1 min) ×39 cycles	350bp	Carbone & Kohn, (1999)[a] Udayanga et al., (2012a)[b]
ITS	ITS1: TCCGTAGGTGAACCTGCGG ITS4: TCCTCCGCTTATTGATATGC	(95 °C : 30 s, 55 °C:50 s, 72 °C:1 min) ×39 cycles	600bp	White et al., (1990)[a] Udayanga et al., (2012a)[b]

The reaction were performed on a Dyad Peltier thermal cycler (Bio Rad) in a 25 µL reaction volume: 10–15 ng genomic DNA, 12.5 µL Quick load Taq 2x Master Mix (New England BioLabs, Ipswich, MA, USA), 1 µL 10 mM of each primer and 1 % DMSO with volumes adjusted to 25 µL with nuclease-free water. PCR products were visualised by electrophoresis in 1 % agarose gels stained with SYBR Safe DNA Gel Stain (Invitrogen, Eugene, OR, USA).

Excess primers and dNTPs were removed from PCR amplification mixtures with ExoSAP-IT (USB Corp., Cleveland, OH, USA) according to the manufacturer's instructions. Amplicons were sequenced using the BigDye Terminator v. 3.1 cycle sequencing kit (Life Technologies, Grand Island, NY, USA) on an Applied Biosystems 3130xl Genetic Analyzer using the same primers used to amplify each of the gene regions.

4.2.3 New primer design and PCR optimisation

Complete failure of amplification of the isolates in the *Diaporthe foeniculina* clade and evidence of non-specific priming in the sequences of the CAL gene region was observed when using the CAL-228F/CAL-737R (Carbone & Kohn, 1999) or CL1/CL2A (O'Donnell, Kistler, Tacke & Casper, 2000) primer sets. Additionally frequent failures of sequencing when using ACT-512F/ACT-783R (Carbone & Kohn, 1990) were encountered in this clade. On closer inspection of ACT and CAL multiple sequence alignments for *Diaporthe*, non-specific binding sites were observed for both ACT-783R and CAL-228F primers (Carbone & Kohn, 1990).

A sequence alignment consisting of both complete and partial sequences of CAL from *Neurospora crassa* (L02964), *Pyricularia grisea* (AF089808), *Apiognomonia errabunda* (DQ313615, DQ313596), *Ophiognomonia clavigignenti-juglandacearum* (GU993756), *Diaporthe lusitanicae* (JX197416), *D. melonis* (JX197417), *D. ampelina* (as *Phomopsis viticola* in GenBank) (AY745032), *D. phaseolorum* (JX197418, JX197419), *D. rudis* (JX197447), *D. sclerotiodes* (JX197420) and *D. eres* (as *Phomopsis* sp. OH-48 in GenBank, AY745025) was generated to design a new internal forward primer (CAL563F) located in the region corresponding to exon 4 in the *N. crassa* calmodulin gene.

A sequence alignment of both complete and partial sequences of the actin gene from *Neurospora crassa* (U78026), *Gaeumannomyces graminis* (AY424309), *Hypocrea orientalis* (JQ238613), *Magnaporthe oryzae* (XM003719823), *Fusarium oxysporum* f. *cubense* (JQ965663), *Thielavia terrestris* (XM003649706), *Nectria haematococca* (XM003050001), *Colletotrichum gloeosporioides* f. sp. *malvae* (AF112537), *Cleistogenes songorica* (FJ972820), *Verticillium alboatrum* (XM003008431), *Phaeosphaeria nodorum* (XM001791742), *Pyrenophora teres* f. *teres* (XM003298028), *Gibberella zeae* (XM387511), *Diaporthe neotheicola* (JQ807344), *D. vaccinii* (JQ807322) and *D. ampelina* (as *Phomopsis viticola* in GenBank) (JN230390) revealed that non-specific binding sites for the ACT-783R (Carbone & Kohn, 1990) primer exist in *Diaporthe* resulting in the frequent failures of amplification and sequencing.

To eliminate these problems a new reverse primer (ACT-878R) was designed. The primer combination of ACT-512F/ACT-878R was used for amplification with isolates in which amplification failed with the primer combination ACT-512F/783R in this study.

Gradient PCR and reagent optimisations were used to develop the standard protocols for amplification of ACT and CAL genes. Twelve reactions across an annealing temperature gradient of 50–65 ˚C for each of the test isolates were performed in three replicates. Optimal annealing temperatures were determined by the intensity of the amplicons visualized by agarose gel electrophoresis. Also the addition of 1 % DMSO to the PCR mix was used to enhance the reaction. Existing and newly designed primers used to amplify ACT and CAL were evaluated for thermal properties, hairpin formation and self-complementarities using the online platforms of OligoCalc (Kibbe, 2007) and the Sequence Manipulation Suite, the analyzing platform for short DNA sequences (Stothard, 2000).

4.2.4 Sequence alignment and phylogenetic analysis

Raw sequences were assembled with Sequencher v. 4.9 for Windows (Gene Codes Corp., Ann Arbor, MI, USA). The assembled consensus sequences were initially aligned with Clustal W (Thompson et al., 1994) and optimised with MAFFT

v. 7 using default settings (Katoh & Standle**y,** 2013) and adjusted manually where necessary.

Newly generated ITS sequences were analysed with all available type-derived sequences listed in Udayanga et al. (2011; 2012a) to determine the preliminary identifications of the isolates. Sequences from isolates recognised as *Diaporthe citri*, *D. cytosporella*, *D. foeniculina* and *D. rudis* were analysed with a selected set of additional ITS sequences available in GenBank identified using the NCBI's BLAST search and authenticated by the publications where sequences were reported. To more fully resolve closely related species, single gene phylogenies were inferred for ACT, CAL, EF1-α, ITS and TUB and a selected set of isolates were subjected to a multi-gene combined analysis. Trees were rooted with *Diaporthe helianthi* (CBS 592.81), which was determined to fall outside of the clades included in this study (trees not shown).

PAUP v. 4.0b10 (Swofford, 2002) was used to perform maximum parsimony analyses. Trees were inferred using the heuristic search option with 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Descriptive tree statistics for parsimony (Tree Length (TL), Consistency Index (CI), Retention Index (RI), Related Consistency Index (RC) and Homoplasy Index (HI)) were calculated for trees generated in a parsimony analysis. Evolutionary models for phylogenetic analyses were selected independently for each locus using MrModeltest v. 2.3 (Nylander, 2004) under the Akaike Information Criterion (AIC) implemented in both PAUP v. 4.0b10 and MrBayes v. 3. Phylogenetic reconstructions of concatenated and individual gene-trees were performed using both Bayesian Inference (BI) Markov Chain Monte Carlo and Maximum Likelihood (ML) criteria. Bayesian reconstructions were performed using MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001). Two simultaneous analyses, each consisting of six Markov chains, were run for 1 000 000 generations with trees sampled every 100 generations resulting in 20 000 total trees. The first 2 000 trees, representing the burn-in phase of the analyses were discarded from each run and the 16, 000 remaining trees were used for calculating posterior probabilities (PP).

Maximum likelihood trees were generated using the software RAxML v 7.4.2 Black Box (Stamatakis, 2006; Stamatakis, Hoover & Rougemont, 2008) in the CIPRES Science Gateway platform (Miller, Pfeiffer & Schwartz, 2010). For the combined dataset all free modal parameters were estimated by RAxML with ML estimate of 25 per site rate categories. The combined five-gene dataset was partitioned by gene region. The RAxML software accommodated the GTR model of nucleotide substitution with the additional options of modelling rate heterogeneity (Γ) and proportion invariable sites (I). These analyses utilised the rapid bootstrapping algorithm in RAxML in XSEDE high performance online computing service**.** Phylogenetic trees and data files were viewed in MEGA v. 5 (Tamura et al., 2011), TreeView v. 1.6.6 (Page, 1996) and FigTree v. 1.4 (Rambaut & Drummond, 2008).

4.2.5 Genealogical Sorting Index

The rooted gene genealogies resulting from each of the single gene analyses of ACT, CAL, EF1-α, ITS and TUB were submitted to the genealogical sorting index (*gsi*) parallel computing resource (Bazinet et al., 2008) for analysis. The *gsi* estimates the degree of exclusive ancestry of individuals in labelled predefined groups in a rooted tree (Cummings, Neel & Shaw, 2008**)**. Values range from 0 to 1 with 0 corresponding to a lack of genealogical divergence from other groups and 1 corresponding to monophyly for the predetermined clade (or species). Each isolate was assigned to a predetermined species based on Genealogical Concordance Phylogenetic Species Recognition (GCPSR) and the *gsi* was calculated for the best tree selected in parsimony analysis and for all trees using 10 000 permutations (Cummings et al., 2008).

The assignment of each tip to groups representing the recognised species was identical for the EF1-α and combined phylogenetic trees (Figure 4.2, 4. 3). Taxa in the ITS tree that were not present in EF1-α and combined trees were not included in the calculation of *gsi*. The *gs*i and each of the probability values (P) corresponding to the species represented by more than one isolate were tabulated. Species with one representative isolate were not subjected to *gsi* analysis. The ensemble genealogical sorting index ($gsi_{T)}$ is the sum of the *gsi* values calculated for all individual gene trees.The novel sequences were deposited in GenBank and the sequence alignments

were submitted to TreeBASE as S14141 (ITS), S14146 (EF1) and S14147 (combined alignment). Taxonomic novelties (MB) and typifications (MBT175959–MBT175968) were registered in MycoBank (Crous et al., 2004a).

4.3 Results

4.3.1 Phylogenetic analysis

Three hundred new sequences were generated in this study from 77 cultures (Table 4.1). Other available sequences were obtained from GenBank. Six alignments were analysed corresponding to single gene analyses of ACT, CAL, EF1-α, ITS and TUB and a combined alignment of the five genes. Comparison of the alignment properties and nucleotide substitution models are provided in Table 4.3. Phylogenetic trees inferred from EF1-α and ITS to show the phylogenetic placement of species and a combined alignment of five genes are presented with annotations for species, host and geographic origin (Figure 4.1–3). Individual gene trees for ACT, CAL & TUB did not markedly differ from the EF1-α and ITS gene trees and are not shown.

4.3.2 ITS phylogenetic analysis

The ITS sequence alignment contained 126 sequences including the outgroup taxon. Maximum parsimony analysis resulted in 45 equally most parsimonious trees (TL=209, CI=0.684, RI=0.977, RC=0.668, HI=0.316). BI and ML trees were identical to the MP tree presented in Figure 4.1. A total of 12 clades were resolved corresponding to the species recognised as *D. alleghaniensis*, *D. australafricana*, *D. canthii*, *D. citri*, *D. cotoneastri*, *D. cytosporella*, *D. foeniculina*, *D. pterocarpi*, *D. pterocarpicola*, *D. rudis*, *D. thunbergii* and *D. vaccinii*. *Diaporthe cynaroidis* was not resolved as distinct from *D. rudis*.

Among the major clades of interest in this study, the *D. foeniculina* clade consists of 48 isolates derived from 21 different hosts in ten countries representing the geographic regions of Australia, Europe, New Zealand, northern South America and South Africa. The isolates from *Citrus* in this clade originated from California (USA), Spain and New Zealand. The ex-type of *D. rhusicola* is also placed within the *D. foeniculina* clade. *Diaporthe cytosporella* is represented by four isolates from *Citrus*

in Spain and *Citrus* and *Vitis* in California (USA). *Diaporthe rudis* comprises 34 isolates derived from 18 different hosts from 13 countries representing Canada, Europe, New Zealand, South America and South Africa, including the epitype culture of *D.viticola*. No isolates of *D. rudis* were reported from *Citrus*.

Table 4.3 Comparison of alignment properties in parsimony analysis of genes, and nucleotide substitution models.

Genes/loci	**ITS**	**EF1-α**	**TUB***	**ACT***	**CAL***	**Combined ITS/EF/ACT /CAL**
Characters included (with gaps)	508	347	454	279	232	2033
Invariable characters	360	155	318	205	129	1337
Parsimony informative characters (%)	97(20%)	189(55%)	132(29%)	68(24%)	95(40%)	659(32%)
Uninformative polymorphic characters	14	3	4	6	8	37
Alignment strategy (MAFFT v6)	FFT-NS-I+manual	FFT-NS-I+manual	FFT-NS-I	FFT-NS-I	FFT-NS-I+manual	-
Number of branches >70 % bootstrap MP/BI and ML analysis	18	17	15	11	11	20
Nucleotide substitution models for Baysien analysis (determined by MrModeltest)	SYM+I+G	GTR+I+G	HKY+G	GTR+G	HKY+G	GTR+I+G

Figure 4.1 The phylogenetic tree generated from the parsimony analysis of the ITS of *D. citri* and related taxa. MP/RAxML bootstrap values/BI ≥70 % are displayed. Ex-type and ex-epitype cultures are in bold. The tree is rooted with *D. helianthi* (CBS 592.81).

Isolates identified as *D. citri* include 19 from various *Citrus* spp. in China, Korea, New Zealand and United States (Florida and Texas).

4. 3.3 EF1-α phylogenetic analysis

The EF1-α data matrix contained 77 sequences including the outgroup and consisted of 347 characters including gaps (Table 4.3). Maximum parsimony analysis yielded a single most parsimonious tree and is presented here as Figure 4.2 (TL=442, CI=0.742, RI=0.964, RC=0.715, HI=0.258). The MP, BI and ML trees generated were identical. The closely related taxa *D. foeniculina* and *D. cytosporella* were clearly distinguished and *D. rhusicola* was placed within *D. foeniculina*. Isolates of *D. australafricana* including the ex-type isolate were placed within the *D. rudis* clade, whereas *D. cynaroidis,* represented by the ex-type isolate, formed a distinct branch. Inspection of EF1-α sequences of *D. australafricana* versus *D. rudis* isolates revealed two base changes including one insertion and one transversion.

4.3.4 Combined analysis of five genes

The combined data matrix consisted of 74 isolates including the outgroup with 2033 characters included in the maximum parsimony analysis (Table 4.3). The maximum parsimony analysis of the alignment yielded a single most parsimonious tree presented here as Figure 4.3 (TL=1302, CI=0.720, RI=0.961, RC=0.692, HI=0.280). The MP, BI and ML trees generated were identical. A total of 13 clades were resolved in the combined phylogenetic tree. *Diaporthe citri* forms a sister clade to a clade containing *D. vaccinii* and *D. cotoneastri. Diaporthe citri* occurs only on *Citrus* in the United States and elsewhere while *D. vaccinii* occurs only on *Vaccinium* in North America. The *Diaporthe rudis* clade includes the taxon previously known as *D. viticola* represented by an ex-epitype culture (CBS 113201) and several authentic isolates previously known as *D. medusaea. Diaporthe australafricana* forms a well-supported clade closely related to *D. rudis*. The multi-gene phylogenetic tree resolves the closely related taxa *D. foeniculina, D. cytosporella, D. pterocarpi* and *D. canthii*. The ex-type of *D. rhusicola* is placed within the *D. foeniculina* clade.

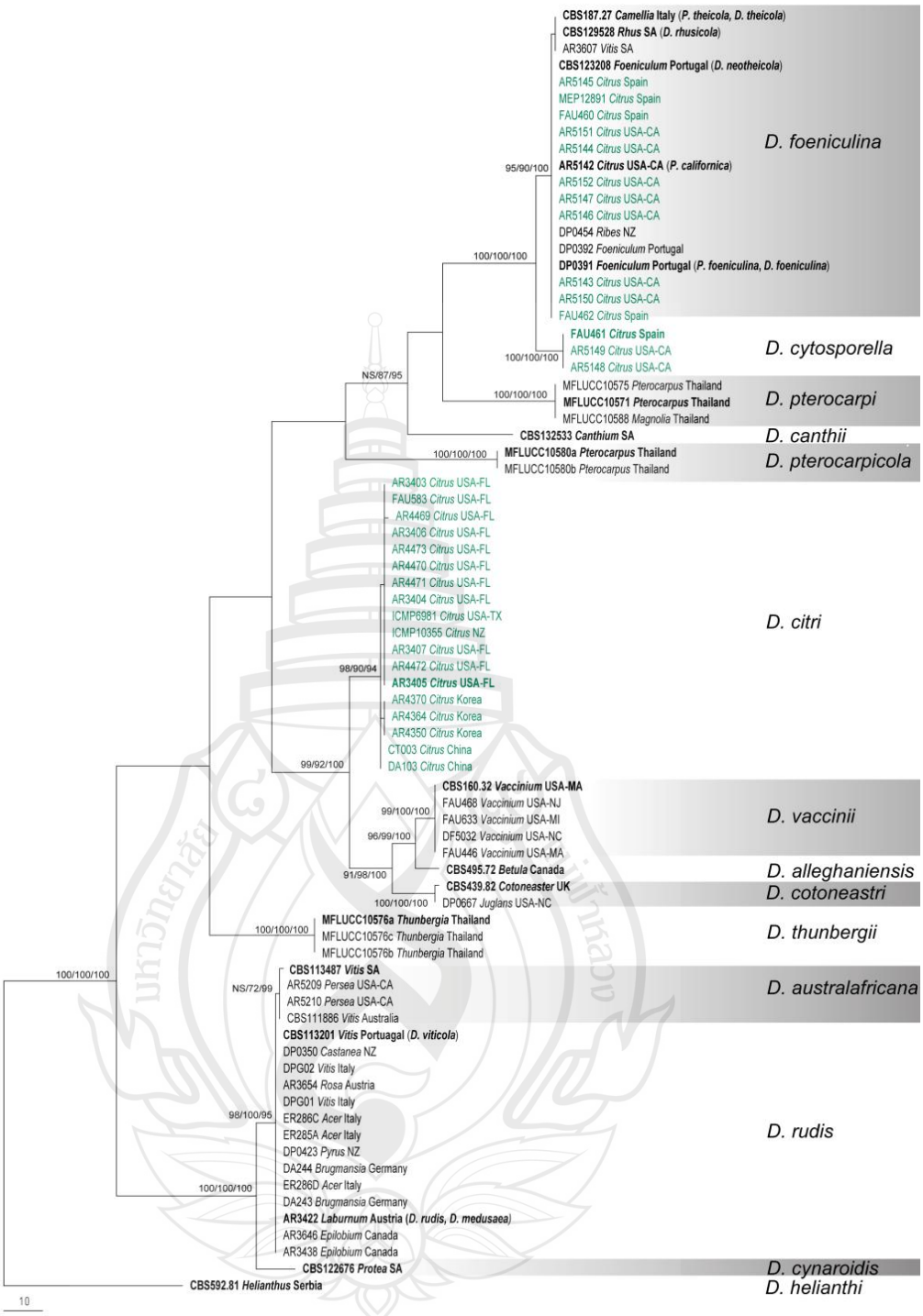


Figure 4.2 The phylogenetic tree generated from the parsimony analysis of the EF1-α of *D. citri* and related taxa. MP/RAxML bootstrap values/BI ≥70 % are displayed above or below each branch. Ex-type and ex-epitype culture numbers are in bold. Isolates from *Citrus* are indicated in green. The tree is rooted with *D. helianthi* (CBS 592.81).

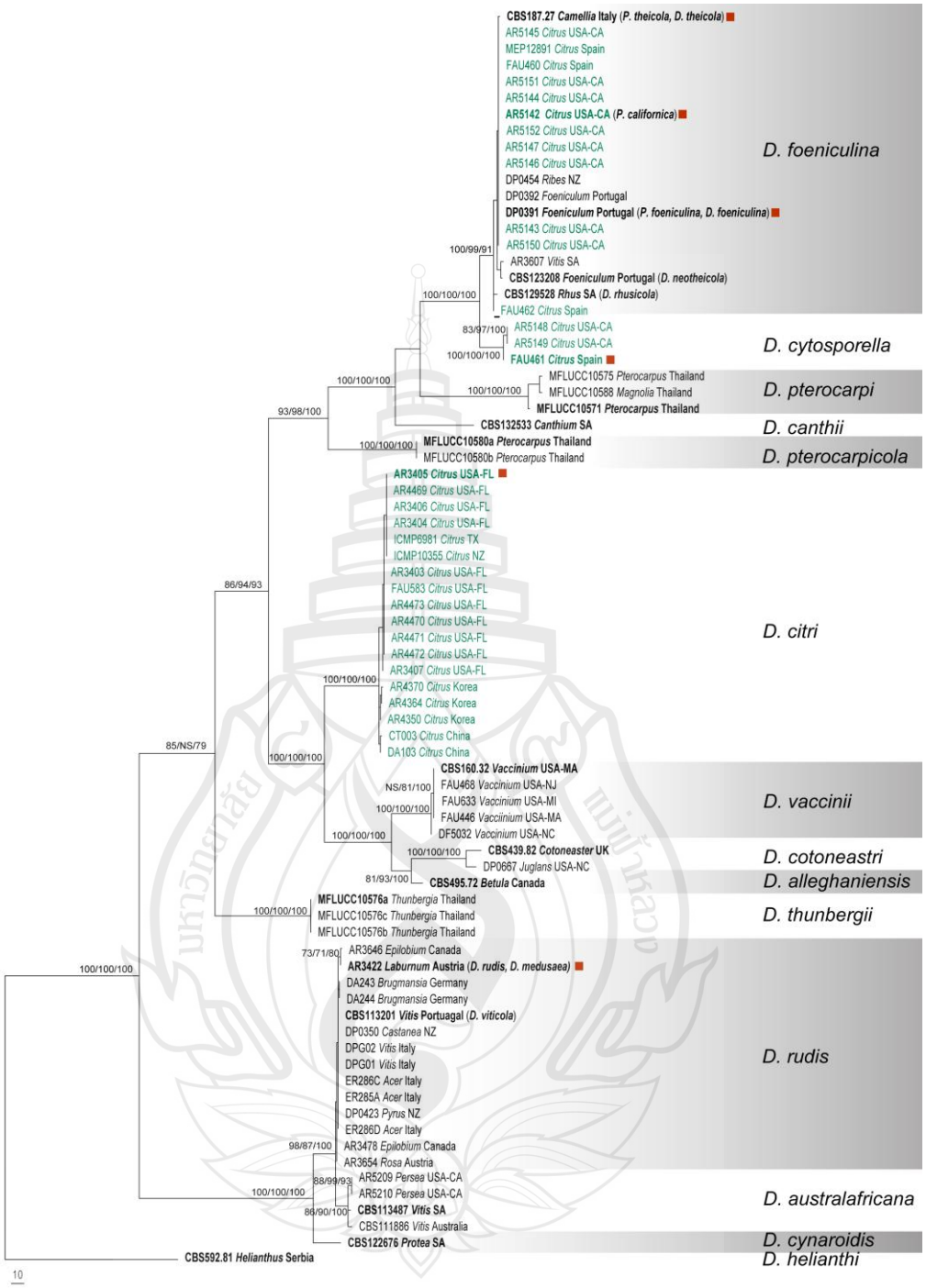


Figure 4.3 The phylogenetic tree generated from the parsimony analysis of the combined ACT, CAL, EF1-α, ITS and TUB. MP/RAxML bootstrap values/BI ≥70 % are displayed. Ex-type and ex-epitypes are in bold. Red squares indicate the epitypes designated. The tree is rooted with *D. helianthi* (CBS 592.81).

Diaporthe foeniculina, *D. cytosporella* and *D. pterocarpi* are all found to occur on multiple, unrelated hosts. *Diaporthe canthii*, represented by a single isolate, is known only from its type host.

4.3.5 Analysis of *gsi* data

All *gsi* values were in range of 0.5–1.0 with the exception of TUB (0.4482) for *D. cytosporella* and TUB (0.4051), CAL (0.2712) and ACT (0.1187) for *D. australafricana* (Table 4.4). Despite minor variation within the ITS1 region in both *D. foeniculina* and *D. rudis*, the *gsi* recognised each as monophyletic for each of the genes, confirming the placement of the ex-type culture of *D. rhusicola* with *D. foeniculina*. Therefore, the observed variation in the ITS regions of these two species is not considered meaningful in terms of species distinction and does not conflict with the other gene regions. Individually, the ITS and EF1-α genes estimated significant measures of exclusive ancestry for all the species including *Diaporthe australafricana* and *D. cytosporella*. The ACT gene resolved all species as monophyletic except *D. australafricana*. The ensemble *gsi* value ($gsi_{T)}$ for all species included indicated significant genealogical divergence from other all species in spite of the conflict observed among genes for *D. australafricana* and *D. cytosporella*. All the other species resolved in the combined phylogeny were supported without conflict.

4.3.6 New primers for *Diaporthe* and protocols for amplification

The evaluation of the thermal properties of the primers by OligoCalc and Sequence Manipulation Suite revealed that the forward CAL primers, CAL-228F (Carbone & Kohn, 1999) and CL1 (O'Donnell et al, 2000), showed potential for self-annealing in each of the tests in addition to issues with non-specific binding sites in the targeted gene region. The newly designed CAL-563F primer and the existing CL2A reverse primer (O'Donnell et al., 2000) were determined to be a suitable primer pair under the criteria given including percentage GC, self-annealing, GC clamp, hairpin formation and length. They were used to eliminate the problems in amplification and sequencing encountered in this study.

Table 4.4 Genealogical Sorting Index (*gsi*) and probability values (P) for gene trees of species resolved in this study.

Species	ITS gsi P	EF1-α gsi P	TUB gsi P	CAL gsi P	ACT gsi P	ALL gsi_T P_T
D. australafricana	1* 0.0004	1* 0.0001	0.4051* 0.001	0.2712* 0.0078	0.1187 0.0483	0.7353* 0.0001
D. citri	1* 0.0004	1* 0.0001	1* 0.0001	1* 0.0001	1* 0.0001	1* 0.0001
D. cotoneastri	1* 0.0005	1* 0.0019	1* 0.0008	1* 0.0079	1* 0.0007	1* 0.0001
D. cytosporella	1* 0.0001	1* 0.0002	0.4492* 0.0003	1* 0.0003	1* 0.0004	1* 0.0001
D. foeniculina	0.869* 0.0001	1* 0.0001	0.9476* 0.0001	1* 0.0001	0.9301 0.0001	0.9598* 0.0001
D. pterocarpi	1* 0.0001	1* 0.0002	1* 0.0001	1* 0.0003	1* 0.0002	1* 0.0001
D. pterocarpicola	1* 0.0014	1* 0.0020	1* 0.0010	1* 0.0088	1* 0.0002	1* 0.0001
D. rudis	1* 0.0001	1* 0.0001	0.9138 * 0.0001	0.8392 0.0001	0.7719 0.0001	0.9332* 0.0001
D. thunbergii	1* 0.0001	1* 0.0001	1* 0.0001	1* 0.0002	1* 0.0001	1* 0.0001
D. vaccinii	1* 0.0004	1 0.0001	1* 0.0001	1* 0.0001	1* 0.0001	1* 0.0001

Notes. The *gsi* statistic is based on a continuum of 0–1, with 0 = lack of genealogical divergence from other groups and 1 = monophyly; (*) = statistically significant P-value ≤ 0.05. The *gsi* is calculated under the null hypothesis that the gene copies labeled as each species assigned are a single group of mixed genealogical ancestry. gsi_T = ensemble *gsi* of 5 gene trees. Species represented by single isolate are excluded in calculation of *gsi*.

Use of this primer pair resulted in an amplicon overlapping approximately 300 bp of the 500-bp CAL-228F/CAL-737R fragment. However, two additional introns, each approximately 60–100 bp in length, are found in the extended sequence obtained using the primers CAL-563F/CL2A. One of these informative introns in *Diaporthe* is not found in either of the *N. crassa* or *P. grisea* reference sequences used as

references for primer design. The newly designed reverse primer for actin (ACT-878R) worked well in combination with ACT-512F for isolates that failed with the ACT-512F/ACT-783R primer combination and resulted in an amplicon of approximately 350 bp in length. The extended 3' region of the newly generated amplicons was not included in the analyses as the majority of the sequences were generated with primer pairs ACT-512F/ACT-783R and it consisted entirely of exon sequence with little variation among isolates.

4.3.7 Taxonomy

In this section we provide modern descriptions and illustrations of the species resolved here based on multi-gene phylogenetic analyses and morphological characters. *Diaporthe citri* occurs only on *Citrus* while *D. cytosporella* and *D. foeniculina* occur on *Citrus* and other woody and herbaceous hosts including high value crops. *Diaporthe rudis* is not known from *Citrus* but was previously confused with those species especially as *D. medusaea* and has a broad host range. Each species is described based on type and other specimens as well as ex-epitype cultures. Synonymous names of *Diaporthe* or *Phomopsis* are reviewed based on protologues, type and other specimens and cultures. When specimens with cultures from similar substrates and localities are available, epitype specimens with ex-epitype cultures are designated for both accepted and synonymous names.

Diaporthe citri (H.S. Fawc.) F. A. Wolf, J. Agric. Res., Washington 33: 625. 1926. Figure 4.4

Basionym. *Phomopsis citri* H.S. Fawc., Phytopathology 2: 109. 1912 nom. conserv. prop. non *Phomopsis citri* (Sacc.) Traverso & Spessi 1910.

= *Diaporthe citrincola* Rehm, Leafl. Philip. Bot. 6: 2269. 1914.

= *Phomopsis caribaea* W.T. Horne, Phytopathology 12: 417. 1922.

Perithecia on decaying twigs, black, globose to conical, 130–200 µm diam, scattered, solitary or in groups, immersed deep in bark with tapering perithecial necks 190–700 µm long. *Asci* unitunicate, 8-spored, sessile, elongate to clavate, (37.3–)40.5–50.5(–55) × (9–)10.5–12(–12.2) µm. *Ascospores* hyaline, two-celled, often 4-guttulate,

with larger guttules at centre and smaller one at ends, elongated to elliptical, (12–)12.4–14(–14.2) × 3.2–3.6(–3.8) μm ($\bar{x}$±SD=13.2±0.8 × 3.3±0.2, n= 30).

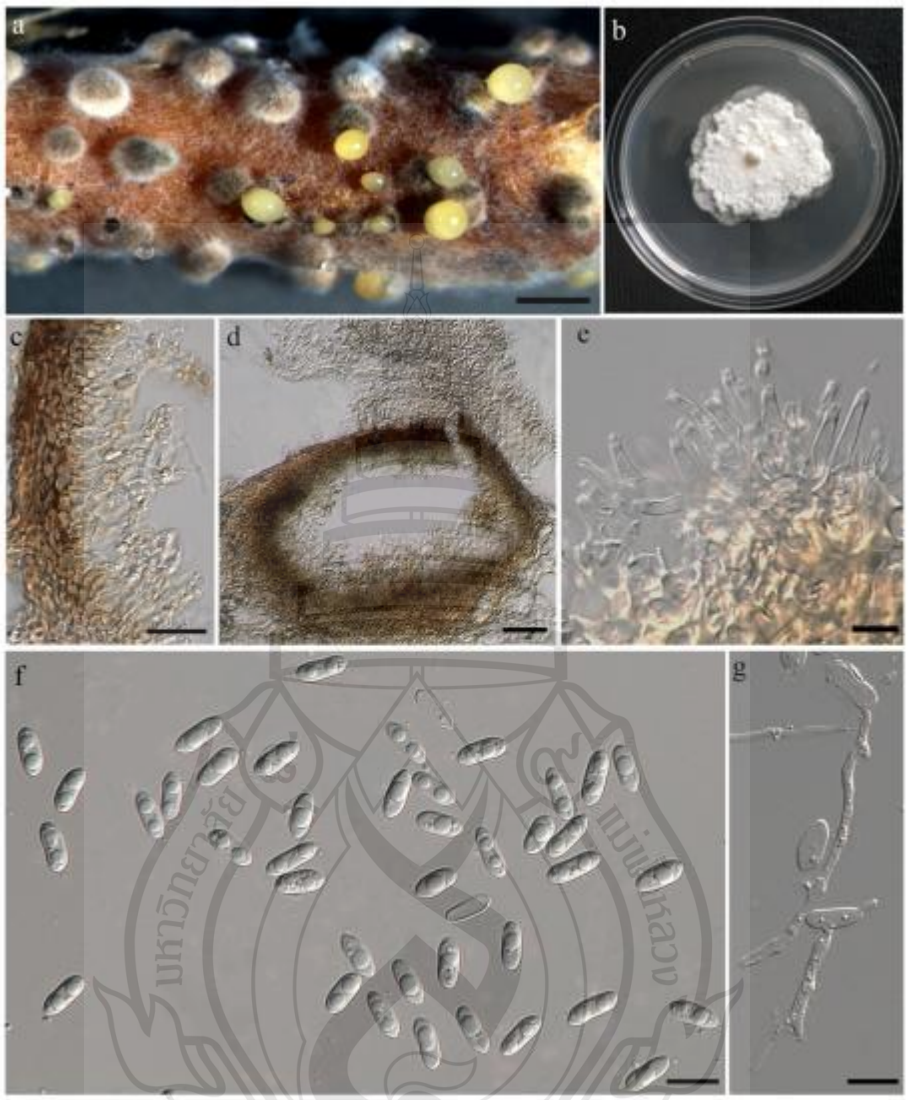
Pycnidia on alfalfa twigs on WA: globose, 200–250 μm diam, later conical, embedded in tissue, erumpent at maturity, up to 450 μm diam, 65–100 μm high, with an elongated black neck, often with a yellowish, spiral conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, straight to sinuous, 10–15 × 1–2 μm. *Conidiogenous cells* phialidic, cylindrical, terminal, slight tapering towards apex, 0.5–1 μm diam. *Paraphyses* abundant among conidiophores, 20–40 × 1–2 μm. *Alpha conidia* aseptate, hyaline, smooth, ovate to ellipsoidal, mono- to biguttulate, rarely 3-guttulate, base subtruncate, (7.6–)8–9(–10.2) × 3–4.2 μm ($\bar{x}$±SD =8.5±0.8 × 3.7±0.2 , n = 30), *Beta and gamma conidia* not observed on alfalfa twigs or in culture.

Cultural characteristics: In dark at 25 ºC for 1 wk, colonies on PDA slow growing, 4.2±0.2 mm/day (n = 8), white, fluffy aerial mycelium, reverse centre greenish yellow pigmentation developing in centre.

Host range: Causing melanose and stem end rot disease, associated with dying or dead twigs of *Citrus* spp. and closely related hosts including *C. aurantiifolia*, *C. aurantium*, *C. maxima* (= *C. grandis*), *C. nobilis*, *C. paradisi*, *C. reticulata*, *C. sinensis*, *C. sudachi*, *C. unshiu*, ×*Citrofortunella microcarpa* (=×*C. mitis*), *Fortunella japonica* (Kobayashi, 2007), *F. margarita*, *Poncirus trifoliata.*

Distribution: Probably throughout *Citrus*-growing regions of the world. Reported from Brazil, China, Cuba, Japan, Korea, New Zealand, Philippines, Puerto Rico and United States (Florida, Texas).

Type material examined: USA, Florida, Lake Alfred, Ana, on twigs of *Citrus* sp, 26 Apr. 2000, L.W. Timmer, dried specimen from culture sporulating on alfalfa stem (Type of *Phomopsis citri* proposed for conservation in Rossman et al. (2013) (BPI 892456, ex-type culture AR 3405 = CBS135422). PHILIPPINES, Los Baños, on dead twigs of *Citrus nobilis*, Oct. 1913, coll. M.B. Raimundo, comm. C. F. Baker, no. 1875 (holotype of *Diaporthe citrincola* S-F52860). CUBA, Isle of Pines, on *Citrus paradisi*, 30 Oct. 1917, Fredrick Maskew, intercepted in San Francisco,



Notes. a. Sporulation on alfalfa stem in WA; b. culture on PDA grown at 25 ˚C in dark for 7 d; c. pycnidial walls lined with paraphyses and conidiophores; d. section through conidiomata; e. conidiophores; f. alpha conidia; g. germinating conidia on a slide.–Scale bars: a = 500 µm, c = 20 µm, d–g = 10 µm.

Figure 4.4 Morphology of *Diaporthe citri* (AR3405 = CBS 135422).

-derived culture sporulating on *Citrus* twig (Lectotype of *Phomopsis caribaea* designated here BPI 358328, Isolectotype NY01097305; MBT175959).

Additional material examined: BRAZIL, Escola Agr., Vicosa, Minas Gerais, on peel of *Citrus* sp., 17 May 1932, P.H. Rolfs (BPI 615855); Intercepted New York #pi 7163, on fruit of *Citrus sinensis*, 22 June 1924, A.C. Hill, det. A.J. Bruman (BPI 358408). JAPAN, Yokohama, intercepted Seattle Washington #pi 4780, on fruit of *Citrus sinensis*, 14 Jan. 1940, A.G. Webb, det. J.A. Stevenson (BPI 358405) - MEXICO, intercepted Brownsville Texas #692229, on leaves of *Citrus* sp., 30 Jan. 1930, Mueller, det. D.J. Smith, A.E. Jenkins, J.A. Watson (BPI 615856); Intercepted Laredo Texas #50818, on leaves of *Citrus* sp., 23 Jan. 1951, Trotter, det. A.H. Lewis, J. A.Watson (BPI 615857). PUERTO RICO, Bayamon, on leaves of *Citrus grandis*, 22 Aug. 1933, C.G. Anderson (BPI 358392). USA, Florida, Orlando, on dead stems of *Citrus aurantifolia*, July 1925, F.A. Wolf (BPI 615860); Florida, Orlando, on dead stems of *Citrus* sp., Jan. 1926, F.A. Wolf (BPI 615959); Florida, Orlando, on leaves of *Citrus sinensis*, Mar. 1922, J.R. Winston (BPI358409); Florida, Eustis, on leaves of *Citrus grandis*, 8 Jan. 1932, H.S. Fawcett (BPI 358391); Florida, St. Nicholas, on stem of *Citrus sinensis*, 28 Nov. 1895, det. F. Albert, W.W. Diehl (BPI 358404); Florida, Fort Myers, on stems of *Citrus sinensis*, 16 Feb. 1924, J.A. Stevenson (BPI 358407); Florida, Gainesville, Florida Agricultural Experiment Station, on stems of *Citrus sinensis*, 16 Mar. 1910, H.S. Fawcett (BPI 358406); Florida, Winter Park, on stems of *Citrus grandis*, 21 Feb. 1923, C.L. Shear (BPI 615868); Florida, Winter Park, on stems of *Citrus grandis*, 20 Jan. 1925, H.E. Stevens, det. C.L. Shear (BPI 615869); Florida, on dead stem of *Citrus* sp., 1913, J.G. Grossenbacker, det. C.L. Shear (BPI 615858); Florida, on dead stem of *Citrus* sp., 8 July 1929, F.A.Wolf, det. C.L. Shear (BPI 615854); Florida, on leaves of *Citrus grandis*, 6 Jan. 1932, H.S. Fawcett **(**BPI 358393) .

Notes: The name *Diaporthe citri* is based on *Phomopsis citri* H.S. Fawc.1912, a later homonym of *Phomopsis citri* (Sacc.) Traverso & Spessa 1910. A conservation proposal has been submitted to continue the use of the widely used name for the species associated with melanose or stem end rot of *Citrus* as *D. citri* (Rossman, Udayanga, Castlebury & Hyde, 2013). *Diaporthe citri* based on the basionym *Phomopsis citri* H.S. Fawc. has priority over the later synonyms *D. citrincola* and *P.*

caribaea. This is also in accordance with the change to unit nomenclature with the older genus *Diaporthe* serving as the correct name for all species in *Diaporthe-Phomopsis* (McNeill et al., 2012). No type specimen for *Phomopsis citri* could be located at BPI or FLAS, leaving only an illustration (Fawcett, l.c.) as a potential, but unsatisfactory, iconotype, thus *P. citri* is proposed for conservation with a new type specimen (Rossman et al., 2013). The type specimens of *D. citrincola* and *P. caribaea* were examined and contributed to the conclusions that these names are synonyms of *D. citri*. A lectotype of *P. caribaea* is designated.

The fruiting structures of *Diaporthe citri* are found on dead twigs, stems and fruits of *Citrus* affected by melanose and stem end rot (Wolf, 1926; Fawcett, 1932; Whiteside & Timmer, 2000b). The fungus generally propagates itself on dead twigs of *Citrus*. A few days after infecting leaf tissue or fruit, the melanose symptoms appear as small, brown, discrete or confluent, sunken spots. A few epidermal cell layers on infected tissue are killed and become impregnated with reddish brown gum that later become raised black pustules (Timmer, 2000). Although pustules on leaves are initially surrounded by yellow halos, they recover and become green again and corky pustules are often the only symptoms (Bach & Wolf, 1928; Nelson, 2008). Fungal structures such as pycnidia or perithecia are never visible in these melanose lesions, therefore, the fungus cannot be observed in the infected leaves or fruit. When the fruiting structures are present on dead twigs or bark of the stems, the pycnidia or ascomata are abundant deep in the tissue.

Diaporthe cytosporella (Penz. & Sacc.) Udayanga & Castleb., *comb. nov.* Figures 4.5, 4.6
MycoBank MB803986

Basionym. *Phoma cytosporella* Penz. & Sacc., Fung. Agron. p. 361. 1887.

≡*Phomopsis cytosporella* (Penz. & Sacc.) H.S. Fawc. & H.A. Lee, Citrus Diseases and their Control, Ed. 1 p. 407. 1926.

Perithecia unknown. *Pycnidia* on alfalfa twigs on WA: globose, 150–200 μm diam, mostly embedded in tissue and erumpent at maturity, up to 450 μm diam, 65–100 high, with an elongated black neck, often with a yellowish conidial cirrus extruding from ostiole; walls parenchymatous consisting of 3–4 layers of medium brown *textura angularis*. *Paraphyses* lacking. *Conidiophores* hyaline, smooth,

branched or unbranched, ampulliform, cylindrical, wider at base, occurring in dense clusters, 7–18 × 1–2 µm,. *Conidiogenous cells* phialidic, cylindrical, terminal, with slight tapering towards apex, 0.5–1 µm diam. *Alpha conidia* aseptate, hyaline, smooth, ovate to ellipsoidal, biguttulate or multi-guttulate, base subtruncate, (6.9–)8–9(–12.6) × (2.3–)2.6–3.2 µm ($\bar{x}$±SD = 7.8±0.9 × 3.0±0.3, n = 30), occasionally larger alpha conidia present in culture and on alfalfa stems. *Beta and gamma conidia* not observed on alfalfa twigs or in culture.

Cultural characteristics: In dark at 25 ºC for 1 wk, colonies on PDA relatively slow growing, 4.0±0.2 mm/day (n = 8). On PDA white, fluffy aerial mycelium, reverse with ash colour pigmentation developing in centre. In a 2-wk-old culture, clusters of black, branched stromata occurring in concentric rings with sporulating pycnidia.

Host range: *Citrus limon*, *C. sinensis* and *Vitis vinifera.*

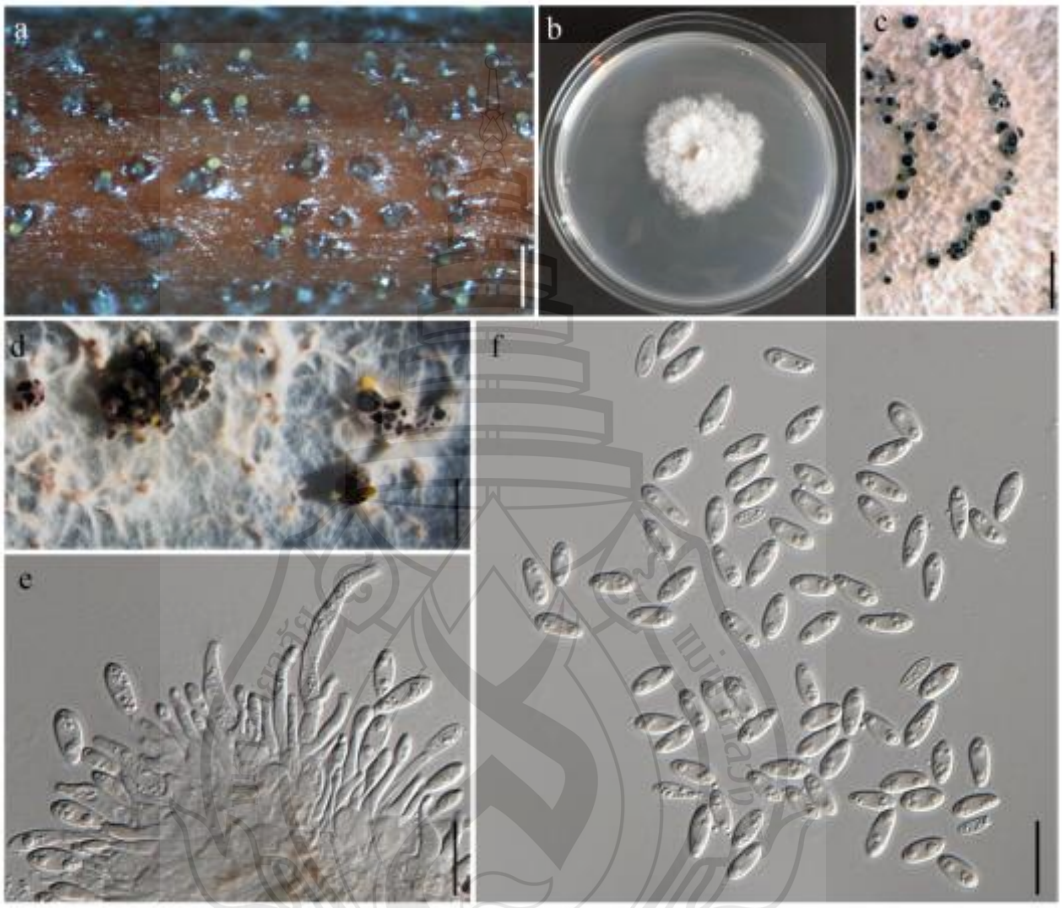
Distribution: Europe (Spain, Italy), United States (California).

Type material examined: ITALY, Rome, Modena, on *Citrus limonia*, Jan. 1886 (holotype of *Phoma cytosporella* BPI 798526). SPAIN, on *Citrus limon*, M.E. Palm, dried culture (epitype of *Phoma cytosporella* designated here, BPI 892459, living culture FAU 461 = CBS 137020; MBT 175960).

Additional material examined: USA, California, on twigs of *Citrus sinensis*, 4 Oct 2011, A. Eskalen UCR1751, dried culture with pycnidia sporulating on alfalfa stems (BPI 892457, living culture AR5149); *ibid*. UCR1750, dried culture with pycnidia sporulating on alfalfa stems (BPI 892458, living culture AR5148).

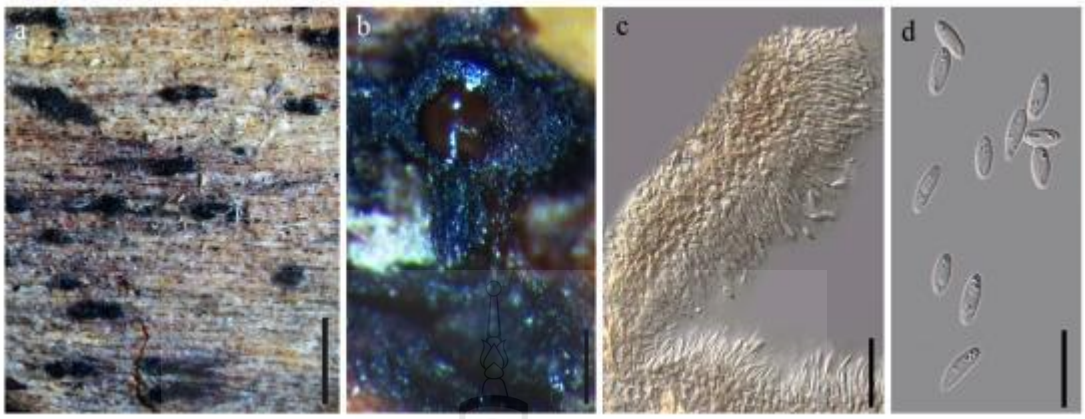
Notes: *Diaporthe cytosporella* is phylogenetically closely related to *Diaporthe foeniculina* but clearly distinguished based on ITS and EF1-α sequences (Figure 4. 1, 2). The species was first described from Italy and later synonymised under *Diaporthe citri.* Although in this study this species is primarily recognized using isolates from *Citrus limon* in Europe (Spain) and United States (California), two ITS sequences (FJ94470, AY745085) from GenBank are 100 % identical suggesting that this species may also occur on *Vitis* and other host species in California (retrieved on 1st of February 2013). At maturity cultures of *D. cytosporella* (AR5148 and AR5149) on PDA produce distinctive black, branched stromata. Perithecia were not

observed in culture. Morphological characters were highly similar among the type specimen (Figure 4.6) and the isolates used in epitype and genetically similar additional materials examined.



Notes. a. Sporulating pycnidia on alfalfa stem; b. culture on PDA (25 ˚C, dark, 7 d); c. concentric pycnidial in rings on culture; d. pycnidia on culture; e. conidiophores; f. alpha conidia.–Scale bars a,c = 2000 µm, c = 3000 µm, e,f = 20 µm.

Figure 4.5 Morphology of *Diaporthe cytosporella* (AR5149).



Notes. a. Pycnidia-bearing bark of *Citrus* sp.; b. branched stroma and sporulating pycnidia; c. section through pycnidia with pycnidial wall and conidiophores; d. alpha conidia.–Scale bars a = 1000 µm, b = 50 µm, c,d = 15 µm.

Figure 4.6 Holotype specimen of *Diaporthe cytosporella* (BPI798526).

Diaporthe foeniculina (Sacc.) Udayanga & Castle., *comb. nov*. Figure 4.7

MycoBank MB803929

Basionym. *Phoma foeniculina* Sacc., Michelia 2: 95. 1880.

≡ *Phomopsis foeniculina* (Sacc.) Sousa da Camara, Agron. Lusit. 9: 104. 1947.

= *Diaporthe theicola* Curzi, Atti Istit. Bot. Univ. Lab. Crittogam. Pavia 3 Sér. 3: 60. 1927.

= *Phomopsis theicola* Curzi, Atti Istit. Bot. Univ. Lab. Crittogam. Pavia 3 Sér. 3: 64. 1927.

= *Phomopsis californica* H.S. Fawc., Phytopathology 12: 419. 1922.

= *Diaporthe neotheicola* A.J.L. Phillips & J.M. Santos, Fungal Diversity 34: 120. 2009.

= *Diaporthe rhusicola* Crous, Persoonia 26: 135. 2011.

Perithecia on decaying twigs black, globose to subglobose (200–)360 × 200 µm, scattered, solitary or in groups, with tapering perithecial necks barely protruding through epidermis. *Asci* unitunicate, 8-spored, sessile, cylindrical to clavate, (40–

)50.5–60.5(–65) × 8–10(–12.2) µm. *Ascospores* hyaline, two-celled, often with four guttules, larger guttules near centre and smaller ones at ends, elongated to elliptical, (9.0–)12.4–14(–15.2) × (3.2–)3.4–3.6(–5.2) µm (x̄±SD = 13.2±0.8 × 3.5±0.1, n = 30).

Pycnidia on alfalfa twigs on WA: globose to subglobose 400–700 µm diam, erumpent at maturity, (300–)500–800(–930) µm high, with an elongated, black neck, mostly embedded in tissue, often with a yellowish, drop-like conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 2–3 layers of medium brown *textura angularis*. *Paraphyses* lacking. *Conidiophores* hyaline, smooth, unbranched, cylindrical, straight to sinuous, 9–15(–18) × 1–2 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, with slight taper towards apex, 0.5–1 µm diam. *Alpha conidia* aseptate, hyaline, smooth, ellipsoidal or fusiform, with none, two or many guttules, rarely with subtruncate base, (7.5–)8.5–9(–9.2) × (2–)2.3–2.5(–2.7) µm (x̄±SD = 8.8±0.3 × x̄±SD=2.4±0.1 µm, n = 30). *Beta conidia* hyaline, aseptate, eguttulate, hamate or slightly curved, abundant, base subtruncate, acute apex, (20–)22–28(–29) × (1.1) –1.4–1.6(–2) µm (x̄±SD =25.1±3.3 × 1.5 ±0.1 µm, n = 30)

Cultural characteristics: In dark at 25 ºC for 1 wk, colonies on PDA slow growing, 5.2±0.2 mm/day (n = 8), white, sparse aerial mycelium, greenish yellow pigmentation developing in reverse centre.

Host range: *Acacia*, *Acer*, *Actinidia* deliciosa**,** *Aspalathus linearis*, *Bougainvillea spectabilis*, *Camellia sinensis*, *Castanea*, *Citrus limon*, *C. limonia*, *Crataegus*, *Diospyros*, *Foeniculum vulgare*, *Fuchsia*, *Hydrangea*, *Juglans*, *Malus*, *Olea*, *Prunus*, *Pyrus*, *Quercus*, *Rhus*, *Ribes*, *Vitis vinifera* and *Wisteria sinensis.* In addition to the hosts on the specimens listed below, these hosts are represented in Figure 4.1 based on the ITS phylogeny and Gomes et al. (2013) as *Diaporthe foeniculacea*).

Distribution: Australia, Argentina, Europe (Greece, Portugal, Spain, Italy), New Zealand, South Africa and USA (California).

Type material examined: FRANCE, on stems of *Foeniculum 'arvensis'*, *Brunaud*, cited in Phillips (2003) with illustration (holotype of *Phoma foeniculina* PAD 281 – unavailable, not examined). PORTUGAL, Madeira, Serra da Agua, at base of 2-yr-old stem of *Foeniculum vulgare*, Aug. 2001, A.J.L. Phillips (epitype of *Phoma foeniculina* designated here LISE 94791, ex-epitype culture from single

ascospores CBS 111553 = DP0391; MBT175961). USA, California, Santa Barbara County, on dead outer bark and decaying fruit of *Citrus limonia*, 3 Mar. 1922, H. S. Fawcett (Holotype of *Phomopsis californica* BPI0358313). USA, California, San Diego, on branch of *Citrus limonia*, 16 Nov. 2012, A. Eskalen (epitype of *Phomopsis californica* designated here BPI 892460, ex-epitype culture AR5142 = CBS135430; MBT 175962). ITALY, on *Camellia sinensis*, Curzi, dried culture specimen (epitype of *Diaporthe theicola* designated here BPI 892462, ex-epitype culture CBS 187.27, same as ex-isotype culture of *Phomopsis theicola*; MBT175963). Illustration in Atti dell'Istituto Botanica della Universita e Laboratoria Crittogamico di Pavia 3 Sér. 3: 60 (1926) [1927] (lectotype of *Phomopsis theicola* designated here; MBT175964). PORTUGAL, Évora, on *Foeniculum vulgare*, Nov. 2007, A.J.L. Phillips (holotype of *Diaporthe neotheicola* CBS-H 20131, ex-holotype culture (Di-C004/5 = CBS 123208).

Additional material examined: PORTUGAL, Madeira, Serra da Agua, at base of 2-year-old stem of *Foeniculum vulgarae*, Aug. 2001, A.J.L. Phillips (LISE 94792 as *Diaporthe foeniculacea*, culture from single ascospores DP0392 = CBS 111554. SPAIN, on fruit of *Citrus limon*, intercepted at Elizabeth, New Jersey, 20 Mar. 1987, C. Markham 001514, M.E. Palm (BPI 1107900, living culture MEP1289-1); *ibid*. (BPI 747926); *ibid*. (BPI 747927); on peel of *Citrus limonia*, intercepted New York #87452, 13 Nov. 1940, Hodson, E.A. Jenkins (BPI 615878); *ibid*., on fruit of *Citrus limon*, M.E. Palm (BPI 892461, culture FAU460).

Notes: *Diaporthe foeniculina* is known to occur on *Citrus* and many other woody plants hosts in temperate and tropical regions. This species causes a stem end rot of lemandarin (*Citrus limonia*) in Europe and the USA (California) and was observed as a saprobe on branches of this host. As *D. neotheicola*, this species has been reported to cause diseases of temperate and tropical fruits from Australia, Europe and South Africa. Our results indicate that isolates from *Citrus* in Spain are conspecific with the type isolate of the recently described *D. neotheicola* from *Foeniculum* as well as isolates from other hosts now considered to be *D. foeniculina*.

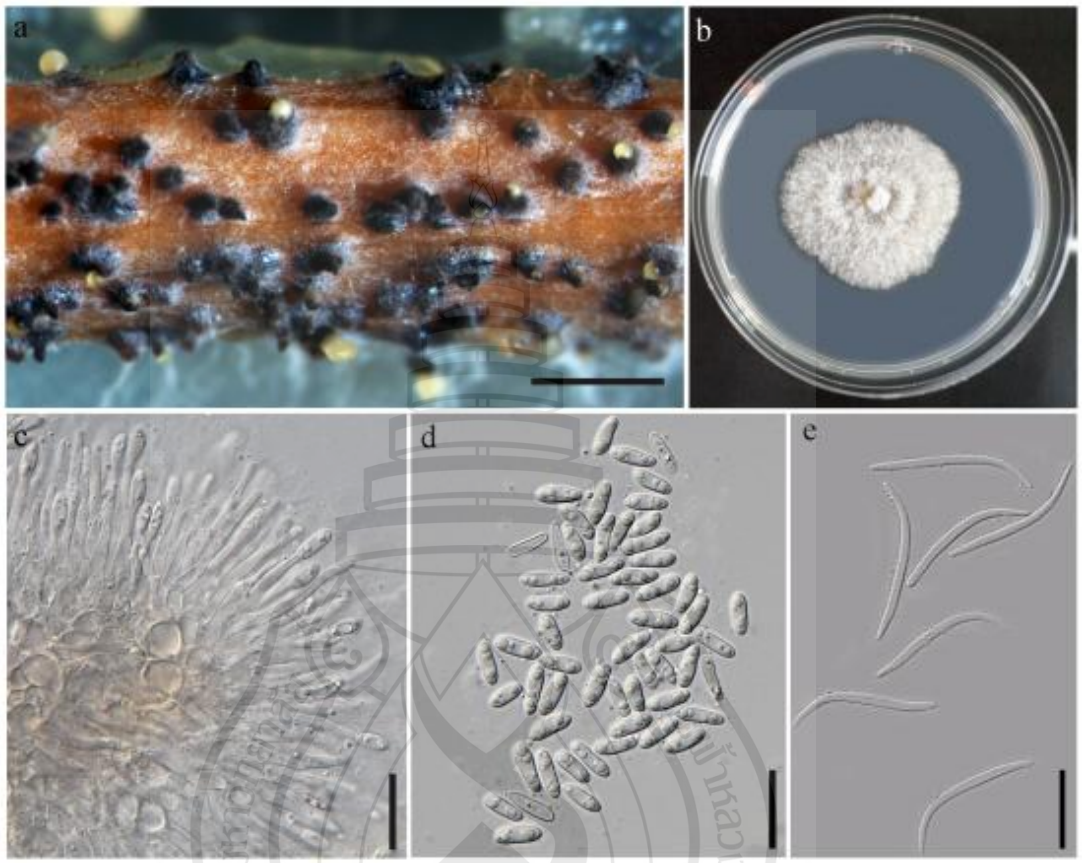
We reviewed the possible synonyms of this species based on available molecular data, living cultures and type specimens. The specimen deposited in LISE 94792 corresponding to the living culture CBS 111554 was selected as the epitype

specimen for *Phoma foeniculina*, now recognised as *D. foeniculina*. Molecular data derived from the epitype of *Phoma foeniculina*, now *Diaporthe foeniculina*, and additional isolates show that this taxon is conspecific with the ex-type isolates of *D. neotheicola* and *P. theicola* (Phillips, 2003; Santos & Phillips, 2009; Gomes et al., 2013) as well as isolates from *Citrus* in Spain. The name *D. neotheicola* has been widely used for this taxon (Santos & Phillips, 2009; Udayanga et al., 2012a; Thomidis et al., 2013).

Phomopsis foeniculina (syn. *Phoma foeniculina*) was considered a synonym of *Diaporthe foeniculacea* (syn. *Sphaeria foeniculacea*) by Phillips (2003). We examined the type specimen of *Sphaeria foeniculacea* and agree with von Arx and Müller (1954) who recognised this species as *Guignardia foeniculacea* (Mont.) Arx & E. Müll. (as *G. foeniculata*).

Gomes et al. (2013) used the name *D. foeniculacea* to refer to this species based on Phillips (2003). However, observation of type specimens of *Sphaeria foeniculacea* confirmed that this name cannot be applied to a species of *Diaporthe*. This is further explained under the excluded species.

Although type specimens of *Phomopsis theicola* and *Diaporthe theicola* could not be located, an ex-type culture of *Phomopsis theicola* exists as mentioned by Santos and Phillips (2009). They stated that *Phomopsis theicola* was not the same as *D. theicola* based on the illustrations in the protologues of these taxa and described the name *D. neotheicola* for the sexual state of *P. theicola*, with an ex-holotype culture from *Foeniculum*. However measurements of asci and ascospores in original description of *D. theicola* in Curzi (1927) are consistent with those of *D. foeniculina* specimens examined in this study. We agree with the opinion of Curzi (1927) that *D. theicola* and *P. theicola* are sexual and asexual morphs of the same fungus, simultaneously described from same specimen and therefore here we epitypify the name *D. theicola* with Curzi's ex-type culture of *P. theicola*.



Notes. a. Sporulation on alfalfa stem in WA; b. culture on PDA (25 ˚C, dark, 7 d); c. conidiophores; d. alpha conidia; e. beta conidia.–Scale bars: a = 2000 µm, all others = 10 µm.

Figure 4.7 Morphology of *Diaporthe foeniculina* (DP0391 = CBS 111553).

Diaporthe rudis (Fr.) Nitschke, Pyren. Germanici 2: 282. 1870. Figure 4.8

Basionym. *Sphaeria rudis* Fr., Elench. fung. (Griefswald) 2: 98. 1828.

≡ *Rabenhorstia rudis* (Fr.) Fr., Summa veg. Scand., Section Post. (Stockholm): 410. 1849.

≡ *Aglaospora rudis* (Fr.) Tul. & C. Tul., Select. fung. carpol. (Paris) 2: 165. 1863.

= *Phoma rudis* Sacc., Michelia 1:257. 1878.

≡ *Phomopsis rudis* (Sacc.) Höhn., Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Kl., Abt.1, 115: 680. 1906.

= *Diaporthe faginea* Sacc., Syll. Fung. 1:619. 1882.

= *Diaporthe macrostoma* Nitschke, Pyren. Germanici 2: 284. 1870.

= *Diaporthe medusaea* Nitschke, Pyren. Germanici 2: 251. 1870.

= *Diaporthe viticola* Nitschke, Pyren. Germanici 2: 264. 1870.

= *Diaporthe silvestris* Sacc. & Berl., Atti Rev. Instit. Venet. Ser. II, 6: 737. 1885.

Perithecia black, clustered, globose, 300–350 µm, with tapering perithecial necks, 200–700 µm long. Asci unitunicate, sessile, elongate to clavate, (50.3–)53.5–58.5(–59.6) × (8.9–)10.6–12(–12.3) µm. *Ascospores* hyaline, two-celled, often tetra-guttulate, with larger guttules at centre and smaller at ends, elongated to clavate, (11.6–)12–14.2(–15) × (2.8–)3.5–3.7(–3.8) µm ($\bar{x}$±SD = 13.2±1.1 × 3.6 ±0.1, n = 30).

Pycnidia on alfalfa twigs on WA: globose 200–250 µm diam, erumpent at maturity, up to 400–500 µm diam; walls 60–150 µm diam, parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* cylindrical, hyaline, smooth, branched, ampulliform, straight to sinuous, 20–45 × 2–2.4 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, with slight tapering towards apex, 0.5–1 µm diam. *Paraphyses* abundant among conidiophores 20–40 × 1–2 µm. *Alpha conidia* aseptate, hyaline, smooth, ovate to ellipsoidal, biguttulate, base subtruncate (6.3–)7–8(8.7) × 2–2.5 µm ($\bar{x}$±SD = 7.5±0.4 × 2.2±0.2, n = 30). *Beta conidia* aseptate, hyaline, smooth, fusiform or hooked, base subtruncate, 27–31(–35.2) × (3–)3.4–3.8(–4.2) µm ($\bar{x}$±SD=29.5±2 × 3.6±2 n = 30). *Gamma conidia* aseptate, hyaline, smooth, fusiform, mostly biguttulate, base subtruncate (10–) 14–15 × 1–2 µm ($\bar{x}$ ±SD = 14.4±0.2 × 1.7±0.24, n = 30).

Notes. a, b. Ectostroma and perithecia on *Laburnum anagyroides*; c. perithecia in transverse section; d. perithecial wall in longitudinal section; e, f. perithecia in longitudinal section; g–j. asci; k. ascospores; l. conidiophores developing on alfalfa stem in culture; m. alpha and beta conidia developing on alfalfa stem in culture; a-k. Epitype specimen BPI 748231. l–n. ex-epitype culture AR3422 = CBS 109292.—Scale bars: a = 2000 µm, b, c =1000 µm, d = 50 µm, e, f = 100 µm, g–k = 25 µm, l–n = 15 µm.

Figure 4.8 Morphology of *Diaporthe rudis*.

Cultural characteristics: In dark at 25 ºC for 1 wk, colonies on PDA relatively slow growing, 4.2 mm/day, white, fluffy aerial mycelium, reverse with yellow pigmentation developing in centre.

Host range: *Acer*, *Asphodelus albus*, *Aucuba japonica*, *Brugmansia*, *Castanea*, *Corylus*, *Dipsacus fullonum*, *Epilobium*, *Eucalyptus*, *Fagus*, *Fraxinus*, *Holcus*, *Hydrangea*, *Ileostylis*, *Laburnum*, *Lupinus*, *Malus*, *Protea*, *Pyrus*, *Rosa*, *Sambucus*, *Salix*, *Vaccinium* and *Vitis vinifera*.In addition to the hosts on the specimens listed below, these hosts are represented in Figure 4.1 based on ITS phylogeny and Gomes et al. (2013) as *Diaporthe viticola*.

Distribution: Australia, Canada, Chile, Europe (Austria, Germany, Italy, Latvia, Portugal, Sweden, Switzerland, Spain), New Zealand, and South Africa.

Type material examined: FRANCE, on a dead branch of *Laburnum anagyroides* (as *Cytisus laburnum)*, ex herb. Guépin no. 163 (holotype of *Sphaeria rudis* UPS F-004948). AUSTRIA, Vienna, 19. 7763/2, Reisenbergbach-Weg, on stem of *Laburnum anagyroides*, 8 Apr. 2000, W. Jaklitsch (epitype of *Sphaeria rudis* designated here BPI748231, ex-epitype culture AR3422 = CBS 109292; MBT175965). GERMANY, Nordrhein-Westfalen, Landkreis Unna, Cappenberg, Schloßgarten zu Cappenberg, on twigs of *Laburnum anagyroides* (syn. *Cytisus laburnum*), 18 Aug. 1866, T. Nitschke (Holotype of *Diaporthe medusaea* B 70 0009168). AUSTRIA, Vienna, 19. 7763/2, Reisenbergbach-Weg, on stem of *Laburnum anagyroides*, 8 Apr. 2000, W. Jaklitsch (epitype of *Diaporthe medusaea* designated here BPI748231, ex epitype culture AR3422 = CBS 109292; MBT175966). GERMANY, Nordrhein-Westfalen, Munsterland, Munster Botanischer Garten, on thin branch of *Fagus sylvatica*, 18 May 1866, T. Nitschke (holotype of *Diaporthe macrostoma* B 70 0009167). GERMANY, Westfalen, Munster, bei der Wienburg, on *Vitis vinifera*, Feb. 1866, Nitschke, (holotype of *Diaporthe viticola* B: not seen), *ibid*. (isotype BPI797316). PORTUGAL, Santo Tirso, Burgaes, on *Vitis vinifera*, 16 Feb. 1998, A.J.L. Phillips (epitype of *D. viticola* designated in Niekerk et al. (2005) CBS-H 7950 not seen, ex-epitype culture STE-U 5683 = CBS 113201). ITALY, "In sarmentis *Vitis viniferae silvestris*, *Cervarese*" (holotype of *Diaporthe silvestris*: PAD 228 not seen). The synonymy of this name is based on Niekerk et al. (2005) in which the holotype specimen was observed and considered to be *D. viticola*.

Additional material examined: AUSTRIA, Vienna, stem of *Rosa canina*, 13 May 2001, W. Jaklitsch (BPI 840948, living culture AR3654); Vienna, stem of *Acer pseudoplatanus*, 31 Mar. 2001, W. Jaklitsch (BPI840940, living culture AR3634). GERMANY, urban residential area, container plant, dead stem of *Brugmansia* sp., 31 Oct. 2012, R. Schumacher (BPI 892463, living culture DA243=CBS 135435). ITALY, on dead stem of *Acer opalus*, 2 May 2012, E. Camporesi ER285 (BPI 892464, living culture ER285A=CBS 135437); *ibid* (ER 286, BPI 892465, living culture ER286D).

Notes: The name *Diaporthe rudis* is based on the oldest epithet of the many synonyms for this species including *D. medusaea*. *Diaporthe medusaea*, originally described from *Laburnum anagyroides* in Germany, has been used as the name for the fungus causing melanose and stem end rot of *Citrus* in North America. We observed holotype material as well as isolates on the same host from Austria in order to recognise the similarities or differences as discussed herein. Wehmeyer, (1933) listed a number of synonyms for *D. medusaea* including *D. citri*, *D. citrincola*, *D. faginea*, *D. rudis* and *D. viticola*. *Diaporthe citrincola* is here recognised as a synonym of *D. citri*. *Diaporthe faginea* was established as a legitimate name for *Sphaeria faginea* Curr., Trans. Linn. Soc., London 22: 281. 1859 nom. illeg. non *S. faginea* Pers. 1794. No specimen as *D. faginea* exists in PAD. Based on an ITS sequence (EF155490) of the isolate from *Fagus* in Germany and a morphological comparison of the protologue, this name is accepted as a synonym of *D. rudis*. Although *Diaporthe viticola* was recognised as a distinct taxon and characterised and epitypified using a specimen on *Vitis* by Niekerk et al. (2005), it is here determined to be a synonym of *D. rudis*.

Excluded species:

Phoma citri Sacc., Nuovo Giorn. Bot. Italiano 8: 200. 1876.

≡ *Phomopsis citri* (Sacc.) Traverso & Spessa, Bol. Da Soc. Brot. 25: 100. 1910 .

Specimens examined: ITALY, Traviso, a Vittorio, on branches of *Citrus limon*, Oct. 1873 (lectotype specimen of *Phoma citri* designated here, Mycotheca Veneto no. 332, FH labeled *Diplodia citri*; MBT175967).

Notes: *Phoma citri* has been confused with *Phomopsis citri*, now regarded as *Diaporthe citri*. Examination of type material of *Phoma citri* confirms that this taxon is not a *Phomopsis* and should be treated as a distinct taxon in the genus *Phoma*.

Diaporthe foeniculacea (Mont.) Niessl, in Thümen, Instituto Rev. Sci. Litt. Coimbra 27: 250. 1879.

Basionym. *Sphaeria foeniculacea* Mont., Annls Sci. Nat., Bot., sér. 3 11: 40. 1849.

≡ *Physalospora foeniculacea* (Mont.) Sacc. [as '*foeniculata*'], Syll. fung. (Abellini) 1: 445. 1882.

≡ *Sphaerella foeniculacea* (Mont.) Cooke [as '*foeniculata*'], J. Bot., Lond. 21: 70. 1883.

≡ *Guignardia foeniculacea* (Mont.) Arx & E. Müll. [as '*foeniculata*'], Beitr. Kryptfl. Schweiz 11(no. 1): 48. 1954.

Material examined: PORTUGAL, Coimbra, on stem of *Foeniculum officinalis*, Jun 1881, (lectotype specimen of *Sphaeria foeniculacea* designated here: in Thümen, Mycotheca Universalis 2260, bound collection in BPI; MBT175968). Isolectotypes: ibid. (BPI 616247, BPI 797288 Shear Types and Rarities).

Notes: One of the names mentioned in Phillips (2003) is *Diaporthe foeniculacea* (Mont.) Niessl (basionym *Sphaeria foeniculacea* Mont.), a name that has been confused with *Phomopsis foeniculina* (basionym *Phoma foeniculina*). We observed three isotype specimens of *D. foeniculacea* in BPI and confirmed the status of this species as a *Guignardia* (sexual morph of *Phyllosticta*) as suggested by Von Arx and Müller (1954), unrelated to *Diaporthe*. One of these specimens is here designated the lectotype.

4.4 Discussion

Melanose and stem end rot of *Citrus* have been reported from the United States since the late 18th century killing twigs and causing a minor form of gummosis by latent infection (Floyd & Stevens, 1912; Fawcett, 1936). A disease of *Citrus* to which the common name melanose is applied was first recognised near Citra, Florida,

by Swingle and Webber, in 1892 (Floyd & Stevens, 1912). The stem end rot disease was investigated in Florida by Burger (1923) and Winston et al. (1923). *Phomopsis citri* was first described from the United States (Florida) as a pycnidial fungus on dead branches and decayed fruits of *Citrus aurantium, C. decumana* and *C. nobilis* (Fawcett, 1912). This name is a later homonym of *Phomopsis citri* (Sacc.) Traverso and Spessa (1910) based on *Phoma citri* Sacc. (1876) originally described from *Citrus limonia* in Italy and now considered to belong in *Phoma*. *Diaporthe citri* (H.S. Fawc.) F. A. Wolf, described as sexual morph of *Phomopsis citri* H.S. Fawc., was originally collected from the United States (Florida) and has since been reported as saprobic or parasitic on leaves, stems and fruits of *Citrus* spp. throughout the world. Fisher (1972) used the concept of Wehmeyer (1933) and considered *Phomopsis cytosporella* as the valid name for *D. citri* based on the chronology of names. However, this interpretation was not adopted by plant pathologists or taxonomists. In our study *P. cytosporella* is determined to be a distinct taxon in *Diaporthe, D. cytosporella* and epitypified based on fresh collections from Europe.

In addition to the association with *Citrus* in United States, *Diaporthe citri* is confirmed here in Brazil, China, Korea and New Zealand and appears to be widespread in Asia, Australasia and South America. Based on sampling in this study, we did not find *D. citri* to occur in Europe or any sequences in public databases corresponding to *D. citri* from Europe. However, our results suggest that this species may be pantropical. The genetic similarities of the isolates of *D. citri* from Asia where *Citrus* originated with those found worldwide suggest a long standing co-existence and the probable widespread movement of the pathogen with its host. *Diaporthe citri* was the dominant species causing melanose and stem end rot symptoms among the recent collections from *Citrus* spp. throughout China (Huang et al., 2013). Two newly described species discovered in north central China (Shaanxi Province) in the same study, *Diaporthe citiasiana* and *D. citrichinensis*, were primarily associated with dead wood of *Citrus unshiu* (satsuma mandarin) and not with melanose and stem end rot diseases (Huang et al., 2013). Gomes et al. (2013) included two species from *Citrus* from Suriname in their analysis, *Diaporthe arecae* and an unidentified species. None of these species were encountered in this study. This indicates that numerous species are associated with *Citrus* worldwide and it is likely that more will be discovered.

Diaporthe foeniculina (referred to as *D. foeniculacea* in Gomes et al., (2013), see taxonomy section) is found to be a pathogen with diverse hosts ranging from crops to temperate woody plants and fruit trees. The recent reports of *Diaporthe foeniculina* (as *D. neotheicola*) causing diseases of temperate cultivated fruit trees including shoot blight of persimmon in Australia and kiwifruit disease in Greece suggest potential for this species to infect a wide range of fruits as an opportunistic pathogen (Golzar, Tan, Shivas & Wang, 2012; Thomidis et al., 2013). Although a number of isolates from *Citrus* in California are identified in this study as *D. foeniculina*, its pathogenicity on *Citrus* in California is unknown. Herbarium specimens previously identified as *D. citri* intercepted at ports in the United States on the fruits of *Citrus limonia* from Spain were identified morphologically as *D. foeniculina* (BPI 615878: intercepted in New York - 1940, BPI 1107900, BPI 747926, BPI 747927: intercepted in New Jersey, 1987). A living culture (MEP1289-1) from BPI 1107900 was used in the phylogenetic analyses in this study and is confirmed as *D. foeniculina*.

Phylogenetic analysis of ITS sequence data was able to resolve the closely related species *D. canthii*, *D. cytosporella*, *D. foeniculina* and *D. pterocarpi*. Although the ITS sequence analysis resolved a clade corresponding to the recently described *D. rhusicola*, this clade was not supported by the ACT, CAL, EF1-α or TUB gene regions, individually or in the multi-gene phylogeny. Inspection of the ITS sequences for this group of isolates (*D. foeniculina* and *D. rhusicola*) indicated that the ITS differences consisted of two deletions and one transition in the ITS1 and two transitions in the ITS2 region. Alternatively, in the case of *D. cynaroidis* and *D. rudis*, ITS sequences do not definitively distinguish the two species although EF1-α, CAL, HIS, and TUB do distinguish the two species.

In the case of *D. rudis* (referred to as *D. viticola* in Niekerk et al., 2005 and Gomes et al., 2013) and *D. australafricana*, the ITS1 region differs by a single transition while the ITS2 region shows minor differences over a span of approximately 7 bp consisting of three transitions, one transversion and one deletion. The CAL gene differs by one transition and isolates of *D. rudis* share a 9 bp insertion in ACT not found in *D. australafricana*. Data from Gomes et al. (2013) show a three bp insertion in isolates of *D. australafricana* not shared by *D. rudis* in HIS. Isolates

with ITS sequences matching that of *Diaporthe australafricana* were previously only known from Africa and Australia on *Vitis* but have recently been found on *Vaccinium* and *Corylus* in Chile and *Persea* from California in USA (Latorre et al., 2012, Elfar et al., 2013). Although closely related, the low but consistent variation found in the sampled genes is considered sufficient to recognise *D. australafricana* as a distinct phylogenetic species in agreement with Niekerk et al. (2005), Udayanga et al. (2012a) and Gomes et al. (2013). However, additional isolates of both taxa would be desirable in order to further investigate population structure and species boundaries.

Much confusion in the literature exists in how to interpret the ITS sequences of closely related species in *Diaporthe* and authors treat observed variation in ITS sequences in different ways (Farr et al. 2002a; 2002b; Murali et al., 2006; Santos et al., 2010). This can result in superfluous, multiple terminal branches in combined gene analyses, even when other gene regions do not support these distinctions. Additionally Santos et al., (2010) showed the occurrence of two phylogenetically distinct ITS populations within an unidentified species of *Diaporthe* based on the sequencing of single ascospore-derived isolates from the same perithecium. Sequence differences were confined to the ITS1 region over a span of approximately 40 bp and are more extensive than those differences noted among isolates of a single species in this study. Sequence differences were not noted in the EF1-α and mating type genes in their analyses and the isolates were fully reproductively compatible (Santos et al., 2010).

While we consider ITS rDNA sequences to be useful as barcodes for identification of known, circumscribed *Diaporthe* species, we suggest that caution is warranted when differences are noted in the absence of other data and that at the minimum EF1-α should be used to confirm identities of *Diaporthe* species. The EF1-α gene region amplified by primers EF1-728F/EF1-986R has given the most consistent results when analysing available ex-type sequences of reliably identified and vouchered species (Castlebury, 2005; Santos et al., 2010; 2011; Udayanga et al., 2012a). Phylogenetic signals observed in protein coding genes are generally considered superior to rRNA genes, although there is less standardisation in terms of universal primers, genes or even regions of genes sequenced for fungi (Schoch et al., 2012). However, results from this study suggest that use of CAL and TUB genes can

be problematic with the potential for incorrect species identification in *Diaporthe* due to non-specific priming and the resulting poor sequence quality (CAL) or potential paralogy (TUB), necessitating critical evaluation of sources of conflict in gene trees.

Epitypification is recognised as the best approach to resolve long standing taxonomic and phylogenetic issues of known taxa that are poorly circumscribed, thus providing a modern interpretive type (Hyde & Zhang, 2008). There is an unprecedented need for mycologists to return to the field to recollect species, fully characterise taxa with DNA sequences and morphological descriptions and epitypify species in *Diaporthe*, which includes a large number of names, not linked to DNA sequence data or ex-type cultures (Hyde et al., 2010a; 2010b; Ko-Ko et al., 2011; Udayanga et al., 2011). Several modern studies aimed at epitypification of important pathogens in *Diaporthe* provide clarification and knowledge of the phylogeny and species boundaries within the genus (Castlebury et al., 2003; Niekerk et al., 2005; Rensburg et al., 2006; Diogo et al., 2010; Udayanga et. al., 2012b; Gomes et al., 2013). Application of genealogical concordance and/or *gsi* in combination with epitypification can provide critical insights into speciation processes, ecology and host associations (Sakalidis, Hardy & Burgess, 2011; Gazis, Rehner & Chaverri, 2011; Mejia, Rossman, Castlebury & White, 2011; Silva et al., 2012b; Walker et al., 2012b).

Species circumscription should only be undertaken in conjunction with rigorous application of multi-gene analyses and genealogical concordance. In addition best practices for introduction of new names should require a thorough investigation of existing names and type specimens. This is particularly important to prevent additional superfluous names in a genus such as *Diaporthe* with an abundance of existing names including those in *Phomopsis*. A total of 2453 results are returned when searching for *Diaporthe* and *Phomopsis* ITS sequences in the NCBI GenBank databases. A search using the terms ‘Phomopsis sp.’ (Organism) AND ‘internal transcribed’ (All Fields) returned 922 sequences; substituting ‘Diaporthe sp.’ returned 341 sequences (retrieved 3 April 2013), which suggests that approximately 51 % of all *Diaporthe* and *Phomopsis* ITS sequences in GenBank are unidentified. Of the 49 % that do have species names, many do not include culture or specimen vouchers and it is likely that the majority are misidentified or named without a proper taxonomic

revision. As sequence data accumulate in public databases, the need for correctly identifying and vouchering sequences, particularly from ex-type or epitype cultures, becomes pressing as these data are being used for identification of pathogens by plant pathologists and in public health and quarantine situations.

4.5 Conclusion

In the present study, the *Diaporthe* species causing melanose and stem end rot diseases of *Citrus* spp. are revised. Three species of *Diaporthe* occurring on *Citrus* are characterised, including *D. citri*, *D. cytosporella* and *D. foeniculina.* Morphology and phylogenetic analyses of the complete nuclear ribosomal internal transcribed spacer regions and partial sequences of actin, beta-tubulin, calmodulin and translation elongation factor 1-alpha were used to resolve species on *Citrus* and related *Diaporthe* species. *Diaporthe citri* occurs on *Citrus* throughout the *Citrus*-growing regions of the world. *Diaporthe cytosporella* is found on *Citrus* in Europe and California (USA). *Diaporthe foeniculina,* including the synonym *D. neotheicola*, is recognised as a species with an extensive host range including *Citrus*. *Diaporthe medusaea*, a name widely used for *D. citri*, was determined to be a synonym of *D. rudis*, a species with a broad host range. *Diaporthe citri* is delimited based on molecular phylogenetic analysis with the inclusion of the conserved ex-type and additional collections from different geographic locations worldwide. *D. cytosporella*, *D. foeniculina* and *D. rudis* are epitypified, fully described and illustrated with a review of all synonyms based on molecular data and morphological studies. Newly designed primers are introduced to optimise the amplification and sequencing of calmodulin and actin genes in *Diaporthe*.

CHAPTER 5

SPECIES LIMITS IN *Diaporthe* II: MOLECULAR RE-ASSESSMENT OF PATHOGENS ON SOYBEAN AND OTHER HERBACEOUS CROPS

5.1 Introduction

Plant pathogenic fungi cause a wide array of diseases on important crops leading to severe losses of yield worldwide (Anderson et al., 2004; Strange & Scott, 2005; Rossman, 2009). Species of *Diaporthe* Nitschke are the causal agents of seed decay, seed rot, pod and stem blight and stem canker of soybean (*Glycine max* (L.) Merrill) in soybean growing countries worldwide, causing more losses than any other single fungal pathogen or species complex on this important crop (Lehman, 1923; Kulik, 1984; Sinclair, 1993; Dorrace & Mills, 2009). The species of *Diaporthe* associated with major diseases have been described in taxonomic revisions based primarily on morphology or characteristic disease symptoms and later based on molecular phylogeny (Hobbs, Schmitthenner & Kuter, 1985; Mengistu, Castlebury, Smith, Ray & Bellaloui, 2009; Santos et al., 2011). Much confusion and instability of species recognition exist regarding *Diaporthe* on soybean due to varying taxonomic opinions based on morphology, host-pathogen interactions, physiological factors and genetic variability (Morgan-Jones, 1989; Udayanga et al., 2011).

A detailed account of stem and pod blight of soybean from North Carolina in the USA was provided by Lehman (1923) include a description of *Diaporthe sojae*. This was the first comprehensive account of *Diaporthe* on soybean

with reports of the sexual and asexual states of these taxa. According to the protologue, this pathogen can infect stems, pods and leaves, however, is seldom found on pods when not also present on some other parts of the plant (Lehman, 1923). Wolf and Lehman (1926) discovered the sexual state of the fungus from overwintered soybean stems following the initial description from culture. Six decades after the discovery of the pod and stem blight pathogen on soybean, Hobbs et al. (1985) described a pathogen causing Phomopsis seed decay (PSD), *Phomopsis longicolla*, from Wooster, Ohio, in the USA, which is the major cause of poor seed quality and loss of yield. PSD has been a considerable thereat in most soybean-growing countries, especially in the mid-southern region of the USA (Li & Chen, 2013). The yield loss due to PSD on a worldwide basis was about 0.19 million metric tons (MMT) in 1994 (Kulik & Sinclair, 1999) and yield losses in USA from 1996 to 2007 ranged from 0.43–0.38 MMT (Wrather & Koenning, 2009, Li, Hartman & Boykin, 2010; Li & Chen, 2013). Due to the prevalence of hot and humid environment conditions in 2009, PSD caused over 12 million bushels of yield loss in 16 states of USA from pod fill to harvest (Koening, 2010).

Hobbs et al. (1985) clearly distinguished the seed decay pathogen, *P. longicolla*, from *Diaporthe sojae* based on morphology and provided pathological and ecological notes about this fungus. PSD of soybean has been recognised as a prototype for the study of seed diseases, with research on its biology, distribution and impact on seed quality (Sinclair, 1993; Zorrilla, Knapp & McGee, 1994, Dorrance & Mills, 2009). Santos et al. (2011) synonymised *P. longicolla* to *Diaporthe longicolla* considering the priority of the older generic name and based on a molecular re-assessment of *Diaporthe* species from Croatia, however, ex-type sequences were not included in their phylogenetic analysis. A sexual morph for *Diaporthe longicolla* has never been reported (Hobbs et al., 1985; Li & Chen, 2013), although both mating type genes (MAT1-1-1 and MAT 1-2-1) were present in the genome of single isolate (Santos et al., 2010; 2011). In contrast, *D. sojae* has been described as a homothallic species with abundant perithecia formed in culture. Seeds infected with *D. longicolla* range from symptomless to shrivelled, elongated, cracked and often appear as chalky white. Although other species can co-occur with PSD, the characteristic disease symptoms are primarily caused by *Diaporthe longicolla* (Hartman et al., 1999).

Diaporthe longicolla is primarily known as a seed-borne pathogen, however, the species has also been isolated from other parts of infected plants in the field including stem and pods. Therefore, *D. longicolla* is generally recognised as a highly aggressive, widely occurring and purely asexual species, with a known ex-type culture and definitive morphological characters (Santos et al., 2011). Thus, the synonymy of *Diaporthe longicolla* under *D. sojae* by Gomes et al. (2013) is uncertain.

Diaporthe phaseolorum is the name linked to the causative agent of pod and stem blight in soybean previously and the species was originally described as *Sphaeria phaseolorum* from *Phaseolus* in the eastern USA. Harter (1917) provided a comprehensive account of the pod blight of Lima beans (*Phaseolus lunatus*) caused by *Diaporthe phaseolorum*, with ecological and taxonomic revision. Wehmeyer (1933) re-defined *Diaporthe phaseolorum* associated with the pod blight of Lima beans in New Jersey and other places eastern USA recognizing *Diaporthe sojae* and *D. batatae* as varieties of *D. phaseolorum*. Wehmeyer (1933) recognised the varieties *D. phaseolorum* var. *sojae* and *D. phaseolorum* var *batatae* (as '*batatis'*) based on the variability of stromatic development and size of pycnidia and conidia as well as differences in hosts. Wehmeyer's recognition of these varieties of *D. phaseolorum* was accepted by subsequent researchers (Hilderbrand & Koch, 1947; Luttrell, 1947; Weltch & Gliman, 1948; Kulik, 1984) although some have been questioned their validity (Morgan-Jones, 1984). For example, until recently, both *Diaporthe phaseolorum* and *D. phaseolorum* var *sojae* has been used for the fungus causing pod and stem blight of soybean (Kmetz, Schmitthenner & Ellett, 1978; Zhang et al., 1997; Mengistu, Smith, Bellaloui, Paris & Wrather, 2010; Sun et al., 2012). *Diaporthe sojae* (≡ *D. phaseolorum* var. *sojae*) is known as the major causative agent of pod and stem blight, however relatively weak pathogen on seeds (Hobbs et al., 1985; Santos et al., 2011). Among the other major species associated with soybean, *Phomopsis glycines* was regarded as a synonym of *D. sojae*, and *P. phaseoli* is considered a *nomen dubium* due to lack of informative structures on the type specimen (Hobbs et al., 1985) and thus is not widely used in recent literature.

In different studies two other taxa have been introduced associated with soybean, namely *Diaporthe phaseolorum* var. *caulivora* (*Dpc*) from Indiana, USA, causing northern stem canker (Athlow & Caldwell, 1954), and *D. phaseolorum* var.

meridionalis (*Dpm*) from Georgia, USA, causing southern stem canker (Fernandez, 1996). Both were previously considered as varieties of *D. phaseolorum*. *Diaporthe phaseolorum* var. *caulivora* was later raised to species rank as *D. caulivora* with a neotype designated (Santos et al., 2011), and *D. phaseolorum* var. *meridionalis* is considered a synonym of *D. aspalathi* (Rensburg et al., 2006). Therefore, the systematics of the southern and northern stem canker pathogens, *D. aspalathi* and *D. caulivora,* is relatively known with the support of recent molecular evidence and phylogenetic placement apart from *D. phaseolorum* and allied taxa within the genus, thus they are not considered in detail in the present study (Santos et al., 2011; Gomes et al., 2013).

Several important species associated with herbaceous crops and weeds are found to be closely related within the species complex resolved in this study, including species occurring on members of the plant families *Apiaceae*, *Asteraceae*, *Convolvulaceae*, *Cucurbitaceae*, *Fabaceae* and *Solanaceae* (Rehner & Uecker, 1994; Castlebury & Mengistu, 2006; Udayanga et al., 2012a). The application of appropriate names to the genetically distinct taxa associated with herbaceous crops has long been problematic due to the absence of distinct morphological and/or cultural characters and biological information (Castlebury et al., 2001). In this study, we revisited the species associated with soybean with molecular phylogenetic analysis and phenotypic characteristics of numerous fresh isolates from USA and elsewhere including ex-type cultures and specimens. Additionally, the species of *Diaporthe arctii* from *Arctium lappa* (*Asteraceae*), *D. batatas* from *Ipomoea batatas* (*Convolvulaceae*) and *D. melonis* (= *Phomopsis cucurbitae*) from *Cucurbitaceae* are also revised.

The aims of this study are 1) to re-define the species limits of soybean pathogens and allied taxa including *Diaporthe arctii*, *D. batatas*, *D. cucurbitae*, *D. longicolla*, *D. melonis*, *D. phaseolorum*, *D. sojae* and a new species described here, *D. ueckerae* based on molecular phylogenetic analysis incorporating ex-type or ex-epitype sequences; 2) to provide modern descriptions and illustrations for the species re–defined and newly described; 3) to re-evaluate the genes used to delineate the species within this complex. For each species accurate scientific names including synonymys are provided along with host range, distribution, and notes on the taxonomy and phylogeny with a comprehensive revision of taxonomic issues of

Diaporthe associated with soybean and other herbaceous crops. The evolutionary relationships inferred in this study and the new taxonomic and nomenclatural clarifications will be useful for scientists working on various aspects of phytopathology, disease control, genomics and development of resistant varieties of soybean.

5.2 Materials and methods

5.2.1 Fungal sampling and morphology

Isolates used in this study are listed in Table 5.1. The illustrations were based on the type and epitype specimens or sporulating pycnidia on WA+alfalfa stem media in culture. Digital images were captured and colony growth rates and characters were determined as described in Udayanga, Castlebury, Rossman and Hyde (2014).

5.2.2 DNA extraction, PCR and sequencing

DNA extraction and the ITS, EF1-α, CAL and TUB genes were amplified following the conditions outlined by Udayanga et al. (2014). HIS (Histone- 3) gene was amplified as described in Gomes et al. (2013) using the primer pair CYLH3F (Crous et al., 2004b) and H3-1b (Glass & Donaldson, 1995).

5.2.3 Sequence alignment and phylogenetic analysis

Raw sequences were assembled with Sequencher 4.9 for Windows (Gene Codes Corp., Ann Arbor, Michigan). The sequences were then initially aligned using MAFFTv.7 (Katoh & Standle**y,** 2013) and optimised manually when needed. Newly generated ITS and EF1- α sequences were analysed with all available type-derived sequences listed in Udayanga et al. (2011; 2012a) and Gomes et al. (2013) to determine the primary identity of the isolates for further phylogenetic analyses.

Table 5.1 Isolates and gene sequences used in this study

Species	Isolate/culture collection	Host family	Host	Country	Collector	GenBank Accessions				
						ITS	EF1-α	TUB	CAL	HIS
D. angelicae	DP0488	*Apiaceae*	*Angelica sylvestris*	Austria	W. Jaklitsch	KJ590735	KJ590775	KJ610890	KJ612132	KJ659217
	CBS 111591, AR 3724	*Apiaceae*	*Heracleum sphondylium*	Austria	W. Jaklitsch	KC343026	KC343752	KC343994	KC343268	KC343510
	CBS 111592, AR3776	*Apiaceae*	*Heracleum sphondylium*	Austria	W. Jaklitsch	KC343027	KC343753	KC343995	KC343269	KC343511
D. arctii	DP0482	*Asteraceae*	*Arctium lappa*	Austria	W. Jaklitch	KJ590736	KJ590776	KJ610891	KJ612133	KJ659218
D. batatas	CBS 122.21	*Convolvulaceae*	*Ipomoea batatas*	USA	L.H. Harter	KC343040	KC343766	KC344008	KC343282	KC343524
D. convolvuli	CBS 124654, DP0727	*Convolvulaceae*	*Convolvulus arvensis*	Turkey	D. Burner	KC343054	KC343780	KC344022	KC343296	KC343538
	FAU649	*Convolvulaceae*	*Convolvulus arvensis*	Canada	J. Ormeno-Nunez	KJ590721	KJ590765	In proc.	KJ612130	KJ659210
D. cuppatea	CBS 117499, STE-U 5431	*Fabaceae*	*Aspalathus linearis*	South Africa	J.C. Janse van Rensburg	KC343057	KC343783	KC344025	KC343299	KC343541
D. cucurbitae	DAOM42078	*Cucurbitaceae*	*Cucumis sativus*	Canada	C.D. McKeen	KM453210	KM453211	In proc.	In proc.	KM453212
	CBS 136.25	*Asteraceae*	*Arctium* sp.	Unknown USA?	W.A. Archer	KC343031	KC343757	KC343999	KC343273	KC343515
D. endophytica	CBS 133811, LGMF916	*Anacardaceae*	*Schinus terebinthifolius*	Brazil	J .Lima	KC343065	KC343791	KC344033	KC343307	KC343549
	LGMF948	*Fabaceae*	*Glycine max*	Brazil	A. Almeida	KC343072	KC343798	KC344040	KC343314	KC343556

Table 5.1 (continued)

Species	Isolate/culture collection	Host family	Host	country	collector	GeneBank Accessions				
						ITS	EF1-α	TUB	CAL	HIS
D. ganjae	CBS 180.91,ILLS 43621	*Cannabaceae*	*Cannabis sativa*	USA:IL	J.C. McPartland	KC343112	KC343838	KC343072	KC343354	KC343596
D. gulyae	BRIP53158	*Asteraceae*	*Helianthus annuus* (wild)	Australia	S.M. Thompson	JF431284	JN645799	-	-	-
	BRIP54025	*Asteraceae*	*Helianthus annuus*	Australia	S.M. Thompson	JF431299	JN645803	-	-	-
D. helianthi	CBS 592.81	*Asteraceae*	*Helianthus annuus*,	Serbia	M. Muntañola-Cvetkovic	AY705842	KC343841	KC344083	KC343357	KC343599
D. hordei	CBS 481.92	*Poaceae*	*Hordeum vulgare*,	Norway	L. Sundheim	KC343120	KC343846	KC344088	KC343362	KC343604
D. infecunda	LGMF912,CPC 20288	*Anacardaceae*	*Schinus terebinthifolius*	Brazil	J .Lima	KC343128	KC343854	KC344096	KC343370	KC343612
	CBS 133812	*Anacardaceae*	*Schinus terebinthifolius*	Brazil	J. Lima	KC343126	KC343852	KC344094	KC343368	KC343610
D. kongii	YPT2011c=BRIP54031	*Asteraceae*	*Helianthus annuus*	Australia	S.M. Thompson	JF431301	JN645797	-	-	-
D. longicolla	FAU599, ATCC 60325	*Fabaceae*	*Glycine max*	USA:OH	T. W. Hobbs	KJ590728	KJ590767	KJ610883	KJ612124	KJ659188
	FAU644	*Fabaceae*	*Glycine max*	USA: MD	unknown	KJ590730	KJ590769	KJ610885	KJ612126	KJ659190
	FAU642	*Fabaceae*	*Glycine max*	USA: MD	unknown	KJ590733	KJ590772	KJ610888	KJ612129	KJ659193
	FAU643	*Fabaceae*	*Glycine max*	USA: MD	unknown	KJ590731	KJ590770	KJ610886	KJ612127	KJ659191
	FAU601	*Fabaceae*	*Glycine max*	USA:OH	T.W. Hobbs	KJ590729	KJ590768	KJ610884	KJ612125	KJ659189

Table 5.1 (continued)

Species	Isolate/culture collection	Host family	Host	country	collector	GenBank Accessions				
						ITS	EF1-α	TUB	CAL	HIS
D. longicolla	FAU601	*Fabaceae*	*Glycine max*	USA:OH	T.W. Hobbs	KJ590729	KJ590768	KJ610884	KJ612125	KJ659189
	FAU645	*Fabaceae*	*Glycine max*	USA:MD	unknown	KJ590732	KJ590771	KJ610887	KJ612128	KJ659192
	FAU657	*Cucurbitaceae*	*Cucumis melo*	USA:OK	?	KJ590727	KJ590766	KJ610882	KJ612123	KJ659187
	PL4, CBS 127267	*Fabaceae*	*Glycine max*	Croatia	K. Vrandečić	KC343199	KC343925	KC344167	KC343441	KC343683
	CBS 116023, STAM 35	*Fabaceae*	*Glycine max*	USA:MS	A. Mengistu	KC343198	KC343924	KC344166	KC343440	KC343682
	CBS 659.78	*Fabaceae*	*Glycine max*	USA	J. Marcinkowska	KC343201	KC343927	KC344169	KC343443	KC343685
D. lucitanicae	CBS 123212, Di-C001/5	*Apiaceae*	*Foeniculum vulgare*	Portuagal	J.M. Santos	KC343136	KC343862	KC344104	KC343378	KC343620
	CBS 123213, Di-C001/5	*Apiaceae*	*Foeniculum vulgare*	Portuagal	J.M. Santos	KC343137	KC343863	KC344105	KC343379	KC343621
D. manihotia	CBS 505.76	*Euphorbaceae*	*Manihot utilissima*	Rwanda	J. Semal	KC343138	KC343864	KC344106	KC343380	KC343622
D. melonis	CBS 507.78	*Cucurbitaceae*	*Cucumis melo*	USA:TX	L. Beraha	KC343142	KC343868	KC344110	KC343384	KC343626
	FAU640	*Cucurbitaceae*	*Cucumis melo*	USA:TX	unknown	KJ590702	KJ590741	KJ610858	KJ612099	KJ659184
	FAU641	*Cucurbitaceae*	*Cucumis melo*	USA:TX	unknown	KJ590701	KJ590740	KJ610857	KJ612098	KJ659183
	FAU626	*Cucurbitaceae*	*Cucumis melo*	USA:TX	unknown	KJ590704	KJ590743	KJ610860	KJ612101	KJ659186
	FAU628	*Cucurbitaceae*	*Cucumis melo*	USA:TX	unknown	KJ590703	KJ590742	KJ610859	KJ612100	KJ659185

Table 5.1 (continued)

Species	Isolate/culture collection	Host family	Host	country	collector	GenBank Accessions				
						ITS	EF1-α	TUB	CAL	HIS
D. neoarctii	AR3450,GB6421=CBS 109490	*Asteraceae*	*Ambrosia trifida*	USA:NJ	G. Bills	KC343145	KC343871	KC344113	KC343387	KC343629
D. novem	4-27/3-1, CBS 127270	*Fabacae*	*Glycine max*	Croatia	T. Duvnjak	KC343156	KC343882	KC344124	KC343398	KC343640
	DP0378	*Apiaceae*	*Daucus carota*	New Zealand	Wadia Kandula	KJ590737	KJ590773	KJ590773	KJ612134	KJ659219
	CBS 127269, 5-27/3-1	*Fabacae*	*Glycine max*	Croatia	T. Duvnjak	KC343155	KC343881	KC344123	KC343397	KC343639
	5/27/3-3, CBS 127271	*Fabacae*	*Glycine max*	Croatia	T. Duvnjak	KC343157	KC343883	KC344125	KC343399	KC343641
	CBS 354.71	*Liliaceae*	*Polygonatum odoratum*	Romania	O. Constantinescu	KC343158	KC343884	KC344126	KC343400	KC343642
D. phaseolorum	AR4203	*Fabaceae*	*Phaseolus vulgaris*	USA:DE	N. F. Gregory	KJ590738	KJ590739	KJ610893	KJ612135	KJ659220
D. schini	CBS 133181, LGMF 921,CPC20297	*Anacardaceae*	*Schinus terebinthifolius*	Brazil	J. Lima	KC343191	KC343917	KC344159	KC343433	KC343675
D. sojae	FAU636	*Fabaceae*	*Glycine max*	USA:OH	unknown	KJ590718	KJ590761	KJ610874	KJ612115	KJ659207
	FAU637,ATCC28463	*Fabaceae*	*Glycine max*	USA:OH	unknown	KJ590720	KJ590763	KJ610876	KJ612117	KJ659209
	FAU635	*Fabaceae*	*Glycine max*	USA:OH	unknown	KJ590719	KJ590762	KJ610875	KJ612116	KJ659208
	FAU604	*Fabaceae*	*Glycine max*	USA:OH	T.W. Hobbs	KJ590716	KJ590759	KJ610872	KJ612113	KJ659205

Table 5.1 (continued)

Species	Isolate/culture collection	Host family	Host	country	collector	GenBank Accessions				
						ITS	EF1-α	TUB	CAL	HIS
D. sojae	PS03	*Fabaceae*	*Glycine max*	Croatia	K. Vrandecˇic	HM347702	HM347670	-	-	-
	Ar2	*Asteraceae*	*Arctium lappa*	Croatia	K. Vrandecˇic	HM347705	HM347679	-	-	-
	FAU499	*Asparagaceae*	*Asparagus officinalis*	USA:NJ	F. A. Uecker	KJ590717	KJ590760	KJ610873	KJ612114	KJ659206
	CBS116019, STAM 30	*Euphorbaceae*	*Caperonia palustris*	USA:MS	A. Mengistu	KC343175	KC343901	KC344143	KC343417	KC343659
	CBS116020, STAM 31	*Asteraceae*	*Aster exilis*	USA:MS	A. Mengistu	KC343176	KC343902	KC344144	KC343418	KC343660
	FAU455	*Asteraceae*	*Stokesia laevis*	USA:MS	C. Wells	KJ590712	KJ590755	KJ610868	KJ612109	KJ659201
	FAU456	*Asteraceae*	*Stokesia laevis*	USA:MS	C. Wells	KJ590713	KJ590756	KJ610869	KJ612110	KJ659202
	FAU457	*Asteraceae*	*Stokesia laevis*	USA:MS	F.A. Uecker	KJ590711	KJ590754	KJ610867	KJ612108	KJ659200
	DP0601, STAM 068	*Fabaceae*	*Glycine max*	USA:MS	A. Mengistu	KJ590706	KJ590749	KJ610862	KJ612103	KJ659195
	DP0616, STAM 167	*Fabaceae*	*Glycine max*	USA:MS	A. Mengistu	KJ590715	KJ590758	KJ610871	KJ612112	KJ659204
	FAU459, ATCC64801	*Asteraceae*	*Stokesia laevis*	USA:MS	unknown	KJ590709	KJ590752	KJ610865	KJ612106	KJ659198
	DP0605, STAM 74	*Fabaceae*	*Glycine max*	USA:MS	A. Mengistu	KJ590707	KJ590750	KJ610863	KJ612104	KJ659196
	FAU458	*Asteraceae*	*Stokesia laevis*	USA:MS	F. A. Uecker	KJ590710	KJ590710	KJ610866	KJ612107	KJ659199

Table 5.1 (continued)

Species	Isolate/culture collection	Host family	Host	country	collector	GenBank Accessions				
						ITS	EF1-α	TUB	CAL	HIS
	DP0623, STAM 204	*Fabaceae*	*Glycine max*	USA:MS	A. Mengistu	KJ590708	KJ590751	KJ610864	KJ612105	KJ659197
D. sojae (=*D. melonis* var. *brevistylophora*)	AR3602, MAFF 410444	*Cucurbitaceae*	*Cucumis melo*	Japan	T. Kobayashi, T. Oosawa	KJ590714	KJ590757	KJ610870	KJ612111	KJ659203
	AR3603, MAFF 410445	*Cucurbitaceae*	*Cucumis melo*	Japan	T. Kobayashi and T. Oosawa	KJ590705	KJ590748	KJ610861	KJ612102	KJ659194
D. sojae (=D. kochmanii)	BRIP54033	*Asteraceae*	*Helianthus annuus*	Australia	S.M. Thompson	JF431295	JN645809	-	-	-
D. stewartii	CBS 193.36	*Asteraceae*	*Cosmos bipinnatus*	USA	A.L. Harison	FJ889448	GQ250324	JX275421	JX197415	-
D. subodinaria	CBS 101711	*Plantaginacea*	*Plantago lanceolata*	New Zealand	B. Alexander	KC343213	KC343939	KC344181	KC343455	KC343697
	CBS 464.90	*Plantaginacea*	*Plantago lanceolata*	South Africa	R. Shivas	KC343214	KC343940	KC344182	KC343456	KC343698
D. tectomae	CBS 100547	*Bignoniaceae*	*Tabebuia* sp.	Brazil	A. Aptroot	KC343215	KC343941	KC344183	KC343457	KC343699
D. terebinthifolii	CBS 133180, LGMF914	*Anacardaceae*	*Schinus terebinthifolius*	Brazil	J. Lima	KC343216	KC343942	KC344184	KC343458	KC343700
D. ueckerae	FAU659	*Cucurbitaceae*	*Cucumis melo*	USA:OK	unknown	KJ590724	KJ590745	KJ610879	KJ612120	KJ659213
	FAU658	*Cucurbitaceae*	*Cucumis melo*	USA:OK	unknown	KJ590725	KJ590746	KJ610880	KJ612119	KJ659214

Table 5.1 (continued)

Species	Isolate/culture collection	Host family	Host	country	collector	GenBank Accessions				
						ITS	EF1-α	TUB	CAL	HIS
D. ueckerae	FAU656	*Cucurbitaceae*	*Cucumis melo*	USA:OK	unknown	KJ590726	KJ590747	KJ610881	KJ612122	KJ659215
	FAU660	*Cucurbitaceae*	*Cucumis melo*	USA:OK	unknown	KJ590723	KJ590744	KJ610878	KJ612121	KJ659212
	LGMF947,CPC 20323	*Fabaece*	*Glycine max*	Brazil	A. Almeida	KC343203	KC343929	KC344171	KC343445	KC343687
	CBS 119639	-	*Homo sapiens*, abscess	Germany	K. Plechulla	KC343202	KC343928	KC344170	KC343444	KC343686
D. vexans	FAU597	*Solanaceae*	*Solanum* sp.	Dominican republic	unknown	KJ590734	KJ590774	KJ610889	KJ612131	KJ659216
	CBS 127.14	*Solanaceae*	*Solanum melanogena*	USA	L.L. Harter	KC343229	KC343955	KC344197	KC343471	KC343713
Diaporthe sp. indet. 1	LGMF946, CPC20322	*Fabaceae*	*Glycine max*	Brazil	A. Almeida	KC343053	KC343779	KC344021	KC343295	KC343537
	CBS 199.39	-	unknown	Italy	G. Goidanich	KC343051	KC343777	KC344019	KC343293	KC343535
	CBS 230.52	*Rutaceae*	*Citrus sinensis*	Suriname	N.J. van. Suchtelen	KC343052	KC343778	KC344020	KC343294	KC343536
Diaporthe sp. indet. 2	FAU501, MD 1038	*Cucurbitaceae*	*Cucumis melo*	Trinidad	Unknown?	KJ590722	KJ590764	KJ610877	KJ612118	KJ659211

Phylogenetic reconstructions of concatenated and individual gene-trees were performed using Maximum Likelihood (ML), Bayesian (BI) and Maximum parsimony (MP) methods as described in section 4. 2. 4. EF1-α phylogenetic tree (see Figure 5.1 in result section), was used for the initial calibration of phylogenetically distinct lineages within the predetermined species complex within the genus and compared with the other single gene phylogenetic trees of ITS, CAL, HIS and TUB. Several well authenticated and ex-type isolates from soybean and sunflower in Australia obtained from recent publications (Santos et al., 2011, Thompson et al., 2010) were used only in the ITS and EF1-α phylogenetic trees, and not included in combined analysis due to the lack of other gene sequences

In order to establish a robust species boundaries within the complex, all the isolates were subjected to a multi-gene analysis of ITS, EF, CAL, HIS, ACT and TUB regions. Individual gene trees were tested for congruency using the 70 % reciprocal bootstrap (BS) threshold method as described by Gueidan, Roux and Lutzoni (2007). Combined trees were tested with and without including ITS to confirm whether there is any significant different within the trees. Phylogenetic trees and data files were viewed in MEGA 5 (Tamura et al., 2011), Treeview (Page, 1996) and Fig tree v1.4 (Rambaut & Drummond, 2008).

5.2.4 Species recognition

In order to determine the species limits, we applied the criteria of GCPSR (Taylor et al., 2000; Dettman et al., 2003). Dettman et al. (2003) emphasise that the species should be recognized if they satisfied one of two criteria: genealogical concordance or genealogical non-discordance. Clades were genealogically concordant if they were present at least some the gene trees and genealogically non-discordant if they were strongly supported (MP≥70%; ML≥70%) in a single gene and not contradicted at or above this level of support in any other single gene tree. This criterion prohibited poorly supported non-monophyly at one locus from undermining well-supported monophyly at another locus. A combined analysis of 5 genes was used to determine the final species boundaries with the support of all single gene trees inferred.

All the novel sequences were deposited in GenBank and the sequence alignments were submitted to TreeBASE. Taxonomic novelties and typifications were registered in MycoBank (Crous et al., 2004).

5.3 Results

5.3.1 Phylogenetic analyses

In total two hundred new sequences were generated in this study from forty isolates with other sequences obtained from GenBank. The alignment properties of the single and combined analyses of five genes used individually and in combination are summarised in Table 5.2.

Table 5.2 Comparison of alignment properties in parsimony analysis of genes, and nucleotide substitution models.

Genes/loci	**ITS***	**EF1-α**	**TUB***	**CAL***	**HIS***	**Combined ITS/EF/TUB /CAL/HIS**
Characters included (with gaps)	562	436	514	585	541	2672
Invariable characters	475	178	319	345	371	1721
Parsimony informative characters (%)	68 (12%)	217 (49%)	143 (29%)	175 (30%)	127 (23%)	730 (27%)
Uninformative polymorphic characters	19	41	52	65	43	221
Number of branches >70 % bootstrap MP/BI and ML analysis	20	26	27	28	28	30
Nucleotide substitution models for Bayesian analysis (determined by MrModeltest2)	GTR+I+G	GTR+I+G	GTR+I+G	HKY+I	GTR+I+G	GTR+I+G

The EF1-α sequence alignment comprises 436 characters including gaps among which 217 are parsimony informative and 41 are variable and parsimony uninformative. The RAxML, MP and Bayesian trees are similar in terms of topology and placement of the isolates in the analysis. The RAxML tree was used as to represent the phylogram of EF1-α (Figure 5.1). The trees were rooted with *Diaporthe manihotia* and *D. ganjae* based on the preliminary analysis within this species complex. Based on the EF1-α phylogeny and sequence alignment, 30 distinct lineages were initially recognised corresponding to the known ex-type sequences and taxonomically authenticated sequences determined.

Single gene trees constructed in three methods were compared with the EF1-α tree to determine the placement of species and the ability of each gene to resolve the species. The distinct lineages recognised in EF1-α tree were concordant with other single gene trees, except a few in which species delimitation is unclear. *Diaporthe arctii*, *D. batatas*, *D. cucurbitae*, *D. longicolla*, *D. melonis*, *D. phaseolorum*, *D. sojae*, and the new species described here, *D. ueckerae* are clearly distinguished based the single EF1-α tree. The species limits of four species closely related to *D. angelicae* including *D. cucurbitae*, *D. gulyae*, *D. stewartii* and *D. subordinaria* were determined with the support of evidence of other genes and combined analysis. The five genes analysed here did not show any conflicts in tree topology among the 70 % reciprocal bootstrap trees, which allowed us to combine them.

The combined alignment comprises 83 taxa, 2672 characters including gaps, among which 1721 characters were constant, 730 are parsimony informative and 221 variable characters are parsimony uninformative. The RAxML, MP and Bayesian trees were similar in topology and branching order. The RAxML tree generated is used to represent the phylogram of combined analysis, with the parsimony bootstrap and Bayesian posterior probability values at the nodes (Figure 5.2). In total, 30 lineages were clearly distinguished including the out group taxa. Five species from soybean were resolved in the analysis including, *D. longicolla*, *D. novem*, *D. sojae*, *D. ueckerae* and *Diaporthe* sp. indet. 1 including one isolate from soybean in Brazil). *Diaporthe phaseolorum* is represented by a single well characterised ex-epitype isolate derived from pod blight symptom of *Phaseolous* in USA. However, the occurrence of *D. phaseolorum* on soybean is yet unknown.

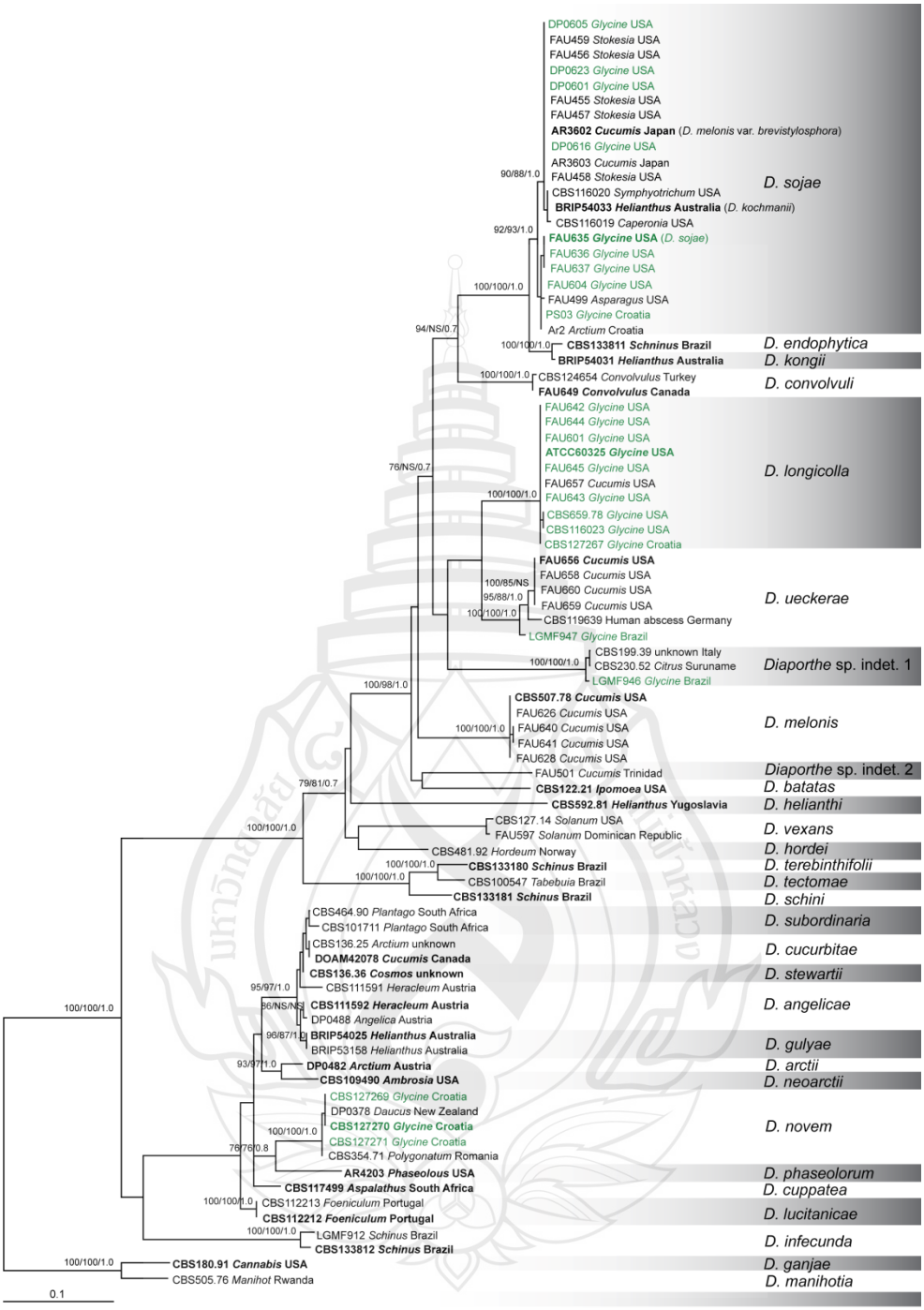


Figure 5.1 The RAxML tree generated from the analysis of the EF1-α. RAxML/MP bootstrap values/Bayesian posterior probabilities ≥70 % are displayed above or below each branch. Ex-type and ex-epitype culture numbers are in bold. Isolates from soybean are indicated in green. The tree is rooted with *D. ganjae* and *D. manihotia.*

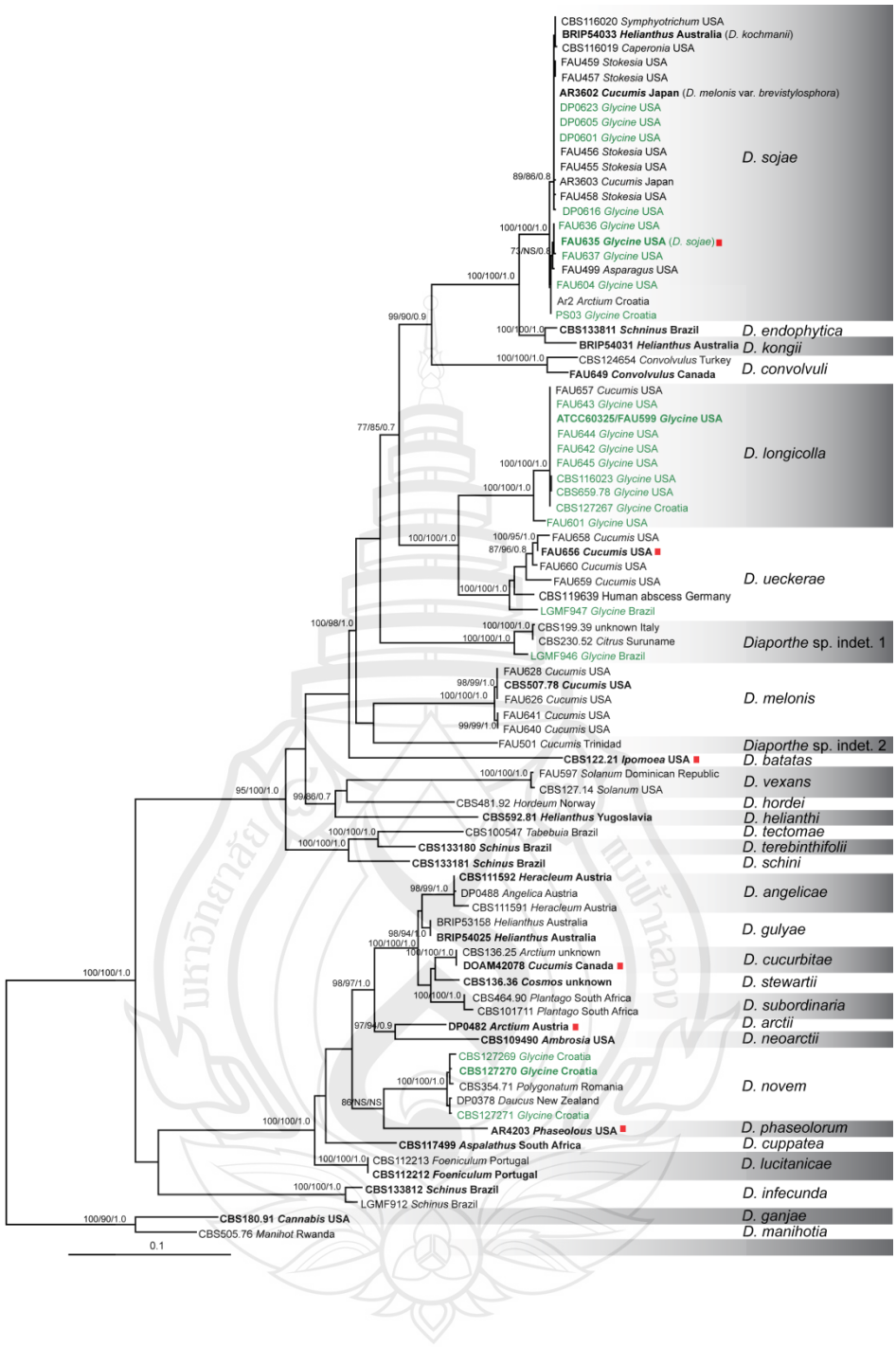


Figure 5.2 The RAxML tree generated from the analysis of the combined ITS, EF1-α, TUB, CAL and HIS. RAxML/MP bootstrap values/Bayesian posterior probabilities ≥70 % are displayed. Isolates from soybean are indicated in green. The tree is rooted with *D. ganjae* and *D. manihotia*. The ex-epi-types designated in this study and ex-type for the new species *D. ueckerae* are indicated by red squares.

The ex-type of *D. melonis* var. *brevistylophora* (AR3602, MAFF 410444) clustered with *Diaporthe sojae*. Therefore it was determined to be conspecific. *D. cucurbitae* and *D. melonis* were determined to be distinct taxa. Thus, *Diaporthe cucurbitae*, *D. melonis*, *D. sojae*, *D. ueckerae* and *Diaporthe* sp. indet 2 were found on the hosts in family *Cucurbitaceae*, mostly from *Cucumis melo*. *Diaporthe arctii* is represented by a well characterised ex-epitype isolate from *Arctium lappa* in Austria, and placed sister to the recently described new species *D. neoarctii*. One isolate from *Arctium* of unknown geographic origin clustered closely with the ex-epitype of *D. cucurbitae*, and the isolate Ar02 from *Arctium* in Croatia was identified as *D. sojae*.

The EF1-α tree and combined tree further confirmed the placement of two pathogens on sunflower (*Helianthus annuus*) in Australia as *Diaporthe gulyae* and *D. kongii* based on the ex-type isolates. Close inspection of available ITS and EF1-α sequences and placement within the combined analysis further revealed that as third sunflower pathogen from Australia, *D. kochmanii*, falls inside *D. sojae* and thus is a synonym of that species. *Diaporthe gulyae* is a distinct species closely related to *D. angelicae*. *Diaporthe endophytica* and *D. kongii* are highly similar with reference to available ITS and EF1-α sequences of ex-type isolates (ITS=99%, EF1-α=99%), however, they are not considered to be the same species in this study until additional sequence data are available for further comparison. The differences between EF1-α sequences of ex-type isolates of *Diaporthe endophytica* and *D. kongii* confined to one transition and two transversions.

5.3.2 Taxonomy

In this section we provide modern descriptions and illustrations of the taxa resolved in the molecular phylogenetic analysis including soybean pathogens and several taxa closely related within the same species complex. The common taxa associated with soybean, *Diaporthe longicolla*, *D. phaseolorum* and *D. sojae*, are re-described based on the fresh collections with notes on their ecology and pathology based on the literature. *Diaporthe arctii*, *D. batatus*, *D. cucurbitae*, *D. melonis* and a new species *D. ueckerae* are closely related species that occur on herbaceous crops for which modern descriptions and illustrations are provided based on ex-type and additional specimens.

Diaporthe arctii (Lasch) Nitschke, Pyrenomycetes Germanici 2: 268. 1870 Figure 5.3

Basionym. *Sphaeria arctii* Lasch, in Rabenh., Klotzsch. Herb. Vivum Mycol. no. 1046. 1846.

≡ *Phomopsis arctii* (Lasch) Traverso, Fl. Ital. Crypt., Pars 1: Fungi. Pyrenomycetae. Xylariaceae, Valsaceae, Ceratostomataceae: 226. 1906.

Pycnidia on alfalfa twigs on WA, globose, 300–500 μm diam, embedded in tissue, erumpent at maturity, with an elongated, black neck 200–300 μm long, often with a yellowish, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers, *Conidiophores* hyaline, smooth, branched, cylindrical to clavate, straight to sinuous, 12–15 × 1–2.5 μm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 μm diam. *Alpha conidia* abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ellipsoidal to fusiform, biguttulate or tetra-guttulate, base subtruncate, (8.5–)9.0–11.0(–12.2) × 2.8–3.2 μm ($\bar{x}$±SD =9.9±0.6 × 3.0±0.3, n=30). *Beta conidia* not seen.

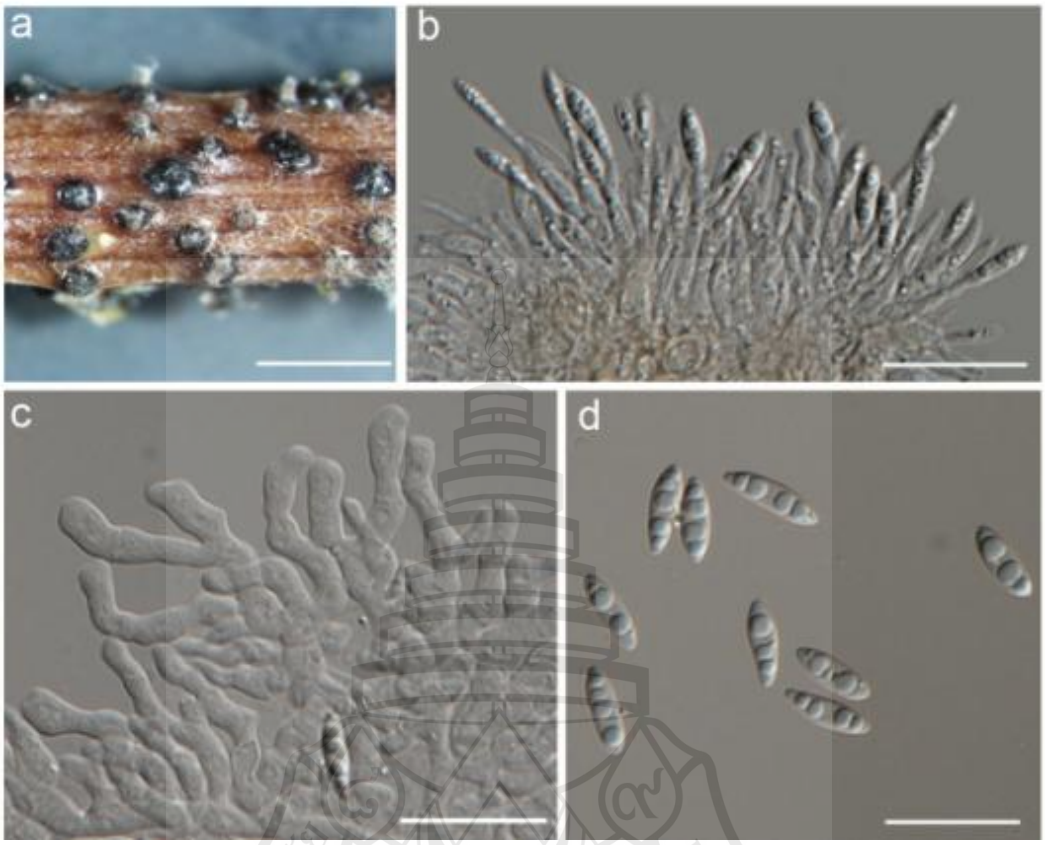
Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.4±0.2 mm/day (n=8), white, fluffy aerial mycelium, reverse centre dark green pigmentation developing in centre.

Host: *Arctium* spp.

Distribution: Austria, Czech Republic, France, Germany, Hungary, Poland, USA, UK including the host range given in Wehmeyer (1933) on *Arctium* spp.

Type material examined: *Sphaeria arctii* – GERMANY, on stem of *Arctium lappa*, Lasch, 1846, (lectotype designated here, Exsiccati specimen from FH, Klotzsch and Rabenhorst, no 1046 in Klotzschii Herbarium Vivum Mycologicum). AUSTRIA, Vienna, 22nd district, Lobau, near Oelhafen, between Lobgrundstrasse and Panozzalacke, Mapping grid square 7865/1, 15 Apr. 2003, W. Jaklitsch WJ 2079 (epitype designated here, BPI 843598, ex-epitype culture DP0482=CBS).

Additional material examined: CZECH REPUBLIC (as Czeckoslovakia), West Beskids, Bank of the Becva, near Vsetin**,** on *Arctium* sp., Sep. 1923, F. Petrak, **(**BPI615658). USA, Michigan, Steinbach Woods, Ann Arbor, on *Arctium* sp., 7 May 1922, L.E. Wehmeyer (BPI615657).



Notes. a. Sporulating pycnidia on alfalfa stem on WA; b. Conidiophores; c. Pre-conidiogenous structures; d. Alpha conidia. a–d. DP0481. Bars: a=1000 μm, b, c = 12 μm, d= 15 μm.

Figure 5.3 Morphology of *Diaporthe arctii*.

Notes: Wehmeyer (1933) considered *Diaporthe arctii* to be a ubiquitous species with 47 host forms included many species as synonyms. Later authors recognised that the broad concept of *D. arctii* is untenable when biological and ecological data are considered (Van der Aa et al., 1990). Some of the names synonymised under *D. arctii* from other different hosts will remain obscure until fresh collections from that host are subjected to molecular analyses. The oldest available epithet listed under the synonyms of *Phomopsis arctii* in *Index Fungorum*, *Sphaeria inquilina Wallr.*, is found to be an illegitimate name (Art. 53.1:McNeil et al., 2012) thus is not considered.

Herein, we designate an epitype for *D. arctii*, based on its definition in the strict sense as occurring on dead stem of *Arctium* spp. in Europe and USA. The observation of the lectotype specimen and additional material confirmed the morphological similarity of these species on *Arctium* with the epitype specimen. Several previous authors have recognised multiple species of *Diaporthe* on *Arctium* using molecular data including *D. angelicae*, *D. eres*, *D. neoarctii*, *D. sojae* and *D. cucurbitae* in this study which are clearly distinguished from *D. arctii* (see Santos et al., 2011, Gomes et al., 2013).

Diaporthe batatas [as '*batatis*'] Harter & E.C. Field, Phytopathology 2: 121. 1912. Figure 5.4

Basionym. *Diaporthe phaseolorum* var. *batatae* [as '*batatis*'] (Harter & Field) Wehm., The genus *Diaporthe* Nitschke and its segregates: 48. 1933.

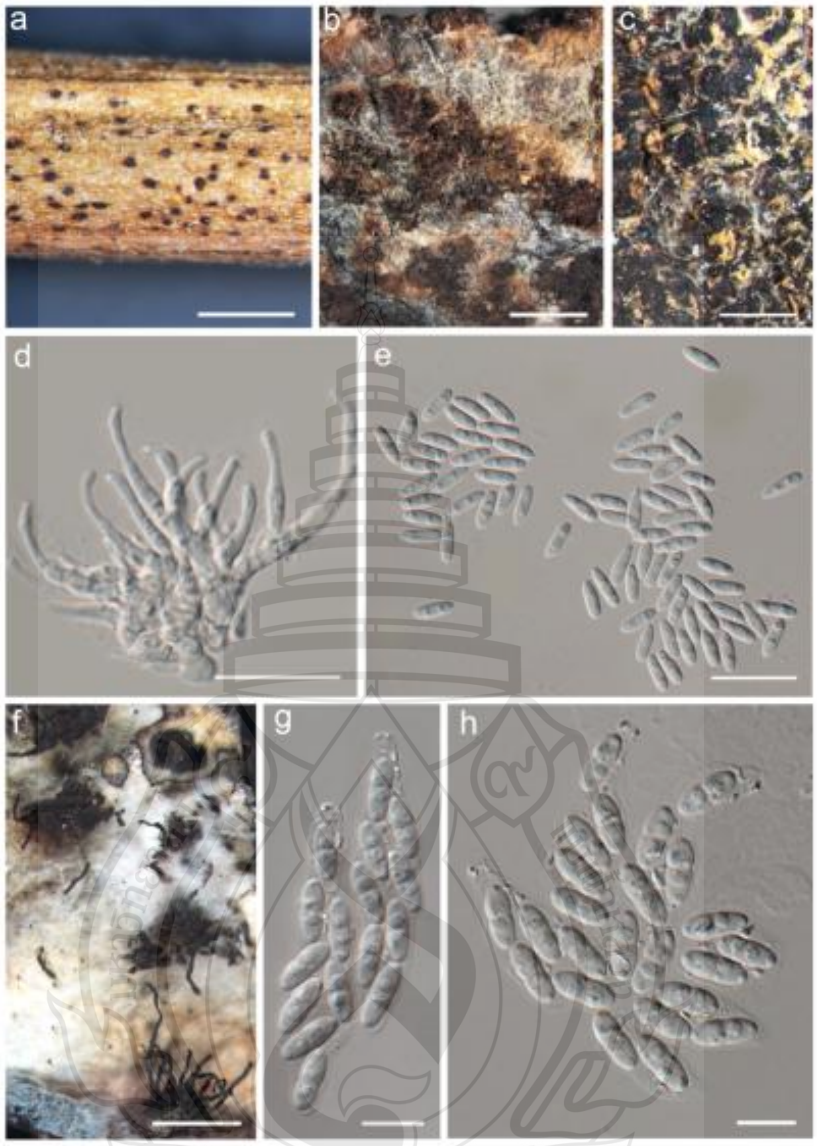
= *Phoma batatas* Ellis & Halst. [as '*batatae*'], New Jersey Agric. Coll. Exp. Sta. Bull. 76: 25 (1890)

≡ *Chorostate batatas* [as '*batatis*'] (Harter & E.C. Field) Sacc., Syll. Fung. 24 (2): 749 (1928)

≡ *Phomopsis batatas* (Ellis & Halst.) Trotter, in P.A. Saccardo, Syll. Fung. 24: 749. 1928.

≡ *Phomopsis batatas* [as '*batatae*'] (Ellis & Halst.) Harter & E.C. Field, Phytopathology 2: 121 (1912) (fide Wehmeyer, 1933)

Perithecia on culture, black, globose, 200–300 µm diam, densely clustered in groups, multiple tapering perithecial necks protruding through substrata, 500–1000 µm long. *Asci* unitunicate, 8-spored, sessile, elongate to clavate, (35–)38.5–48.5(–50) µm × (6.5–)7–9 (–11) µm ($\bar{x}$±SD=43.0±5.0 × 8.0±0.7, n = 30). *Ascospores* hyaline, two-celled, often biguttulate, elliptical to fusiform (8–)8.2–9.5(–9.6) × 3–4 µm ($\bar{x}$±SD=8.9±0.3 × 3.3±0.3, n = 30). *Pycnidia* on stems of *Ipomoea batatus*, black, globose, 200–250 µm diam, embedded in tissue, and irregular ectosroma; walls parenchymatous, consisting of 3–4 layers of medium black *textura angularis*.



Notes. a. Pycnidia on stem of *Ipomoea batatus*; b. Pycnidia in culture on CMD; c. Infected root surface; d. Conidiophores; e. Alpha conidia; f. Perithecia in culture; g. Asci; H. Ascospores. a–e. BPI615746, f–h. BPI615747. Bars: a= 1000 μm, b= 2000 μm, c= 1000 μm, d= 25 μm, e= 15 μm, f= 1200 μm, g, h= 10 μm.

Figure 5.4 Morphology of *Diaporthe batatas.*

Conidiophores hyaline, smooth, branched or unbranched, straight to sinuous, 8–16 × 1.5–2 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.4–1 µm diam. *Paraphyses* absent. *Alpha conidia* abundant on stems and infected roots, aseptate, hyaline, smooth, ellipsoidal, often biguttulate, base subtruncate, (5.5–)6.0–7.5(–7.7) × 2–3 µm ($\bar{x}$±SD =6.9±0.3 × 2.5±0.3 , n=30). *Beta conidia* not seen.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 4.5±0.2 mm/day (n=8), white, fluffy aerial mycelium, reverse centre dark pigmentation developing in centre.

Host: *Ipomoea batatus*

Distribution: USA (Alabama, District of Columbia, Indiana, Mississippi, New Jersey, North Carolina, Virginia) (Harter & Field, 1912).

Type material examined: *Diaporthe batatas* – USA, District of Columbia, on stem and tubers of *Ipomoea batatus*, 1910, L.L. Harter (holotype, BPI615746, with dry culture include BPI615747). USA, District of Columbia, on *Ipomoea batatus*, unknown collection dates, L.L. Harter (epitype designated here, BPI In Proc., ex-epitype culture CBS 122.21). *Phoma batatas* – USA, New Jersey. Middlesex Co. New Brunswick, on *Ipomoea batatae*, Sep. 1890, B.D. Halsted, # s.n. (holotype NY00937309). USA, District of Columbia, on *Ipomoea batatus*, unknown collection dates, L.L. Harter (epitype designated here, BPI In Proc., ex-epitype culture CBS 122.21).

Additional material examined: USA, New Jersey, substrate undetermined, 21 Jan. 1944, E.A. Walker (BPI615748).

Notes: *Diaporthe batatas* is known to cause dry rot of *Ipomoea batatas* (sweet potato) and is reported from several states in the USA (Harter & Field, 1912) and occasionally outside the USA. The disease has originally reported by Halsted (1890), from *Ipomoea batatas* in New Jersey, which was attributed to an unknown species of *Phoma*. In several publications (Seymour, 1929; Wehmeyer, 1933; Weiss, 1950; Cash, 1954) the transfer of *Phoma batatas* Ellis & Halsted to *Phomopsis batatas* was ascribed to Harter and Field (1912). But these authors did not introduce a combination in *Phomopsis* for *Phoma batatas* (Harter & Field, 1912). Therefore, the first person to publish the name *Phomopsis batatas* is Trotter in Saccardo (1928). The type specimen

of *Phoma batatae* (in NY) has been observed by Harter and Field (1912) who confirmed that the characteristics of *P. batatas* are identical to *Diaporthe batatas.* We have chosen the isolate CBS 122.21 deposited by L.L. Harter in 1921 as the epitype of *Diaporthe batatas* and *Phoma batatas* considering its historic authenticity and morphological similarity. *Diaporthe batatas* is distinguished from *Phomopsis ipomoeae* Petrak, reported from tubers of *Ipomoea* sp., by the conidial morphology and from *P. ipomoeae-batatas* Punith., cause of leaf blight of sweet potato, by its occurrence on leaves (Punithalingam, 1982). Based on the illustrations in Punithalingam (1982), the latter species appears to belong in *Phyllosticta*.

Diaporthe cucurbitae (McKeen) Udayanga & Castl. comb. nov. Figure 5.5

Basionym. *Phomopsis cucurbitae* McKeen, Canad. J. Bot. 35: 46. 1957.

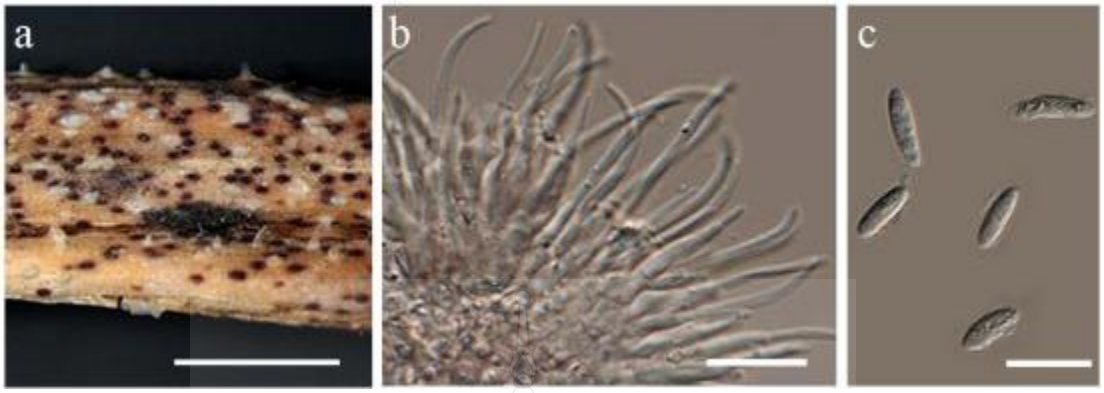
Pycnidia on dead twigs of *Cucumis*, globose, 200–400 µm diam, embedded in tissue, erumpent at maturity, evenly distributed in infected area causing blight; walls parenchymatous, consisting of 3–4 layers, *Conidiophores* hyaline, smooth, branched, cylindrical to clavate, straight to sinuous, 9–14 × 1–2 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 µm diam. *Alpha conidia* abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ellipsoidal, biguttulate, base subtruncate, (6–)8.0–11.0(–14.) × 2.8–4.2 µm ($\bar{x}$±SD =10.1±0.5 × 3.0±0.3, n=30). *Beta conidia* abundant. Aseptate, hyaline, smooth, elongated falcate, hooked at the apex and base subtruncate, 14–25 × 0.8–1.5 ($\bar{x}$±SD =18.1±3.0 × 1.0±0.3, n=30).

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 4.2±0.2 mm/day (n=8), white, fluffy aerial mycelium, reverse centre dark green pigmentation developing in centre.

Host range: *Arctium* spp., *Cucumis* spp.

Distribution: Canada

Type material examined: *Phomopsis Curcurbitae* – CANADA, Legmington, Ontario, on *Cucumis* sp., 1951, C.D. McKeen (holotype, DAOM 41000); CANADA, Leamington, Ontario, on seeds of *Cucumis* sp., Apr. 1956, C.D. McKeen, MEE M-151 (epitype designated here, BPI In Proc., ex-epitype culture DAOM 42078).



Notes. a. Pycnidia on stem of *Cucumis* sp.; b. conidiophores; c. alpha conidia. a–c. DAOM 41000, Bars. a= 1000 μm, b= 15 μm, c= 10 μm.

Figure 5.5 Morphology of *Diaporthe cucurbitae.*

Additional material examined: Unknown: on *Arctium* spp., A.W. Archer, Sep. 1985, living culture CBS 136.25.

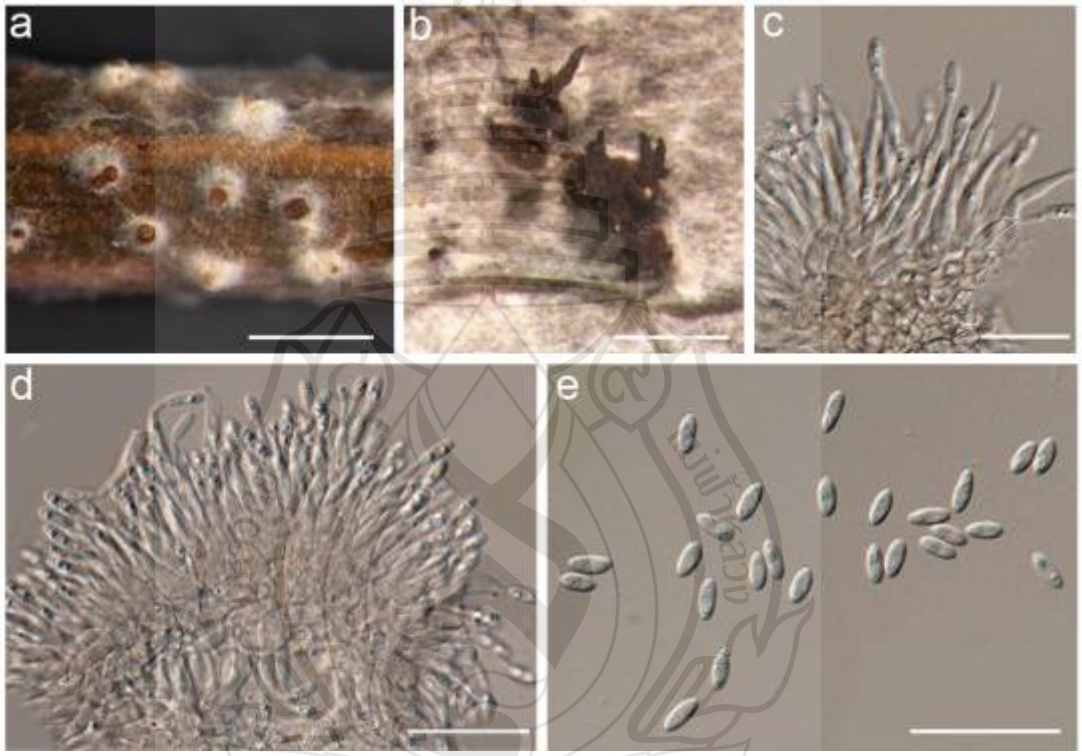
Notes: *Diaporthe cucurbitae* based on *Phomopsis cucurbitae* was described from Ontario, Canada, as the cause of black rot of greenhouse-grown cucumbers. The species has been considered to be a synonym of *D. melonis* solely based on host association (Beraha & O'Brien, 1979). The disease was observed in greenhouses in 1950 and 1954. In addition to black rot, the pathogen was observed to decay of fruits. Pathogenicity experiments revealed that this species infects cucurbits and tomatoes under experimental conditions (McKeen, 1957).

Diaporthe longicolla (Hobbs) J.M. Santos, Vrandečić & A.J.L. Phillips, Persoonia 27: 13. 2011. Figure 5.6

Basionym. Phomopsis longicolla Hobbs, Mycologia 77: 542. 1985.

Pycnidia on alfalfa twigs on WA: globose, 220–250 μm diam, embedded in tissue, erumpent at maturity, with an elongated, numerous pycnidia produced in in cluture with branched, elongated black neck 200–300 μm high, often with a yellowish, conidial cirrus extruding from ostiole as yellowish translucent drops; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*.

Conidiophores hyaline, smooth, unbranched, cylindrical to subcylindrical, straight,6–12 × 1–3 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 µm diam. *Paraphyses* absent. *Alpha conidia* abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ellipsoidal, often biguttulate, base subtruncate, (5–)6–7.2(–8) × (1.6–) 2–2.7 (–2.9) µm ($\bar{x}$±SD =6.4±0.3 × 2.3±0.1 , n=30). *Beta conidia* not seen.



Notes. a. Pycnidia on alfalfa stem on WA; b. Ectostroma on PDA; c,d. Conidiophores; e. Alpha conidia. a–e. BPI877419, BPI358745. Bars. a= 900 µm; b= 800 µm; c,d=12 µm ; e=20 µm.

Figure 5.6 Morphology of *Diaporthe longicolla*.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.5±0.2 mm/day (n=8), white, floccose, dense, fluffy aerial mycelium, producing abundant, black clusters of stroma with elongated necks at maturity. Reverse initially greenish yellow and black pigmentation developing rapidly with age.

Host range: Reported as *Phomopsis longicolla* or *Diaporthe sojae* on *Abutilon theophrasti* (Li, Bradley, Hartman & Pedersen, 2001; Vrandecˇ ic´ , C´osic´, Riccioni , Duvnjak & Jurkovic, 2004), *Acer truncatum* (Sun et al., 2011), *Ambrosia trifida*, *Euphorbia maculata*, *Rumex crispus* and *Xanthium strumarium* (Roy, Ratnayake & McLean, 1997), *Arachis hypogaea* (Sanogo & Etarock, 2009), *Aster exilis*, *Caperonia palustris*, *Desmanthus illinoensis*, *Eclipta prostrata*, *Euphorbia nutans*, *Ipomoea lacunose*, *Polygonum aviculare* and *Sida spinosa* (Mengistu & Reddy, 2005), *Solanum melongena* (Shu, Chen, Huang, He & Zhou, 2014), *Chamaesyce nutans* (Mengistu et al., 2007), *Cucumis melo*, *Glycine max* (Hobbs et al., 1985; Zhang et al., 1997; Santos et al., 2011; Gomes et al., 2013), *Vigna unguiculata* (Roy & Rathnayake 1997), *Trichilia elegans* (Flores, Pamphile, Sarragiotto & Clemente, 2013).

Distribution: Australia (Ash et al., 2010), Croatia (Vrandecˇic´ et al., 2004; Santos et al., 2011), China (Shan et al., 2012; Shu et al., 2014), Greece (Holevas et al., 2000), New Mexico (Sanogo & Etarock, 2009), USA – Arkansas and Missouri (Zhang et al., 1997) and Illinois, Iowa, Mississippi and Ohio (Hobbs et al., 1985) – and former Yugoslavia (Vidic´, Jasnic´ & Stojšin, 1996).

Type material examined: *Phomopsis longicolla* – USA, Wayne Co., Wooster, Ohio Agricultural Research and Development Center, on seeds of *Glycine max* cv. Wells, 5 day old dried culture on PDA, 13 Nov. 1983, T.W. Hobbs P 74 (holotype, BPI358745, ex-type culture ATCC 60325, FAU599).

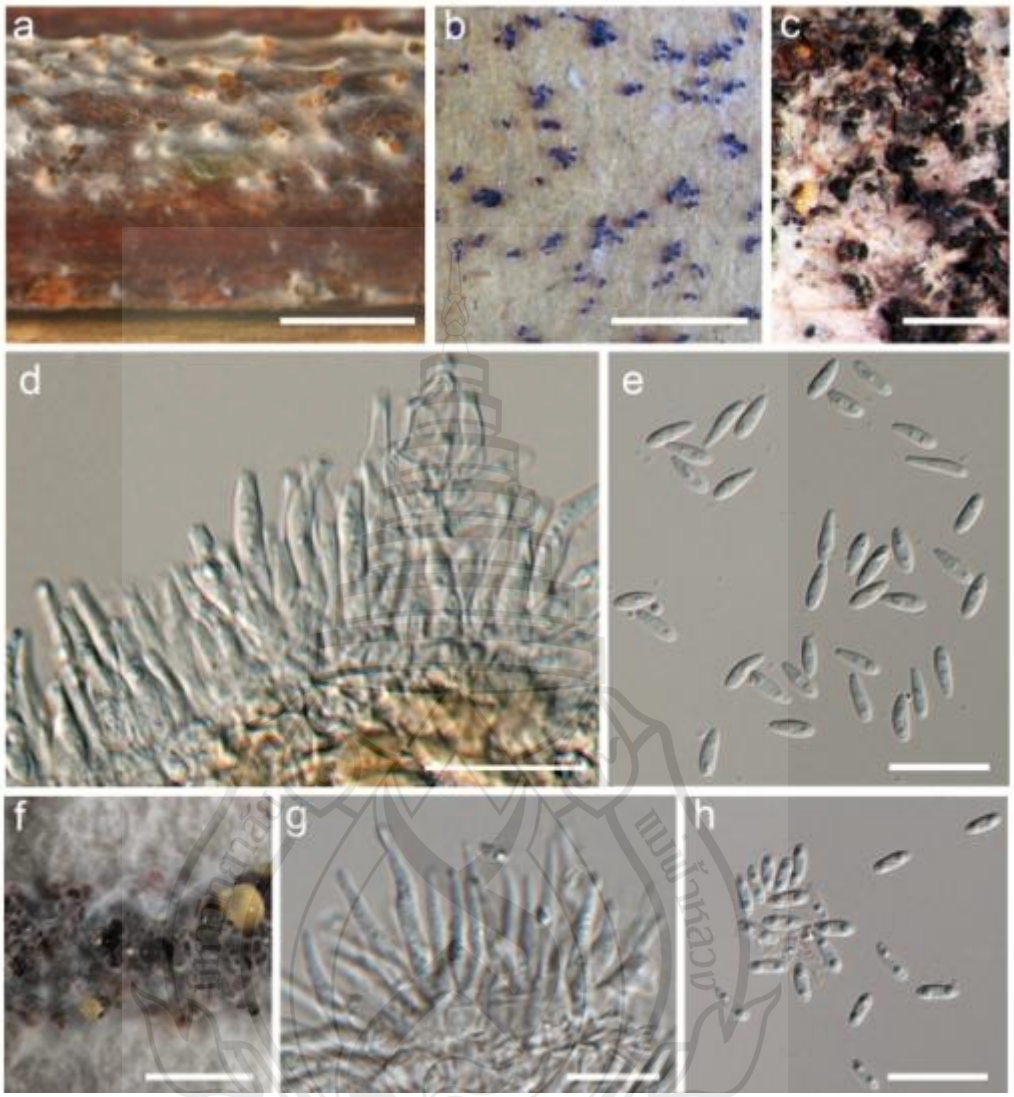
Additional material examined: USA, Wooster. Ohio Agricultural Research and Development Center, Wayne country, on seeds of *Glycine max* cv. Wells, 5 day old dried culture on PDA, 13 Nov. 1983, T.W. Hobbs, living culture FAU601; Mississipi, Stoneville, Experimental field, on *Glycine max*, 2003, Alemu Mengistu (STAM 35) (BPI877419, living culture CBS 116023; Mississipi, Stoneville, Experimental field, on *Ipomoea lacunosa*, 2003, Alemu Mengistu (STAM 27) (BPI877412), living culture CBS 121120; Mississipi, Stoneville, Experimental field, on *Chamaesyce nutans*, 2003, Alemu Mengistu (STAM 28) (BPI877413), living culture CBS 116017;

Maryland, on stem of *Glycine max*, unknown collection data, (BPI748005) living culture FAU642, *ibid* FAU643, *ibid* (BPI748006) living culture FAU644, *ibid* (BPI748007) living culture FAU645; Oklahoma, on crown of *Cucumis melo,* living culture FAU657; on *Glycine max*, Dec. 1978, J. Marcinkowska, living culture CBS 659.78, NRR13656.

Notes: Several species of *Diaporthe* occur on soybean often growing together and causing similar symptoms. *Diaporthe longicolla* is a seed borne pathogen, which can also infect other parts of a soybean plant (Harrington, Steimel, Workneh & Yang, 2000). This may mislead field pathologists identifying the causal agent especially if only morphology or disease symptoms are considered. In studying the seed decay pathogen, Hobbs et al. (1985) clearly distinguish the two kinds of *Diaporthe* isolates based on morphology, ecology and pathogenicity, one group of which is *Phomopsis longicolla*. The other group producing abundant perithecia was determined to be a homothallic species that agree with the concept of *Diaporthe sojae* as described by Lehman (1923). Because the ex-type culture of *D. longicolla* is available and has been widely studied. Based on its morphology including examination of type specimens and considering the historical literature, we determined that *D. longicolla* is not conspecific with *D. sojae*, therefore, the synonymy proposed by Gomes et al., (2013) appears to be incorrect. Using morphology, infection strategy and biology, *D. longicolla* can be distinguished from *D. sojae* and other species on soybean. *Diaporthe longicolla* also has been reported from weeds associated with soybean growing fields (Mengistu et al., 2007) and as a human pathogen for immuno-suppressed patients (Garcia-Reyne et al., 2011).

Diaporthe melonis Beraha & M.J. O'Brien, Phytopathol. Z. 94, 3: 205. 1979. Figure 5.7

Pycnidia on alfalfa twigs on WA: globose, 150–200 μm diam, embedded in tissue, erumpent at maturity, with an elongated, black neck 200–500 μm high, often with a yellowish, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, straight to sinuous, 9–14 × 2–3 μm.



Notes. a. Pycnidia on alfalfa stem; b. Perithecia on CMD in culture; c. Pycnidia on sterile stem of *Glottidium* sp.; d. Conidiophores; e. Alpha conidia. a–e BPI 615367, NY00921853. f–h FAU641, Bars. a, b=3000 µm, c=1000 µm;d= 22 µm, e=15 µm, f=3500 µm, g= 12 µm, h= 20 µm.

Figure 5.7 Morphology of *Diaporthe melonis.*

Conidiogenous cells phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 µm diam. *Paraphyses* absent. *Alpha conidia* abundant in culture and on sterile twigs, aseptate, hyaline, smooth, ovate to ellipsoidal, biguttulate, base subtruncate, (5.3–)5.7–7.2(–7.6) × (2–)2.2–2.8(–2.9) µm ($\bar{x}$±SD =6.5±0.3 × 2.4±0.2 , n=30). *Beta conidia* not seen.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.3±0.2 mm/day (n=8), white, fluffy aerial mycelium, reverse centre light yellow pigmentation developing in centre; producing abundant, clusters of black stroma with elongated necks at maturity.

Host range: *Cucumis melo*, *Glycine max*

Distribution: Indonesia (Gomes et al., 2013), USA

Type material examined: *Diaporthe melonis* – USA, Texas, on *Cucumis melo*, type specimen made on *Glottidium* stem inoculated with mycelium and conidia of asexual state on PDY agar, 08 Sep. 1978, L. Beraha, 71906 (holotype BPI 615367), *ibid*, Mar. 1979, a twig on culture with sporulating pycnidia (isotype NY 00921853), a culture on CMD from isotype (NY 00921854)

Additional material examined: USA, Texas, on *Cucumis melo*, unknown collector and date (BPI748003, living culture FAU 640), *ibid* (BPI748004 living culture FAU 641); Texas, on *Cucumis melo*, unknown, living culture FAU626; Texas, on *Cucumis melo*, unknown, living culture FAU628.

Notes: *Diaporthe melonis* is known to cause soft rot, also called Phomopsis fruit decay on cantaloupe in Texas, USA. The early symptoms of the Phomopsis decay on cantaloupe are slightly soft, circular, sunken areas on the rind of ripe fruit (Beraha & O'Brien 1979). The species is closely related to *D. sojae* and *D. longicolla* based on the molecular phylogeny, and also able to infect soybean causing the disease symptoms on stem (Harington et al., 2000; Li, 2011).

Diaporthe phaseolorum (Cooke & Ellis) Sacc., Syll. Fung. 1: 692. 1882 Figure 5.8

Basionym. *Sphaeria phaseolorum* Cooke & Ellis, Grevillea 6, 39: 93. 1878.

Perithecia on dead twigs, black, globose, subglobose or irregular, 300–500 µm diam, solitary, deeply immersed in host tissue with elongated, tapering perithecial necks protruding through substrata, 300–700 µm long. *Asci* unitunicate, 8-spored, sessile, clavate, (33–)39–44(–48) µm × (4.5–)5–7 (–8) µm ($\bar{x}$±SD=41.0±2.1 × 6.0±0.7, n= 30). *Ascospores* hyaline, two-celled, often 4-guttulate, with larger guttules at centre and smaller one at ends, elliptical or fusiform (9.7–12 × 3.5–4.3µm ($\bar{x}$±SD=11.0±0.7 × 3.7±0.3, n= 30).

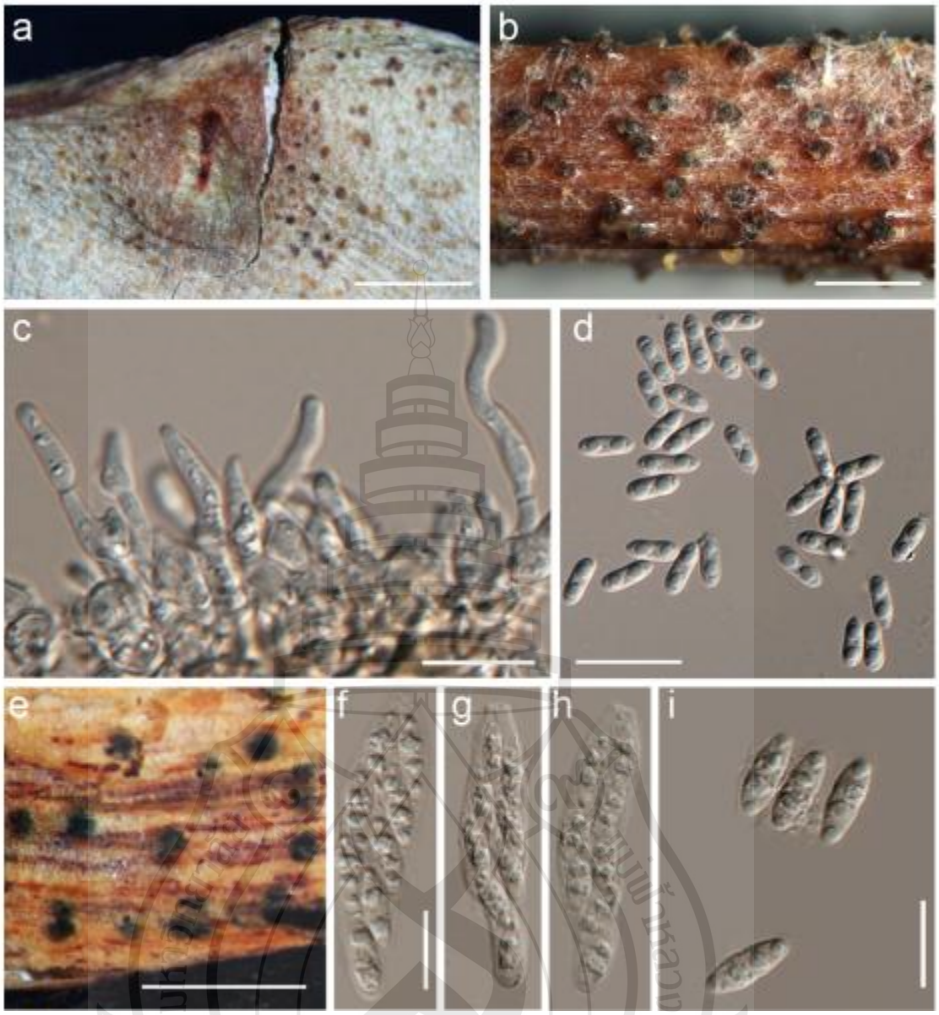
Pycnidia on alfalfa twigs on WA: globose, 200–250 µm diam, embedded in tissue, erumpent at maturity, with an elongated, black neck 200–300 µm long, yellowish, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, straight to sinuous, 7–12 × 2–3 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.4–1 µm diam. *Paraphyses* absent. *Alpha conidia* abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ellipsoidal, biguttulate, base subtruncate, (7.3–)8.2–9.7(–10.3) × 2.8–3.5 µm ($\bar{x}$±SD =8.8±0.4 × 3.1±0.1 , n=30). Beta not seen.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.3±0.2 mm/day (n=8), white, fluffy aerial mycelium, reverse centre dark pigmentation developing in centre.

Host range: *Phaseolus* spp.

Distribution: Cuba, Jamaica, USA (Delaware, New Jersey, Ohio and probably in other Atlantic states and along the Pacific Coast) (Harter, 1917).

Type material examined: *Sphaeria phaseolorum* – USA, on old *Phaseolus vulgaris* vines, 1878, Cooke and Ellis, (lectotype designated here, in Ellis North American Fungi. Century II, No.188 in Exsiccati bound collection in BPI), *ibid*. (paratype, BPI616862); USA, Delaware, Kent C., Townsend, on bean pod and leaf of *Phaseolus vulgaris*, 4 July 2005, N.F. Gregory (epitype designated here BPI879942, ex-epitype culture AR4203 = CBS).



Notes. a. Infected lesion on the pod of *Phaseolus* sp.; b. Sporulating pycnidia on alfalfa stem on WA; c. Conidiophores; d. Alpha conidia; e. Perithecia on stem of *Phaseolus* sp; f–h. Asci; i. Ascospores. a. BPI879942. b–d.AR4203 e–i. No. 188, in Ellis North American Fungi. Century II (1878). Bars. a=4000 μm, b=1000 μm, c=15 μm, d=12 μm, e=4000 μm, f–h=10 μm, i=14 μm.

Figure 5.8 Morphology of *Diaporthe phaseolorum.*

Additional material examined: USA, New Jersey, New Field, on old vines of *Phaseolus vulgaris*, July 1896, unknown collection data (BPI616861), *ibid.* (BPI 616865). USA, New Jersey, on pods of *Phaseolus lunatus*, 25 May 1983, M.M. Kulik (BPI358899). CUBA, Santiago de Las Vegas, on pods of *Phaseolus lunatus*, 29 Aug. 1919, S.C. Burner (BPI358117). USA, Ohio, Cincinnati, on stems of *Phaseolus limensis*, 10 July 1924, R. Sprague (BPI616863), *ibid.* (BPI616864); JAMAICA, on *Phaseolus lunatus*, 4 Feb. 1971, L. McClain, intercepted at Miami airport APHIS 001431 (BPI0358471).

Notes: *Diaporthe phaseolorum* is a species originally described from *Phaseolous* in the eastern USA, however, the name is widely applied to species of *Diaporthe* on soybean, in the sense of *D. phaseolorum* var. *sojae* (Kemtz, Ellett & Schmitthenner 1974; Kemtz et al., 1978; Zhang et al., 1999; Castlebury, Rossman, Jaklitsch & Vasilyeva, 2002), which is redefined here as *Diaporthe sojae*. The morphological distinctiveness of historical collections of *Diaporthe* from *Phaseolus* noted by Wehmeyer (1933), and subsequent collections urge the need of resolving this taxon with a fresh collection from eastern USA. The epitype designated here, is a specimen received from a home garden in Delaware, with infected lesions on pod, stem and leaves of *Phaseolus*, is highly similar to the lectotype material, and and additional material examined. Harter (1917), reported the occurrence of pod blight of lima beans (*Phaseolus lunatus*) along the states of Atlantic and pacific seaboard, caused by *Diaporthe phaseolorum*. The disease symptoms on pod blight of bean, asexual and sexual morphs described in Harter (1917), is also similar to the epitype material. However, the name *Diaporthe phaseolorum* is widely applied to collections from soybean in the recent literature, therefore, a careful interpretation is required when consulting disease notes and host associations.

Diaporthe sojae Lehman, Ann. Missouri Bot. Gard. 10: 128. 1923 Figure 5.9

≡ *Diaporthe phaseolorum* var. *sojae* (Lehman) Wehm., The genus *Diaporthe* Nitschke and its segregates 47: 1933.

= *Phomopsis sojae* Lehman, J. Elisha Mitchell Sci. Soc. 38: 13. 1922.

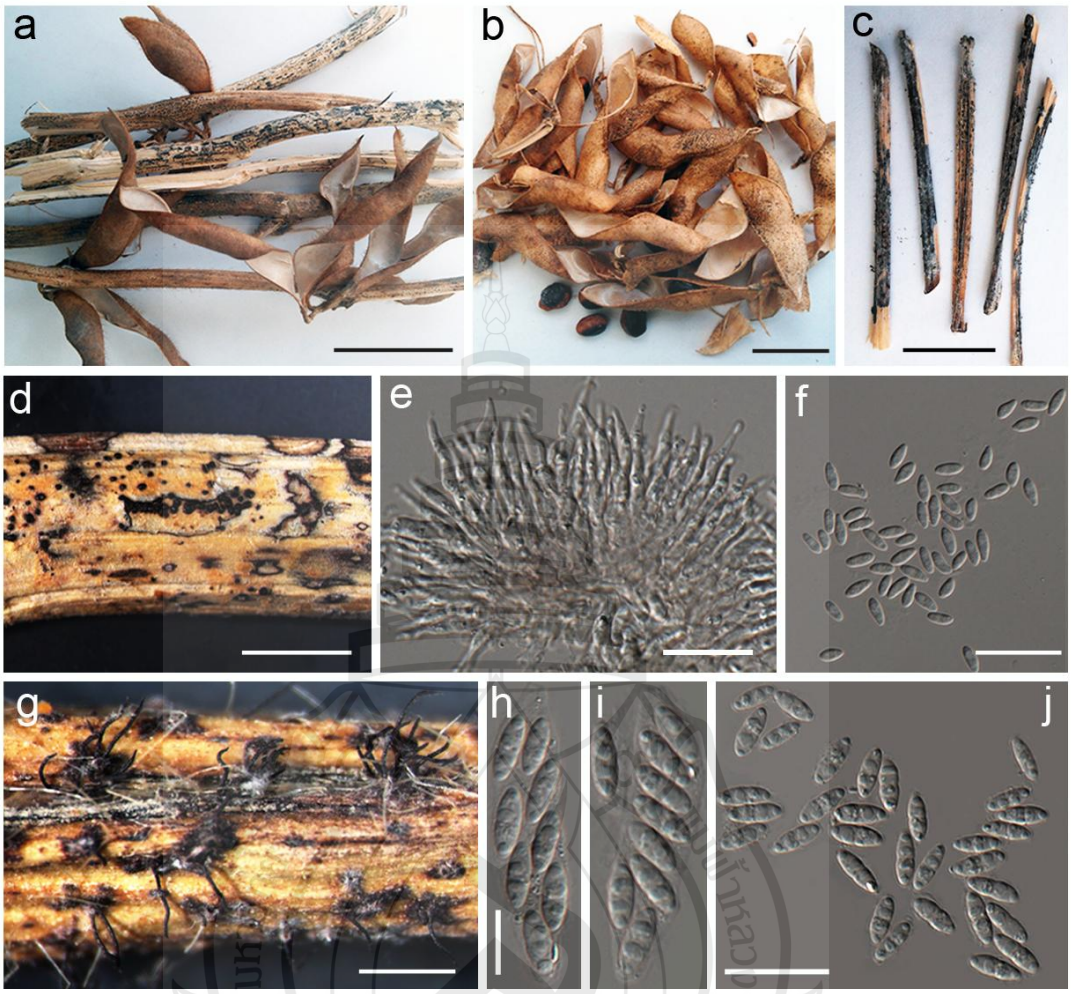
= *Phomopsis glycines* Petr., Ann. Mycol. 34: 240. 1936 (fide Hobbs 1985)

= *Phomopsis camptothecae* C.Q. Chang, Z.D. Jiang & P.K. Chi, Mycosystema 24: 145. 2005 (fide Santos et al. 2011)

= *Diaporthe kochmanii* R.G. Shivas, S.M. Thomps. & A.J. Young, in Thompson, Tan, Young, Neate, Aitken & Shivas, *Persoonia*, Mol. Phyl. Evol. Fungi 27: 86 (2011)
= *Diaporthe melonis* var. *brevistylospora* Tak. Kobayashi et Tak. Ohsawa in Ohsawa & Kobayashi, Ann. phytopath. Soc. Japan 55(4): 416 (1989)

Perithecia on soybean stems in culture, black, globose, 200–300 µm diam, densely clustered in groups, deeply immersed in host tissue with elongated, tapering perithecial necks protruding through substrata, 400–600 µm. *Asci* unitunicate, 8-spored, sessile, elongate to clavate, (35–)38.5–46.5(–48) µm × 7–9 (–12.0) µm ($\bar{x}$±SD=44.0±5.0 × 8.0±0.7, n= 30). *Ascospores* hyaline, two-celled, often 4-guttulate, with larger guttules at centre and smaller one at ends, elongated to elliptical, slightly or not constricted at septum (9–)9.5–11.9(–12) × 3–4 µm ($\bar{x}$±SD=10.5±0.6× 3.4±0.2, n= 30)

Pycnidia on soybean stems ostiolate, black, subglobose, 200–250 µm diam, embedded in tissue, erumpent at maturity, with an elongated, black neck 200–400 µm high, evenly scattered on infected stems and pods as black pustulate structures; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* densely aggregated**,** hyaline, smooth, unbranched, ampulliform, straight to sinuous, (8–)12–16 (–18) × 2–4 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, base enlarged, slightly tapering towards apex, 0.5–1 µm diam. *Paraphyses* absent. *Alpha conidia* abundant, aseptate, hyaline, smooth, ovate to ellipsoidal to clavate, often biguttulate, base subtruncate, (5–)5.3–7.3(–7.8) × 2–3 µm ($\bar{x}$±SD =6.4±0.5 × 2.5±0.3 , n=30). *Beta conidia* not seen.



Notes. a. Infected stems of soybean; b. Infected and shattered pods; c. Sterile soybean stems with perithecia produced in culture; d. Stem blight symptoms of soybean with pycnidia; e. Conidiophores; f. alpha conidia; g. Perithecia on soybean stems in culture; h, i. Asci; j. Ascospores. a–j. BPI615412. Bars. a= 4000 µm, b= 1500 µm, c= 1000 µm, d= 3000 µm, e=20 µm, f=15 µm, g= 1100 µm, h,i= 12 µm, j= 20 µm.

Figure 5.9 Morphology of *Diaporthe sojae*.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.6±0.2 mm/day (n=8), white, fluffy aerial mycelium, reverse centre yellowish pigmentation developing in centre later becoming darkly striate, eventually entirely black with age.

Host range: Probably as *Diaporthe phaseolorum* and *D. phaseolorum* var. *sojae*, *Camptotheca acuminata* (Chang et al., 2005), *Capsicum annuum* (Pennycook, 1989), *Cucumis melo*, *Glycine max* (Lehman, 1923; Zhang et al., 1997; Santos et al., 2011), *Helianthus annuus* (Vrandecˇ ic´, C´osic´, Jurkovic´, Duvnjak & Riccioni, 2009; Thompson et al., 2011), and *Stokesia laevis* (Sogonov, Castlebury, Rossman, Mejia & White, 2008).

Distribution: Argentina, Brazil, Canada, Italy, Puerto Rico, Senegal and Taiwan (Richardson, 1990), Bermuda, China, Colombia, Cuba, Egypt, Guyana, India, Israel, Japan, New Zealand, Nigeria, Sierra Leone and Zaire (Punithalingam & Holliday, 1972), Australia (Thompson et al., 2011), Croatia (Vrandecˇ ic´ et al., 2009; Santos et al., 2011), France (Bernaux, 1981), Hungary (Ersek, 1978), Japan (in this study), Korea (Zhang et al., 1997), Malawi (Peregrine & Siddiqi, 1972), Malaysia (Raeisi , Puteh & Sijam, 2011), Nepal (Dahal, Amatya & Manandhar, 1992), Russia (Kozireva, Primakovskaja & Skripka, 1982), South Africa (Gorter, 1977), Tanzania (Ebbels & Allen, 1979), USA – Arkansas (Zhang et al., 1997), Florida (Alfieri, Langdon, Wehlburg & Kimbrough, 1984), Georgia (Hanlin, 1963), Illinois (Gerdemann, 1954), Indiana, Louisiana, New Jersey and North Carolina (Wehmeyer, 1933), Iowa (Gilman 1949), Kentucky and Minnesota (Richardson, 1990), Mississippi (Johnson & Kilpatrick, 1953), Northe Carolina (Lehman, 1923), Ohio (Kmetz et al., 1974) and Oklahoma (Preston, 1945) and the former Yugoslavia (Jasnic´ & Vidic,´ 1985).

Type material examined: *Diaporthe sojae* – USA, Raleigh, North Carolina, on stems and pods of *Glycine max*, 1 Sept. 1920, S.G. Lehman, Perithecia developed on sterilised petioles of soybean after inoculation of pure culture of strain 17 with stems and pods (lectotype designated here, BPI615412); Raleigh, North Carolina, on stems and pods of *Glycine max*, Late summer 1920, S.G. Lehman, (isolectotype designated here, BPI615415); Ohio, on *Glycine max*, unknown dates and collection data **(**epitype designated here, BPI748002) ex-epitype culture FAU635. *Diaporthe melonis* var.

brevistylospora – JAPAN, Shizuoka, on *Cucumis melo*, Nov. 1985, T Ohsawa, (holotype, FF-PRI:LFP-DB-41, TFM:FPH 7097 not seen, ex-type culture FF-PRI:LFP-DB-41=AR3602 /MAFF 410444 (observed). *Diaporthe kochmanii* – AUSTRALIA, Queensland, Lawes, on *Helianthus annuus* Experimental Line, 25 Nov. 2010, S.M. Thompson (holotype, BRIP 54033, includes ex-type culture).

Additional material examined: USA, Mississippi, on *Stokesia laevis*, Sep. 1986, (BPI747968) living culture, FAU456, *ibid* (BPI747969) living culture FAU 458; Mississippi, Picayune, on *Stokesia laevis*, Sep. 1986, F.A. Uecker, W. Cibula, C. Wells, (456) (BPI746622); Mississippi, Picayune. Crosby Arboretum, Interpretive Center, on *Stokesia laevis*, Sep. 1986, F.A. Uecker, W. Cibula, C. Wells, (456) (BPI746625); Mississippi, Picagune, Crosby Arboretum, on *Stokesia laevis*, Sep. 1986, C. Wells 455 (BPI749206), living culture FAU455=ATCC64805; Mississippi, Hillside Bog, Hancock Co., on *Stokesia laevis*, Sep. 1986, F.A. Uecker 457 (BPI749208), living culture FAU457=ATCC64803; Florida, on *Glycine max*, unknown dates and collection data **(**BPI747998), living culture FAU625=CBS In Proc; Ohio, *Glycine max*, unknown collection data, living culture FAU636; Ohio, *Glycine max*, unknown collection data, living culture FAU637=ATCC 28463; Ohio, *Glycine max*, unknown collection, T.W. Hobbs, living culture FAU604; New Jersey, *Asparagus officinalis*, unknown date, F.A. Uecker, FAU499; Mississippi, *Stokeasia laevis*, unknown date, FAU459=ATCC64801; Mississippi, on *Glycine max*, unknown date, Alemu Mengistu, STAM 68, living culture DP0601; Mississippi, on *Glycine max*, unknown date, Alemu Mengistu, STAM 167, living culture DP0616; Mississippi, on *Glycine max*, unknown date, Alemu Mengistu, STAM 74, living culture DP0605; Mississippi, on *Glycine max*, unkonwn date, Alemu Mengistu, STAM 204, living culture DP0623. JAPAN, on *Cucumis melo*, Nov. 1985, T. Kobayashi & T. Oosawa, DA-2, MAFF 410445.

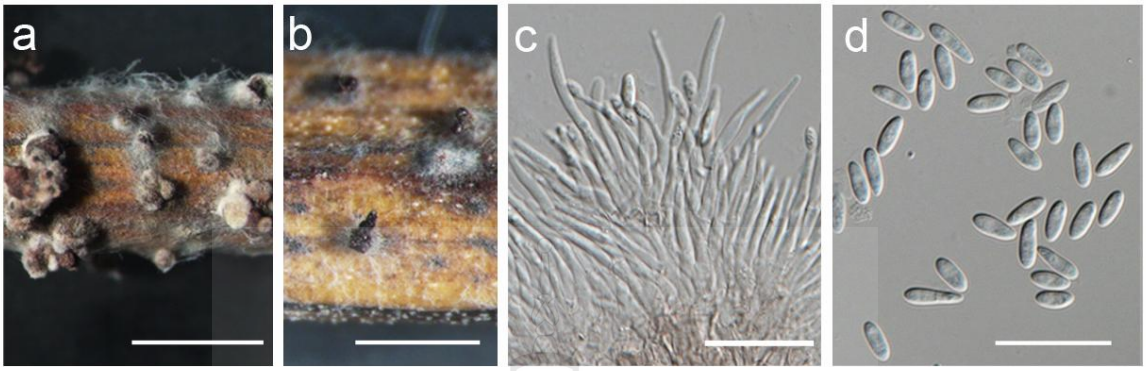
Notes: The original description of *Diaporthe sojae* and a comprehensive account of pod and stem blight of soybean provide the morphological characteristics of the species linked to disease symptoms (Lehman, 1923). The protologue did not indicate the connection with the previously described *Phomopsis sojae*, therefore it is not considered as the basionym. Many authors have used this name based on the Wehmeyer's treatment of *Diaporthe* (1933), of recognizing it as *D. Phaseolorum* var.

sojae. We revisited the taxonomic status of this name including observation of available type material and determined that the species that causes pod and stem blight should be called *Diaporthe sojae*. An epitype for this name is designated herein. The isolates from *Glycine max* and *Stokesia laevis* of *D. sojae* produce sexual and asexual morphs similar to the type specimens observed, unlike *Diaporthe longicolla* (see under *D. longicolla*). We confirmed that the ex-type isolate of *Diaporthe melonis* var. *brevistypospora* (AR3602 /MAFF 410444), causing the concave rot of melon in Japan (Ohsawa & Kobayashi, 1989) and *Diaporthe kochmanii* (BRIP54033) described from sunflower in Australia (Thompson et al., 2011), are conspecific with *Diaporthe sojae* based on molecular phylogeny and therefore regard as synonyms of *D. sojae*. Authentic strains of *Phomopsis brevistylospora* from *Cucumis melo* fruit in Japan (MAFF410446/AR3595, MAFF410450/AR3597) were found to be species not closely related to *Diaporthe sojae* based on preliminary phylogenetic analysis (tree not shown).

Diaporthe ueckerae Udayanga & Castl. sp. nov. Figure 5.10

Etymology: In honor to the mycologist Francis A. Uecker, who greatly contributed to the collections of specimens, taxonomy and phylogeny of the genus *Diaporthe*.

Pycnidia on alfalfa twigs on WA: globose, ostiolate, occurring as clusters, 150–200 µm diam, embedded in tissue, erumpent at maturity, with an elongated, black neck 200–300 µm high, often with a yellowish, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, long, slender, (9–)12–28(–30) × 1.5–2.5 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 µm diam. *Paraphyses* absent. *Alpha conidia* abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ellipsoidal, often biguttulate, base subtruncate, (6–)6.4–8.2(–8.6) × (2–)2.3–3.0 µm ($\bar{x}$±SD =7.2±0.4 × 2.6±0.2, n=30). *Beta conidia* not seen.



Notes. a. Pycnidial clusters on alfalfa stem; b. Pycnidia with long protruding necks on alfalfa stem on WA; c. Conidiophores; d. Alpha conidia. a–d. BPI748011, BPI748011. Bars: a= 1000 µm, b= 1200 µm, c = 20 µm, d= 15 µm

Figure 5.10 Morphology of *Diaporthe ueckerae*

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.0±0.2 mm/day (n=8), white, fluffy aerial mycelium, reverse centre dark pigmentation developing in centre.

Type material examined: USA, Oklahoma, on crown of *Cucumis melo*, unknown collection data (holotype, BPI748011, ex-type culture FAU656 = CBS).

Additional material examined: USA, Oklahoma, on *Cucumis melo*, unknown collection data (BPI748012, living culture FAU660, *ibid* living cultures FAU658, FU659.

Notes: *Diaporthe ueckerae* is based on isolates from *Cucumis* in Oklahoma that represent a new species. This species is distinct from other species of *Diaporthe* on this host such as *D. cucurbitae* and *D. melonis* that are considered in this study. The disease symptoms caused by *D. ueckerae* on *Cumcumis melo* are not known, however, these isolates were initially identified as *Diaporthe melonis*. The species is clusters with two isolates (CBS119639: human abscess from Germany, LGMF937: *Glycine max* form Brazil) included in Gomes et al., (2013), as *Diaporthe* sp. 1.

Therefore this species is a ubiquitous pathogen that can infect humans and opportunistic plant hosts including soybean.

Excluded species from the complex:

Phoma phaseoli Desm., *Annls Sci. Nat.*, Bot., sér. 2 6: 247 (1836)

≡ *Phomopsis phaseoli* (Desm.) Sacc., *Nuovo G. bot. ital.* 22: 47 (1915)

Type material of *Phoma phaseoli*: ITALY, on stems of *Phaseolus lunatus*, 1836, Desmaziers, (lectotype designated here, in Plants cryptogames de France, Fascicule XVII, (1836), bound Exsiccati no. 843 in BPI). NETHERLANDS, on stems of *Phaseolus vulgaris*, Oct. 1950, Goossens (epitype designated here, BPI In Proc) ex –epitype culture CBS 422.50.

Notes: The name *Phomopsis phaseoli* is associated with species of *Diaporthe* on soybean and was considered to be the asexual morph of *D. phaseolorum*. Two later homonyms of *Phomopsis phaseoli* are illegitimate. One was described in Grove (1917) from stems of *Phaseolous* in the UK and the other in Petch (1922) from and dead stems of soybean seedlings in Sri Lanka. Based Hobbs et al. (1985), this name is considered doubtful. This name has not been widely used and then only as a synonymy of *D. phaseolorum* or *D. sojae*. Morphological characteristics of the conidia and conidiophores on Desmaziers' type collection, here designated as lectotype, is similar to the asexual morph of the plurivorous species *Diaporthe eres.* It is not similar to *D. phaseolorum* or *D. sojae*. The ex-epitype culture CBS 422.50 of *P. phaseoli* has been well characterised as *Diaporthe eres* (Gomes et al., 2013), therefore, it is considered a synonym of *D. eres* nom. cons. prop. and is not associated with any of the species of *Diaporthe* on soybean.

5.4 Discussion

Accurate identification of fungal pathogens and their precise naming is an essential step for virtually all aspects in phytopathology including disease surveillance, control and plant health inspection (McCartney, Foster, Fraaije & Ward, 2003; Anderson et al., 2004). Several groups of important pathogenic fungi associated with major crops have historically been difficult to identify and properly named (Zhang et al., 1997; Damm et al., 2013; Woudenburg, Groenewald, Binder & Crous,

2013; Luo & Zhang, 2013; Crouch, 2014). Several species complexes in *Diaporthe* have been studied, specifically those associated with *Glycine max* (soybean) (Zhang et al., 1998, 1999; Hobbs et al., 1985), *Helianthus annuus* (sunflower) (Thompson et al., 2010), *Citrus* (Huang et al., 2013a; Udayanga et al., 2014), (*Vitis* spp.) grapevine (Niekerk et al., 2005; Baumgartner et al., 2013), and rosaceous and ericaceous hosts (Farr et al., 2002a). Although the most important species of *Diaporthe* associated with soybean were originally described from the USA, their occurrence has been assessed in Argentina (Pioli, Morandi & Bisaro, 2002), Croatia (Santos et al., 2011), China, Korea (Sun et al., 2013), Italy, Indonesia, Brazil (Costamilan et al., 2008), Malaysia (Raeisi et al., 2011), Yugoslavia (Nevena et al., 1997) and worldwide (Zhang et al., 1997; 1998).

In this study the species of *Diaporthe* on soybean and related taxa were determined based on the principles of genealogical concordance phylogenetic species recognition (GCPSR) linked with morphological characteristics. The individual and combined analysis of five genes revealed a monophyletic clade that was also supported based other studies of the genus using molecular sequence analyses (Rehner & Uecker, 1994; Udayanga et al., 2012a; Gomes et al., 2013). The clade assessed here included 83 isolates that resulted in recognition of 30 well resolved species, the majority of which are linked to the ex-type sequences including several epitypes designated in this study. *Diaporthe longicolla* has been recognised as an important pathogen for its importance in agriculture as a pathogen of soybean. We incorporated multiple DNA sequences of the ex-type culture and a modern description and illustrations of type material of *D. longicolla* in this study, which can be used for future identification of this species.

The name *Diaporthe phaseolorum*, has conventionally been used to recognise species of *Diaporthe* from soybean. However, it is essential to designate an epitype which can reasonably be recognised as reference material for the taxon. *Diaporthe phaseolorum*, the pathogen causing pod blight of Lima bean in the USA is exclusively reported from that host, is well characterised in terms of host, symptoms, disease distribution and morphology (Harter, 1917). The epitype specimen designated here for *D. phaseolorum* is exactly similar with the morphology, disease symptom and

geographical origin with previous reports of Lima bean pathogen, which is reliably linked with available molecular data.

Wehmeyer's concept (1933) of varieties of *D. phaseolorum* has been challenged in several authors in based on different aspects. Morgan-Jones (1985) reported the separation of *D. phaseolorum* into varieties based on morphological characters including colony appearance, size of stromata, arrangement of perithecia, presence of an anamorph, presence of alpha- and beta-conidia. Sinclair and Backman (1989) used symptomology and virulence on soybean. However, Morgan-Jones, (1989) stated that of the variability found in morphology, physiology and host relationships in the *D. phaseolorum* complex, classification at varietal level is considered unsatisfactory. Re-defining *D. phaseolorum*, *D. sojae* and *D. longicolla* in relation to their biology, ecology and taxonomic significance will leads to provide meaningful grounds to study for plant pathologists as well as various other aspects of applied sciences. It is possible that Lehman (1923) could have been collected more than one fungus co-occurring with similar symptoms in his study from North Carolina soybean fields, however the protologue of *D. sojae* well defined for a homothallic species which produce sexual state in culture and linked to the asexual morph observed in plant material. The molecular phylogenetic analysis and through observation of type materials with additional collections confirmed the assignment of correct names for *D. longicolla*, *D. phaseolorum* and *D. sojae*, eventually leads to understand the natural history associated with these pathogenic fungi.

Host association and ecology within the species complex provide an overview of the distribution of the pathogens included in this study. *Diaporthe sojae*, which is widely associated with soybean, has also been extensively sampled from *Stokesia laevis* (*Asteraceae*) in USA and *Helianthus annuus* in Australia (as *D. kochmanii*). *Diaporthe sojae,* which is conspecific with *D. kochmanii* from Australia, is known as a less virulent on sunflower causing stem canker compared to *D. gulyae* and *D. helianthii*. The *Diaporthe* species associated with hosts of *Cucurbitaceae*, mostly edible gourds, pumpkins and melons, are found to be closely related to soybean pathogens. The inclusion of molecular data with the clarification of *Diaporthe cucurbitae, D. melonis* and a novel species *D. ueckerae* will allow the accurate

diagnosis of common pathogens, which threaten agricultural commodities where these pathogens do not yet occur.

Although many of the species included in study are well known as pathogens of important crops, the extensive sampling from various geographic locations and different hosts has revealed that *D. longicolla* and *D. sojae* can be endophytic in tropical trees and mangroves (Cheng et al., 2008; Sebastianes et al., 2012, Rönsberg et al., 2013). The closely related taxa *D. endophytica*, *D. schini* and *D. terebinthifolii* were originally described from *Schinus* in Brazil as endophytes (Gomes et al., 2013). Species of *Diaporthe* are known to crossover from fungal plant pathogens to infect humans, also known as uncommon medically important pathogens. The phenomenon has been widely discussed due to recent outbreaks and occasional reports (Walsh & Groll, 1999; Gauthir & Keller, 2013; Cariello et al., 2013). For instance, within the *D. sojae* species complex, *Diaporthe longicolla* is known to cause a cutaneous infection in an immuno-suppressed patient in Guinea (Garcia-Reyne et al., 2011), *D. sojae* (as *D. phaseolorum*) was reported from an infection on the hands and on a foot of a renal transplanted Brazilian farmer (Mattei et al., 2013), and one isolate of *D. ueckerae* was from a human abscess (CBS 119639). Mengistu et al. (2007) observed the infection and effect of *Diaporthe longicolla* (as *Phomopsis longicolla*) and *D. sojae* (as *D. phaseolorum*) on soybean that had been originally isolated from weeds. The isolates of *D. longicolla* from *Ipomoea lacunosa* (pitted morning glory) and *Chamaesyce nutans* (nodding spurge), *D. sojae* from *Desmanthus illinoensis* (Illinois bundle flower), *Caperonia palustris* (Texas weed), and *Aster exilis* (slender Aster) infected soybean including seeds in pathogenicity experiments. Molecular detection of the species from various weeds and their potential to infect soybean provide the possibility of the transfer of inocula from less common hosts and survival of pathogens despite crop rotation. Accurate knowledge of the host range of a fungal pathogen is useful in selecting replacement crops in rotation in the field as well as for significant control of weeds, which can be opportunistic hosts. The weeds associated with field crops, the stubble accumulating follow the harvesting as well as various other plants in the vicinity of crop fields can be reservoirs of the survival of most of the species associated with soybean and other crops.

Species of *Diaporthe* on soybean were evaluated by retrieving ITS sequences in GenBank under search syntax of ("Diaporthe"[Organism] OR "Phomopsis" [Organism]) AND "Glycine max"[All Fields] AND “internal transcribed” [All Fields]) (retrieved on 2014 March 16). A total of 172 ITS sequences were returned and the highest values of “Top Organisms” column in GenBank is summarised as: *D. longicolla* as *Phomopsis longicolla/D. sojae* (39), *D. caulivora* (36), *D. sojae as D. phaseolorum* (22), *D. phaseolorum* var. *meridionalis*/*D. aspalathi* (11), *D. eres* (7), *D. novem* (7), *D. foeniculina* (2), *D. rudis* (1), *D. endophytica* (1) and all other taxa as *Diaporthe* sp./*Phomopsis* sp. Several sequences under specific isolate codes (55) can belong to one of above well defined species or to several undescribed taxa. This reveals the potential association of at least nine species with soybean in various geographic regions of the world including two species, *D. foeniculina* and *D. rudis,* associated with an extensive range of hosts as opportunistic pathogens (Udayanga et al., 2014). *Diaporthe novem*, a species originally described from Croatia, was not found from soybean in USA or elsewhere (Santos et al., 2011). Instead, this study reveals the first record of *D. novem* associated with *Daucus carota* in New Zealand suggesting that the taxon can be found from a wide range of hosts. The accurate identification of fungal pathogens using a robust phylogenetic framework will facilitate the implementation of better strategies for developing resistant varieties as well as understanding the potential for cross infection of closely related species to soybean in the broad scope of phytopathological and agronomic concerns.

5.5 Conclusion

Phytopathogenic species of *Diaporthe* are associated with the serious diseases including seed decay, pod and stem blight and stem canker of soybean leading to considerable loss of crop production worldwide. Accurate identification of the species that cause these diseases has been difficult due to the lack of a recent comprehensive phylogenetic and taxonomic revision. In this study, we revised the phylogenetic placement of the soybean seed decay and pod and stem blight pathogens, *D. longicolla* and *D. sojae,* as well as *Diaporthe phaseolorum* and closely related taxa.

Species boundaries were determined based on combined phylogenetic analysis of five gene regions: partial sequences of calmodulin (CAL), beta-tubulin (TUB), histone-3 (HIS), and translation elongation factor 1-α (EF1-α), and the nuclear ribosomal internal transcribed spacers (ITS). Phylogenetic analyses revealed that this species complex is comprised of soybean pathogens as well as species associated with hosts in *Apiaceae*, *Asteraceae*, *Convolvulaceae*, *Cucurbitaceae*, *Fabaceae* and *Solanaceae*, including mostly vegetables and other herbaceous field crops. *Diaporthe arctii*, *D. batatas*, *D. phaseolorum* and *D. sojae* are epitypified and synonyms are clarified. The seed decay pathogen *Diaporthe longicolla* was determined to be distinct from *D. sojae*. *Diaporthe phaseolorum* associated with stem and leaf blight of Lima bean was not found to be associated with soybean in this study. In addition, *Diaporthe cucurbitae*, the pathogen causing black rot of cucumbers, was distinguished from *D. melonis* causing soft rot of cantaloupe. The taxon *D. phaseolorum* var. *brevistylospora*, causing concave rot of melon in Japan, is conspecific with *D. sojae*. A new species, *D. ueckerae*, associated with *Cucumis melo* from Oklahoma, is introduced with full description and illustrations.

CHAPTER 6

GENEALOGICAL CONCORDANCE PHYLOGENETIC SPECIES RECOGNITION IN THE *Diaporthe eres* SPECIES COMPLEX

6.1 Introduction

In the last two decades tremendous progress has been made in the ability to define fungal species incorporating the use of molecular data (Hibbett & Taylor, 2013; Hyde et al., 2013). Circumscribing species within cryptic species complexes that have complicated life histories is essential for determining patterns of speciation and potential hyperdiversity within a genus (Bickford et al., 2007; Silva et al., 2012; Fekete, Irinyi, Karaffa, Árnyasi & Asadollahi, 2012; O'Donnell et al., 2013). Genealogical Concordance Phylogenetic Species Recognition (GCPSR) as an approach for defining species primarily of fungi was proposed by Taylor et al. (2000), based on Avise and Ball's (1990) genealogical concordance species concept requiring the analysis of several unlinked genes. This approach is often used alternative to morphological and biological species recognition (Dettman et al., 2003a). However, there have been relatively a few evaluations of the utility of genes to delineate closely related species in genera with broad host ranges and wide geographic distributions (Giraud, Refregier, de Vienne, Le Gac & Hood 2008; Dupis, Roe & Fah, 2012; Groenwald et al., 2012; Salgado-Salazar, Rossman & Chaverri, 2013).

The genus *Diaporthe* comprises pathogenic, endophytic and saprobic species with both temperate and tropical geographic distributions (Rehner & Uecker, 1994; Rossman et al., 2007; Udayanga et al., 2011). Species recognition criteria in

Diaporthe have evolved from morphology and host associations (Wehmeyer, 1933) to the more recent use of phylogenetic species recognition (Castlebury et al., 2003; Santos & Phillips 2009; Santos et al., 2011; Udayanga et al., 2012a; 2012b; Gomes et al., 2013; Udayanga et al., 2014). *Diaporthe eres* Nitschke, the type species of the genus was originally described by Nitschke (1870), from *Ulmus* sp. collected in Germany. Under *D. eres*, Wehmeyer (1933) listed a large number of synonyms with approximately 70 host associations belonging to a wide range of plant families based on morphological characters. Despite Wehmeyer's (1933) broad concept of *D. eres* and various later opinions based on morphological data and molecular phylogenetics, a comprehensive study of the species has not been attempted (Udayanga et al., 2011; Gomes et al., 2013). Few of the synonyms listed in Wehmeyer's taxonomic treatment have been accepted by later studies or re-examined with molecular data. The oldest name associated with *D. eres* is apparently *Phomopsis velata* (Sacc.) Traverso and the editors of *Index Fungorum* have recently listed *D. eres* as a synonym of *P. velata* along with many other synonyms including names belonging to *Chorostate*, *Cucubitaria*, *Diatrype*, *Phoma*, *Phomopsis*, *Sphaeria*, *Sclerophoma*, *Sclerophomella* and *Valsa*. Rossman, Udayanga, Castlebury, Hyde (2014) proposed to conserve the name *Diaporthe eres* considering its status as the generic type its wide appearance in literature and include a list of all the available older names to be suppressed.

Diaporthe eres has also been regarded as a minor pathogen causing leaf spots, stem cankers and diseases of woody plants in diverse families including the *Ericaceae*, *Juglandaceae*, *Rosaceae*, *Sapindaceae*, *Ulmaceae*, *Vitaceae* and others, mostly in temperate regions worldwide (Vrandecic, Jurković & Ćosić, 2010; Anagnostakis, 2007; Thomidis, 2009; Baumgartner et al., 2013) and considered to be a pathogen with plant health inspection and quarantine significance (Cline & Farr 2006). Several recent disease reports of *D. eres* include cane blight on blackberry in Croatia (Vrandecic, Jurkovic, Cosic, Postic & Riccioni, 2011), as a pathogen of butternut (*Juglans cinerea*) in Connecticut (Anagnostakis, 2007), a shoot blight and canker disease of peach in Greece (Thomidis, 2009) and stem canker of *Salsola tragus* in Russia (Kolomiets et al., 2009) and association with wood cankers of grapevines in Croatia (Kaliterna**,** Milicevici & Cvjetkovic**,** 2012) and USA (Baumgartner et al., 2013). It is reported less frequently on herbaceous plant such as

the Cucurbitaceae (Garibaldi, Bertetti, Poli & Gullino, 2011; Gomes et al., 2013). Wehmeyer (1933) based on his species concept on morphology rather than host distinction, he observed that *Diaporthe eres* might be regarded as a species complex (Bryford, 1990; Cline & Farr, 2006). Barr (1978) recognised three sections of *Diaporthe* based on ascospore morphology, one of which was *Diaporthe* section *Diaporthe* which was based on the type species *D. eres*. Although the group species concept has historically been associated with *D. eres*, the lacking of ex-type or ex-epitype for the generic type species has been a major issue regarding the circumscription of the species. The available identifications in disease reports and other recent phylogenetic studies are solely based on morphology or more recently based on similarity comparison of authentic sequences in AFTOL (ID 935). Despite the previous taxonomic opinions based on morphology and host association the limits of *Diaporthe eres* species complex was unknown.

The principles of GCPSR are based on the assumption that recombination within a lineage likely to be the reason for conflict within gene trees, with the transition from conflict to congruence representing the species boundaries (Taylor et al., 2000). Selecting multiple genes with strong phylogenetic signals, the absence of significant incongruence and the applications of standard criteria to determine the species boundaries are the most important factors in the best phylogenetic practices resolving the cryptic species complexes (O' Donell, Nirenberg, Aoki & Cigelnik, 2000; Bischoff, Rehner & Humber, 2009; Watanambe et al., 2011, Salichos & Rokas, 2013; Damm et al., 2013). Dettman et al. (2003a) further upgraded operational criteria of GCPSR with the implementation of two step processes to resolve the complex phylogenetic problems in fungi. In the first step, independent evolutionary lineages were recognised by genealogical concordance and non-discordance and these lineages were subsequently subjected to the genetic differentiation and exhaustive subdivision process to determine the species limits (Dettman et al., 2003a; 2003b). The methods were further implemented in species complexes in model ascomycete *Neurospora* (Dettman et al., 2003b, 2006) and various other groups of fungi including important fungal genera (O' Donell, Ward, Geiser, Kistler & Aoki, 2004; Taylor et al., 2000; Cai et al., 2011; Laurence, Summerell, Burgess & Liew, 2014).

The aims of this study are as follows: 1) to define the species limits of *D. eres* and closely related species based on multi-gene genealogies coupled with morphological characters when possible; 2) to designate epitypes for *Diaporthe eres* and related species including *D. alnea*, *D. bicincta*, *D. celastrina*, *D. helicis* and *D. pulla* providing modern descriptions and illustrations with a revision of potential synonyms; and 3) to critically evaluate the phylogenetic species concepts in *Diaporthe* in relation to issue in *D. eres*, providing insights of utility of various genes within this species complex.

6.2 Materials and methods

6.2.1 Sampling and morphology

Sources of isolates are listed in Table 6.1. Additional fresh specimens and cultures were obtained from various contributors also listed in Table 6.1. Specimens of *D. eres* were initially collected from *Ulmus* in Germany and subsequent collections were made from the same host tree to identify both the sexual and asexual morphs. Morphological descriptions were based on type or epitype specimens. Descriptions were based on the type specimens and pycnidia made from WA+alfalfa stem media in culture. Digital images were captured and culture characteristics were observed as described in Udayanga et al. (2014).

6.2.2 DNA extraction, PCR and sequencing

DNA was extracted and the ITS, EF1-α, CAL, TUB and ACT genes were amplified following the protocols outlined by Udayanga et al. (2014). The FG1093 (60S ribosomal protein L37) was amplified using the universal primers for Ascomycota, E1F1 and E3R1 (Walker et al., 2012) following the touch-down PCR protocol outlined by the same study. HIS (Histone- 3) genes were amplified as described in Gomes et al. (2013) using the primer pair CYLH3F (Crous et al., 2004b) and H3-1b (Glass & Donaldson, 1995). Apn2 primers for *Diaporthe* were designed and the conditions were optimised as described below in this study.

Table 6.1 Isolates and sequences used in this study.

Species	Isolate/culture collection	Host	Collector	GenBank Accessions							
				ACT	Apn2	CAL	EF1-α	FG1093	HIS	ITS	TUB
D. alleghaniensis	CBS 495.72*	*Betula alleghaniensis*	R. H. Arnold	JQ807299	KJ380963	KC343249	GQ250298	KJ381045	KC343491	FJ889444	KC843228
D. alnea	CBS 146.46*	*Alnus* sp.	S. Truter	KJ420774	KJ380969	KC343250	KC343734	KJ381037	KC343492	KC343008	KC343976
	CBS 159.47	*Alnus* sp.	S. Truter	KJ420775	KJ380970	KC343251	KC343735	KJ381038	KC343493	KC343009	KC343977
	LCM22b.02a	*Alnus* sp.	L. Mejia	KJ420776	KJ380971	KJ435020	KJ210557	KJ381039	KJ420883	KJ210535	KJ420825
	LCM22b.02b	*Alnus* sp.	L. Mejia	KJ420777	KJ380972	KJ435021	KJ210558	KJ381040	KJ420884	KJ210536	KJ420826
D. bicincta	DP0659 = CBS 121004*	*Juglans* sp.	A.Y. Rossman	KJ420771	KJ380976	KC343376	KC343860	KJ381042	KC343618	KC343134	KC344102
D. celastrina	CBS 139.27*	*Celastrus* sp.	L. E. Wehmeyer	KJ420769	KJ380974	KC343289	KC343773	KJ381041	KC343531	KC343047	KC344015
D. citri	AR3405*	*Citrus* sp.	L. W. Timmer	KC843234	KJ380981	KC843157	KC843071	KJ381049	KJ420881	KC843311	KC843187
D. citrichinensis	ZJUD034A= CBS 134242*	*Citrus* sp.	F. Huang	KJ420779	KJ380980	KC843234	KC843071	KJ381048	KJ420880	KC843311	KC843187
	ZJUD034B =M1040	*Citrus* sp.	F. Huang	KJ420778	KJ380979	KJ435042	KJ210562	KJ381047	KJ420879	KJ210539	KJ420829
D. eres	AR5193*	*Ulmus* Sp.	R. Schumacher	KJ420760	KJ380958	KJ434999	KJ210550	KJ381003	KJ420850	KJ210529	KJ420799
	AR5196	*Ulmus* sp.	R. Schumacher	KJ420766	KJ380932	KJ435006	KJ210554	KJ381021	KJ420866	KJ210533	KJ420817
	DP0438	*Ulmus minor*	W. Jaklitch	KJ420765	KJ380935	KJ435016	KJ210553	KJ381020	KJ420886	KJ210532	KJ420816
	LCM114.01a	*Ulmus* sp.	L. Mejia	KJ420754	KJ380919	KJ435027	KJ210545	KJ380988	KJ420837	KJ210521	KJ420787
	LCM114.01b	*Ulmus* sp.	L. Mejia	KJ420754	KJ380918	KJ435026	KJ210544	KJ380987	KJ420836	KJ210520	KJ420786
	FAU483	*Malus* sp.	F. A. Uecker	JQ807326	KJ380933	KJ435022	JQ807422	KJ381031	KJ420874	KJ210537	KJ420827
	DAN001A =M1115	*Daphne laureola*	unknown	KJ420750	KJ380914	KJ434994	KJ210540	KJ380982	KJ420831	KJ210516	KJ420781
	DAN001B =M1116	*Daphne laureola*	unknown	KJ420751	KJ380915	KJ434995	KJ210541	KJ380983	KJ420832	KJ210517	KJ420782
	AR5197	*Rhododendron*	R.Schumacher	KJ420764	KJ380931	KJ435014	KJ210552	KJ381016	KJ420863	KJ210531	KJ420812
	CBS 439.82	*Cotoneaster* sp.	H. Butin	KC843231	KJ380920	JX197429	GQ250341	KJ380989	KC343574	FJ889450	JX275437

Table 6.1 (continued)

Species	Isolate/culture collection	Host	Collector	GenBank Accessions							
				ACT	Apn2	CAL	EF1-α	FG1093	HIS	ITS	TUB
D. eres	AR3519	*Corylus avellana*	W. Jaklitsch	KJ420758	KJ380922	KJ435008	KJ210547	KJ380991	KJ420839	KJ210523	KJ420789
	FAU506	*Cornus florida*	F. A. Uecker	JQ807328	KJ380925	KJ435012	JQ807403	KJ380994	KJ420842	KJ210526	KJ420792
	FAU570	*Oxydendrum arboreum*	F. A. Uecker	JQ807335	KJ380926	KJ435025	JQ807410	KJ380996	KJ420844	KJ210527	KJ420794
	AR3723	*Rubus fruticosus*	W. Jaklitsch	JQ807273	KJ380941	KJ435024	JQ807354	KJ380995	KJ420843	JQ807428	KJ420793
	FAU522	*Sassafras albida*	F. A. Uecker	JQ807331	KJ380924	KJ435010	JQ807406	KJ380993	KJ420841	KJ210525	KJ420791
	DP0666	*Juglans cinerea*	S. Anagnostakis	KJ420756	KJ380921	KJ435007	KJ210546	KJ380990	KJ420838	KJ210522	KJ420788
	DP0667 =CBS 135428	*Juglans cinerea*	S. Anagnostakis	KC843232	KJ380923	KC843155	KC843121	KJ380992	KJ420840	KC843328	KC843229
	AR3560	*Viburnum* sp.	W. Jaklitch	JQ807270	KJ380939	KJ435011	JQ807351	KJ380998	KJ420846	JQ807425	KJ420795
	AR5224	*Hedera helix*	R. Schumacher	KJ420763	KJ380961	KJ435036	KJ210551	KJ381006	KJ420853	KJ210530	KJ420802
	AR5231	*Hedera helix*	R. Schumacher	KJ420767	KJ380936	KJ435038	KJ210555	KJ381022	KJ420867	KJ210534	KJ420818
	AR5223	*Acer nugundo*	R. Schumacher	KJ420759	KJ380938	KJ435000	KJ210549	KJ380997	KJ420845	KJ210528	KJ420830
	CBS 109767 =AR3538	*Acer* sp.	W. Jaklitsch	JQ807294	KJ380940	KC343317	KC343801	JF319006	KC343559	DQ491514	KC344043
	DLR12A =M1117	*Vitis vinifera*	L. Phillipe	KJ420752	KJ380916	KJ434996	KJ210542	KJ380984	KJ420833	KJ210518	KJ420783
	DLR12B =M1118	*Vitis vinifera*	L. Phillipe	KJ420753	KJ380917	KJ434997	KJ210543	KJ380985	KJ420834	KJ210519	KJ420784
	AR4347	*Vitis vinifera*	S.K. Hong	JQ807275	KJ380929	KJ435030	JQ807356	KJ381009	KJ420856	JQ807430	KJ420805
	Di-C005/1	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250334	-	-	GQ250203	-

Table 6.1 (continued)

Species	Isolate/culture collection	Host	Collector	GenBank Accessions							
				ACT	Apn2	CAL	EF1-α	FG1093	HIS	ITS	TUB
D. eres	Di-C005/2	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250335	-	-	GQ250204	-
	Di-C005/3	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250336	-	-	GQ250205	-
	Di-C005/4	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250342	-	-	GQ250208	-
	Di-C005/5	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250343	-	-	GQ250209	-
	Di-C005/6	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250344	-	-	GQ250210	-
	Di-C005/7	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250345	-	-	GQ250211	-
	Di-C005/8	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250337	-	-	GQ250206	-
	Di-C005/9	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250346	-	-	GQ250212	-
	Di-C005/10	*Hydrangea macrophylla*	J. M. Santos	-	-	-	GQ250347	-	-	GQ250213	-
	AR4355	*Prunus* sp.	S. K. Hong	JQ807278	KJ380942	KJ435035	JQ807359	KJ381001	KJ420848	JQ807433	KJ420797
	AR4367	*Prunus* sp.	S. K. Hong	JQ807283	KJ380962	KJ435019	JQ807364	KJ381029	KJ420873	JQ807438	KJ420824
	AR4346	*Prunus mume*	S. K. Hong	JQ807274	KJ380955	KJ435003	JQ807355	KJ381027	KJ420872	JQ807429	KJ420823
	AR4348	*Prunus persici*	S. K. Hong	JQ807276	KJ380952	KJ435004	JQ807357	KJ381015	KJ420862	JQ807431	KJ420811
	AR3669= MAFF 625029	*Pyrus pyrifolia*	S. Kanematsu	JQ807340	KJ380930	KJ435002	JQ807415	KJ381012	KJ420859	JQ807466	KJ420808
	AR3670 = MAFF 625030	*Pyrus pyrifolia*	S. Kanematsu	JQ807341	KJ380950	KJ435001	JQ807416	KJ381011	KJ420858	JQ807467	KJ420807
	AR3671=MAFF 625033	*Pyrus pyrifolia*	S. Kanematsu	JQ807342	KJ380954	KJ435017	JQ807417	KJ381018	KJ420865	JQ807468	KJ420814

Table 6.1 (continued)

Species	Isolate/culture collection	Host	Collector	GenBank Accessions							
				ACT	Apn2	CAL	EF1-α	FG1093	HIS	ITS	TUB
D. eres	AR3672= MAFF 625034	*Pyrus pyrifolia*	S. Kanematsu	JQ807343	KJ380937	KJ435023	JQ807418	KJ381023	KJ420868	JQ807469	KJ420819
	DP0177	*Pyrus pyrifolia*	W. Kandula	JQ807304	KJ380945	KJ435041	JQ807381	KJ381024	KJ420869	JQ807450	KJ420820
	DP0591	*Pyrus pyrifolia*	W. Kandula	JQ807319	KJ380946	KJ435018	JQ807395	KJ381025	KJ420870	JQ807465	KJ420821
	AR4369	*Pyrus pyrifolia*	S. K. Hong	JQ807285	KJ380953	KJ435005	JQ807366	KJ381017	KJ420864	JQ807440	KJ420813
	DP0180	*Pyrus pyrifolia*	W. Kandula	JQ807307	KJ380928	KJ435029	JQ807384	KJ381008	KJ420855	JQ807453	KJ420804
	DP0179	*Pyrus pyrifolia*	W. Kandula	JQ807306	KJ380944	KJ435028	JQ807383	KJ381007	KJ420854	JQ807452	KJ420803
	DP0590	*Pyrus pyrifolia*	W. Kndula	JQ807318	KJ380951	KJ435037	JQ807394	KJ381014	KJ420861	JQ807464	KJ420810
	AR4373	*Ziziphus jujuba*	S. K. Hong	JQ807287	KJ380957	KJ435013	JQ807368	KJ381002	KJ420849	JQ807442	KJ420798
	AR4374	*Ziziphus jujuba*	S. K. Hong	JQ807288	KJ380943	KJ434998	JQ807369	KJ380986	KJ420835	JQ807443	KJ420785
	AR4357	*Ziziphus jujuba*	S. K. Hong	JQ807279	KJ380949	KJ435031	JQ807360	KJ381010	KJ420857	JQ807434	KJ420806
	AR4371	*Malus pumila*	S. K. Hong	JQ807286	KJ380927	KJ435034	JQ807367	KJ381000	KJ420847	JQ807441	KJ420796
	FAU532	*Chamaecyparis thyoides*	F. A. Uecker	JQ807333	KJ380934	KJ435015	JQ807408	KJ381019	KJ420885	JQ807333	KJ420815
	CBS113470	*Castanea sativa*	K.A. Seifert	KJ420768	KJ380956	KC343388	KC343872	KJ381028	KC343630	KC343146	KC344114
	AR4349	*Vitis vinifera*	S.K. Hong	JQ807277	KJ380947	KJ435032	JQ807358	KJ381026	KJ420871	JQ807432	KJ420822
	AR4363	*Malus* sp.	S. K Hong	JQ807281	KJ380948	KJ435033	JQ807362	KJ381013	KJ420860	JQ807436	KJ420809
	DNP128 (=BYD1,M1119)	*Castaneae mollissimae*	S.X. Jiang	KJ420762	KJ380960	KJ435040	KJ210561	KJ381005	KJ420852	JF957786	KJ420801
	DNP129 (=M1120)	*Castaneae mollissimae*	S.X. Jiang	KJ420761	KJ380959	KJ435039	KJ210560	KJ381004	KJ420851	JQ619886	KJ420800

Table 6.1 (continued)

Species	Isolate/culture collection	Host	Collector	GenBank Accessions							
				ACT	Apn2	CAL	EF1-α	FG1093	HIS	ITS	TUB
	CBS 587.79	*Pinus pantepella*	G. H. Boerema	KJ420770	KJ380975	KC343395	KC343879	KJ381030	KC343637	KC343153	KC344121
D. helicis	AR5211*	*Hedera helix*	A. Gardiennet	KJ420772	KJ380977	KJ435043	KJ210559	KJ381043	KJ420875	KJ210538	KJ420828
D. neilliae	CBS 144. 27	*Spiraea* sp.	L.E. Wehmeyer	KJ420780	KJ380973	KC343386	KC343870	KJ381046	KC343628	KC343144	KC344112
D. pulla	CBS 338.89*	*Hedera helix*	M. Cevetcovic	KJ420773	KJ380978	KC343394	KC343878	KJ381044	KC343636	KC343152	KC344120
D. vaccinii	CBS 135436 =DF5032	*Vaccinium* corymbosum	D.F. Farr	JQ807303	KJ380964	KC849457	JQ807380	KJ381032	KJ420877	AF317570	KC843225
	FAU633	*Vaccinium* macrocarpon	F. A. Uecker	JQ807338	KJ380966	KC849456	JQ807413	KJ381034	KJ420878	U11360,U11414	KC843226
	FAU446	*Vaccinium* macrocarpon	F. Caruso	JQ807322	KJ380967	KC849455	JQ807398	KJ381035	KJ420882	U11317,U11367	KC843224
	CBS 160.32*	*Vaccinium* macrocarpon	C. Shear	JQ807297	KJ380968	KC343470	GQ250326	KJ381036	KC343712	AF317578	JX270436
	FAU 468	*Vaccinium* macrocarpon	F. A. Uecker	JQ807323	KJ380965	KC849458	JQ807399	KJ381033	KJ420876	U113327,U11377	KC843227

Notes. CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands ; AR, DF, DP,DLR, FAU, DNP , M, LCM : culture collection in Systematic Mycology and Microbiology, Beltsville, Maryland, USA ; MAFF : Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan.

PCR reaction mixtures were similar to section 4.2.2 and the conditions for amplification of Apn2 gene were 95 C° for 1 min, (95 °C: 30 s, 54 °C: 50 s, 72 °C: 1 min) ×39 cycles, 72 °C for 10 min extension. The product purification and sequencing is as described in section 4.2.2.

6.2.3 Apn2 (DNA lyase) primer design and assessment

Kanematsu et al. (2007) defined the structure of mating type genes with the complete sequences of two mating types (MAT1-1-1 and MAT 1-2-1) in *Diaporthe* with the presence of Apn2 (DNA-lyase) and SLA2 (transmembrane protein) genes flanking MAT gene as previously observed in some other filamentous ascomycetes (Butler et al., 2004; Debuchy & Turgeon, 2006). A DNA-lyase gene is coded at the 3' end of MAT1-1-3 in MAT1-1 and MAT1-2 of *Diaporthe* W- and G-types. Therefore, the mating-type loci of *Diaporthe* W- and G-types and other ascomycetes are thought to be orthologous. An alignment of the complete four sequences of Apn2-Mat genes of *Diaporthe* W and G types in Kanematsu et al. (2007) (AB199324-27), with a selected set of homologous Apn2 genes available in GenBank including *Colletotrichum caudatum* (JX076930-32), *C. cerale* (EU365102, 365045, 365117), *C. fragariae* (FR719119), *C. fructicola* (FR719124), *C. gloeosporioides* (FR719121-22, FR719126), *C. siamense* (FR719125), and *Thielavia terrestris* chromosome A (XM003651303), *Myceliopsthora thermophila* Chromosome 1 (CP003002), and the mating type A locus from *Neurospora terricola* (HE600070), *N. pannonica* (HE600067) and *N. africana* (HE600066) were used to design the forward and reverse primers to amplify the Apn2 gene. The target fragment contained the Apn2 gene approximately 800 bp including an intron region of 70-100 bp. The forward primer (apn2fw2: GCMATGTTYGAMATYCTGGAG) and the reverse primer (apn2rw2: CTT GGTCTCCCAGCAGGTGAAC) were designed based on the proximal end of first exon and the distal end of the second exon region relatively conserved across the alignment. The selected primers were then evaluated for thermal properties, GC content, hairpin formation, melting temperatures (T_m) and self-complementarities using the online platforms of OligoCalc (Kibbe, 2007) and the Sequence Manipulation Suite v. 2 (SMS v. 2) (Stothard, 2000).

Gradient PCR and reagent optimisations were used to develop the standard protocols for amplification. Twelve reactions across an annealing temperature gradient of 65–50 ˚C for each of the test isolates were performed in three replicates. The optimal annealing temperature was determined by the intensity of the amplicons visualised by agarose gel electrophoresis.

Primers were initially tested against a panel of 20 species selected from a broad range of *Diaporthe* species (derived from ex-type cultures) and closely related taxa (Table 6.1) including the representative isolates of *Ophiodiaporthe cyathea* (AR5192) and *Mazzantia galii* (AR4658). PCR products were purified and sequenced using the protocols detailed section 4.2.2.

6.2.4 Sequence alignment and phylogenetic analysis

Raw sequences were assembled with Sequencher 4.9 for Windows (Gene Codes Corp., Ann Arbor, Michigan). The consensus sequences were then initially aligned using online source of MAFFTv.7 (Katoh & Standley 2013) and optimised in the SATEv.2.2.7 (Simultaneous Alignment and Tree Estimation) high throughput alignment platform (Liu et al., 2012). Newly generated ITS and EF1-α sequences were analysed with all available type-derived sequences listed in Udayanga et al., (2011; 2012a) and Gomes et al. (2013) to determine the primary identity of the isolates within the species complex. Analysis of the EF1-α alignment was used for the initial identification of phylogenetically distinct lineages as it was presumed that the ITS may obscure the species delineation within the *D. eres* species complex. ML, MP and Bayesian gene-trees were estimated using the methods described in the section 4.2.4. All isolates were subjected to a multi-gene analysis of seven genes including Apn2, EF, CAL, HIS, FG1093, ACT and TUB regions, excluding the ITS region from the combined analysis. Individual datasets were tested for congruency using the reciprocal 70 % bootstrap (BS) threshold method as described by Gueidan et al. (2007). Phylogenetic trees and data files were viewed using the softwares mentioned in section 4.2.4.

6.2.5 Phylogenetic species recognition

In order to accurately determine the species boundaries, we applied the criteria previously described by Dettman et al. (2003a). Clades were genealogically concordant if they were present at least some the gene trees and genealogically non-discordant if they were strongly supported (MP≥70%; ML≥70%) in a single gene and not contradicted at or above this level of support in any other single gene tree. This criterion prohibited poorly supported non-monophyly at one locus from undermining well-supported monophyly at another locus. In addition, species limits were determined conclusively if resolved with strong support (PP≥.95; ML≥70%; MP≥75%) in all analyses for either the combined dataset seven genes (excluding ITS). Since the variability of ITS sequences within the *D. eres* clade resulted confusion, also confirmed by Santos et al. (2010), we opted to use the combined seven gene alignment to reconstruct the evolutionary relationships.

When deciding which independent evolutionary lineages should be ranked as phylogenetic species, genetic differentiation and exhaustive subdivision criteria were applied (Dettman et al., 2003a; 2006). Genetic Differentiation required that lineages be well differentiated, preventing minor terminal lineages from being recognised as phylogenetic species. Exhaustive subdivision required that all individuals be classified into phylogenetic species and no individuals were to be left unclassified. The technique involved tracing from the terminals of the tree, collapsing all lineages that were not subtended by an independent evolutionary lineage (Dettman et al., 2006; Laurence et al., 2014).

6.2.6 Testing phylogenetic informativeness

To determine loci most suitable for species level phylogenetic inference in closely related species within *Diaporthe* we employed the phylogenetic informativeness profiling method (Townsend, 2007) implemented in PhyDesign (Lopez-Giraldez & Townsend, 2011). Phylogenetic informativeness (PI) was measured from a partitioned combined dataset of 10 ex-types / taxonomically authenticated species for the ITS, EF1-α, TUB, CAL, ACT, HIS, FG1093 and Apn2 genes. The maximum likelihood tree from RAxML analysis of the concatenated data set was ultrametricised using Mesquite (Maddison & Maddison, 2011).

Per gene and per site informativeness for all partitions were determined using PhyDesign and the rates of change for each site determined under the HyPhy criteria (Pond, Frost & Muse, 2005). The resulting topology and informativeness profiles are presented.

6.3 Results

6.3.1 DNA Sequencing, Apn2 new primers and phylogenetic analyses

Four hundred new sequences were generated in this study (Table 6.1) from 68 living cultures of *Diaporthe* for eight genes (ACT, Apn2, CAL, EF1-α, HIS, FG1093, ITS and TUB). Additional sequences were obtained from GenBank.

The Apn2 primers were newly designed and used in this study. Analysis of the newly designed Apn2 primers (apnfw2/apanrw2) revealed that the melting temperatures (Tm) of apn2fw2= 49-56 °C and apn2rw2=58.6 °C with GC content of apn2fw2= 38-57% and apn2rw2=59%. No hairpin formation or self-complementarities were found. The optimal annealing temperature for the primer pair was determined to be 54 °C by the by gradient PCR with PCR amplification conditions as outlined in materials and methods. Amplification and sequencing of 20 different ex-type isolates of *Diaporthe* outside of the *D. eres* species complex (GenBank accessions KM016673-KM016694) including additional isolates of *Ophiodiaporthe cyathea* (AR5192, KM016693) and *Mazzantia galii* (AR4658, KM016692) were successful. The standard blastx search for resulted Apn2 sequences revealed that the genes are homologous to the putative conserved domains in EEP (exonuclease/endonuclease/phosphatase) super-family of proteins which include large number of catalytic enzymes including AP endonucleases.

Eight different alignments corresponding to each individual gene, a combined alignment of all eight genes, and a combined alignment of the seven genes excluding the ITS were analysed. Comparison of the alignment properties and nucleotide substitution models are provided in Table 6.2. Phylogenetic trees inferred from EF1-α and ITS sequences of total isolates, summary of the results of GCPSR in RAxML

cladogram and a Phylogram of combined analysis of seven genes are presented with annotations for species, host and geographic origin (Figures 6.1, 2, 3).

Table 6.2 Alignment properties

Genes/loci	ITS	EF1-α	CAL	TUB	ACT	FG1093	HIS	Apn2	combined 7 genes
Characters included in analysis (with gaps)	548	369	429	517	259	379	501	769	3193
Characters excluded in analysis	-	-	101	-	-	-	-	-	101
Invariable characters	479	266	309	434	186	263	381	645	2563
Parsimony informative characters (%)	43(8)	62(16)	45(10)	69(13)	40(15)	44(11)	69(13)	78(10)	427(13)
Uninformative variable characters	26	41	75	14	33	72	51	46	203
Nucleotide substitution models	GTR+I+G	HKY+G	GTR+I+G	HKY+G	GTR+I+G	HKY+G	GTR+I+G	HKY+G	GTR+I+G

6.3.2 Single gene analysis and comparison

The ITS and EF1-α sequence alignment comprised of 548 and 369 characters respectively, with 78 isolates with the out group. Phylogenetic trees obtained from maximum likelihood (ML), parsimony (MP), and Bayesian (BI) analysis were compared for the placement of each isolate, topology of the tree and clade stability. The topology of the ML tree inferred from RAxML was identical to BI and MP trees with reference to the major sub clades an ML tree was used to present the ITS and EF1-α phylogenetic trees (Figure 6.1).

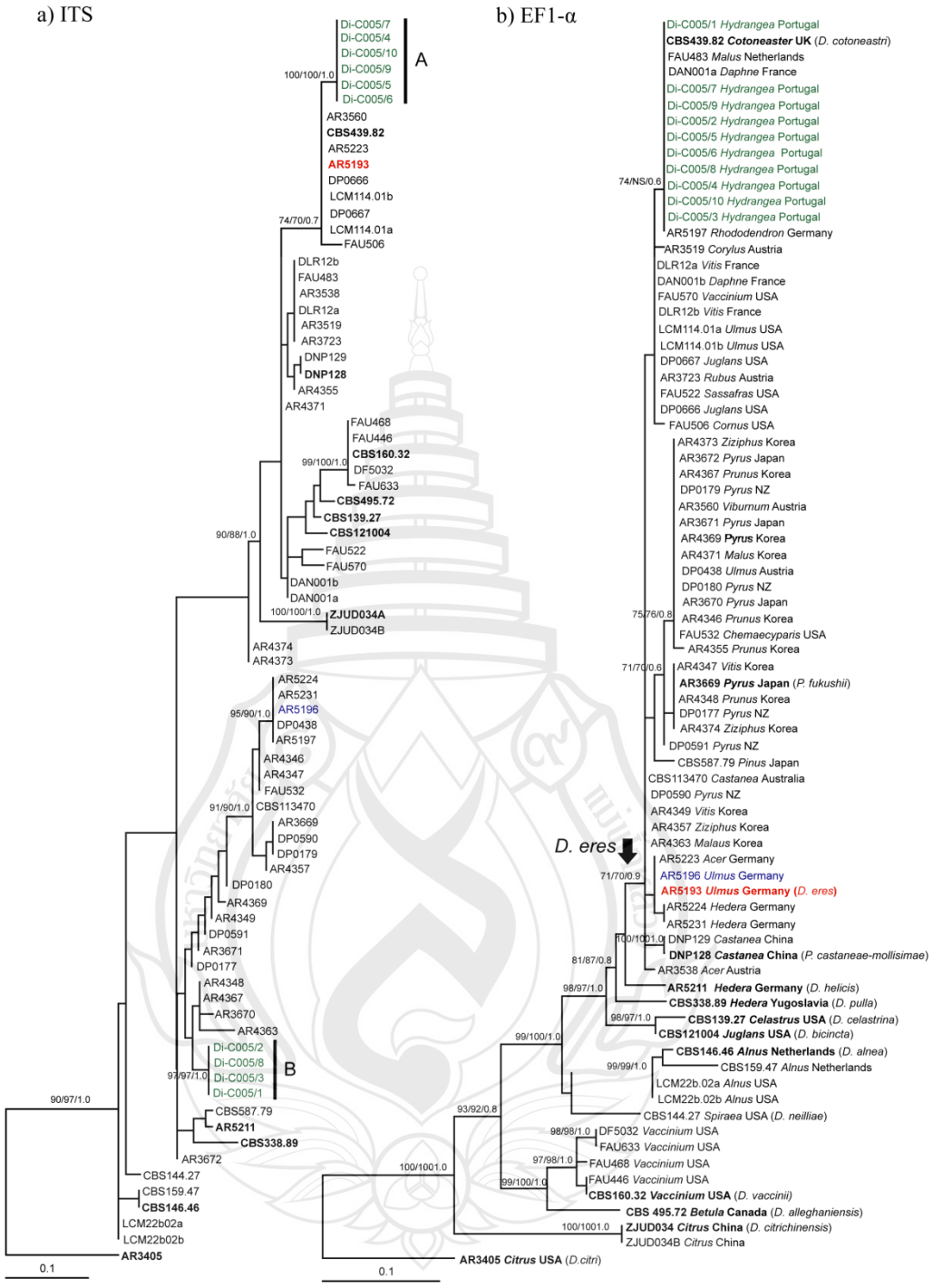


Figure 6.1 The phylograms inferred from RAxML analysis of A) ITS and B) EF1-α. The ML, MP bootstrap values ≥70%, PP≥0.75 are indicated. The trees are rooted with *Diaporthe citri* (AR3405). The sequences of Di-C005/1-10 (green) were obtained from Santos et al. (2010). Ex-type and ex-epitype cultures are in bold.

The other alignment properties resulted in parsimony analysis were shown in Table 6.2. The preliminary analysis and the similarity search through recent studies used to select the taxa clustered with this group of interest and the selection of basal lineages as out groups. The high degree of homoplasy was observed in within each of the single gene alignments therefore large numbers of most parsimonious trees were usually generated. The visible comparison of the alignments confirmed that the identification based on either barcode ITS region or any other gene is difficult.

The ITS phylogeny has limited resolution within the species complex often resulting in an inconclusive branching order and lack of bootstrap support at the internodes, resulting in two major clusters. Analysis of each region of the ITS sequences of *Diaporthe eres* with the reference annotated sequence (KC343073) revealed an approximately 176 bp span for ITS1 and 161 bp for ITS2 region with the intermediate 5.8s rDNA partition spanning approximately 157 bp. The differences within two ITS1 clusters were consistent although the two clusters were not completely congruent with the ITS2 region.

We obtained two different isolates (AR5193, AR5196) derived from two twigs of *Ulmus* collected from the same tree at the same time in Germany that were determined to be *D. eres* based morphology and host association. However, the single ascospore derived isolate (AR5193), had a different ITS sequence from the single conidial derived isolate (AR5196) similarly clustered as different groups. However, they were always determined as a single species based on EF1-α and all the other genes. Also according to the principles of genealogical non-discordance, these ITS variations only can be recognised as either poorly supported or non monophyletic grouping and therefore do not support as distinct phylogenetic species (Figure 6.1).

Inspection of the ITS alignment also revealed that isolates can share similarity in the ITS1 and ITS2 regions both within and between species in this complex. The ITS1 region of *Diaporthe vaccinii* is identical to most of the isolates identified as *D. eres*. In contrast, the ITS2 region is different within the two corresponding isolates. *Diaporthe citri* and *Diaporthe citrichiensis* share ITS partitions with the other species in the complex. However they are strongly divergent when compared to the other genes and therefore regarded as outgroup taxa in the analyses.

As opposed to the ITS, the EF1-α phylogenetic tree clearly distinguished the species boundaries except in a few closely related species, which were only recognised in the combined analyses. The EF1-α phylogenetic tree was used as an initial guide to determine the species limits and tested with all the other genes and in various combinations. Therefore, nodes of which the EF1-α phylogenetic tree significantly supported (≥70 %) for the major lineages were initially recognised as the species limits and then later confirmed by the other species recognition criteria were used. However, species limits were not confirmed until the genealogical concordance, non-discordance, genetic differentiation and exhaustive subdivision criteria are applied.

Comparison of each single gene phylogenetic tree revealed that the *D. eres* isolates recognised by the EF1-α phylogenetic tree cluster together with a significant bootstrap support of the other genes; however a minor genetic variation was always present in regard to the species recognised in combined tree.

6.3.3 Genealogical concordance phylogenetic species recognition

The combined sequence alignment of seven genes comprised 3293 total characters from 68 isolates. An ambiguously aligned region of 100 bp (2677-2777) in the combined alignment was excluded from the analysis. The phylogenetic tree inferred from ML analysis was identical to the Bayesian and parsimony trees in terms of major clades and branching order. A total of 25 independent evolutionary lineages were recognised based on given criteria of the ML/MP ≥70% bootstrap support in single genes, are summarised on to the combined cladogram (Figure 6.2). The lineage number 11 only supported by the tubulin gene tree and contradicted by all other 7 gene trees (including ITS) and number 13 only poorly supported by combined tree and contradicted with all single gene trees. Therefore the two lineages were excluded under genealogical non-discordance criterion. The all other lineages were supported with more than one genes at the same level as in the EF1-α tree (Figure 6.1) and not contradicted when not supported, therefore selected under genealogical concordance criterion for further analysis to determine the species limits.

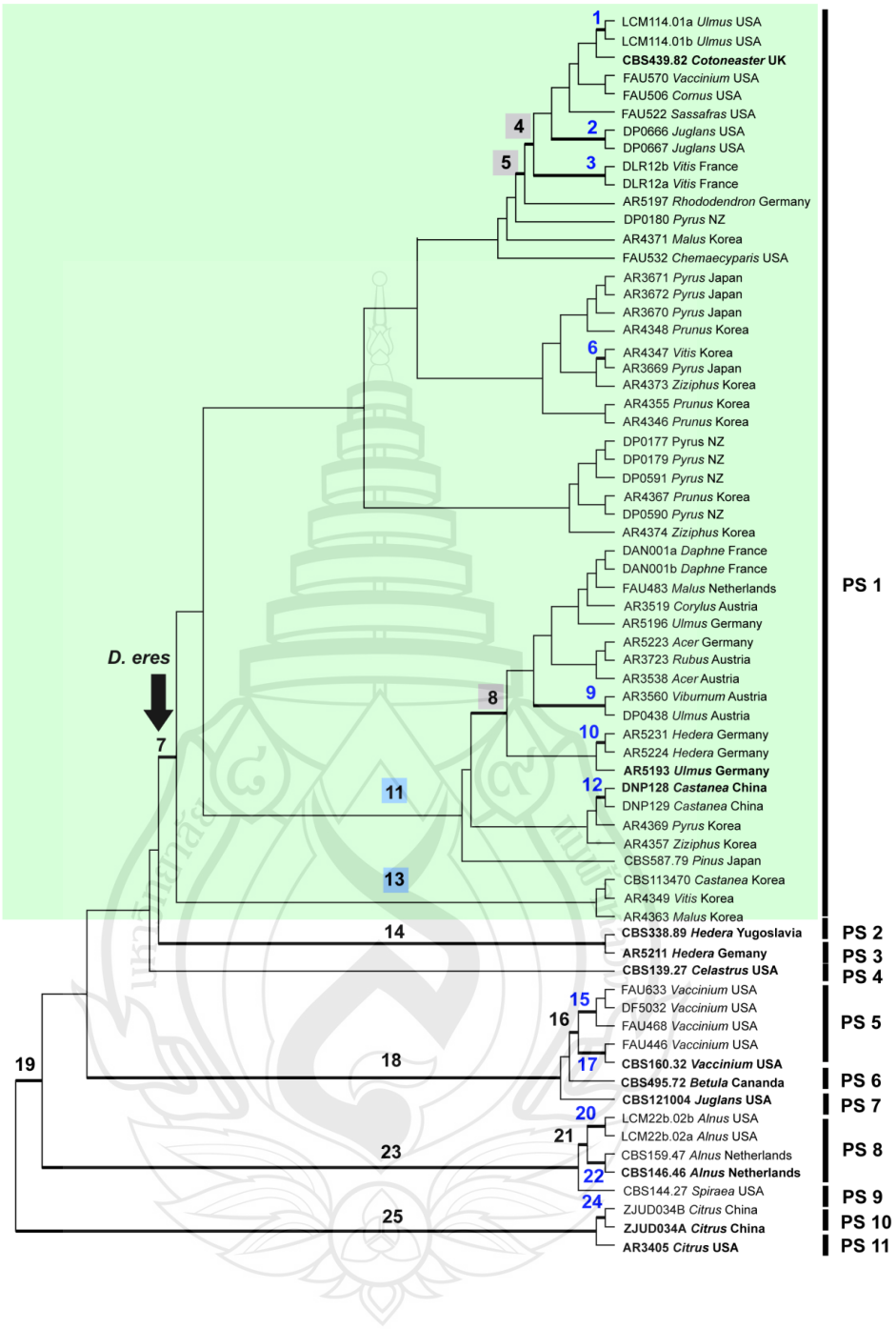


Figure 6.2 The summary of GCPSR: The RAxML cladogram of combined 7 genes. The blue filled squares (11 and 13) indicate the branches supported in combined analysis but either poorly supported or contradicted in single gene trees. The terminal numbers (blue) were excluded from the ranking process under the genetic differentiation criterion. PS 1- PS 11 indicates the phylogenetic species recognised.

To fulfill the genetic differentiation criterion, the terminal lineages 1, 2, 3, 6, 9, 10, 11, 12, 15, 17, 20, 22 and 24 (blue numbers) resulted in combined analysis were excluded from exhaustive subdivision process (Figure 6.2). The remaining 11 lineages were used in exhaustive subdivision process, involved tracing from the terminals of the tree. All lineages not subtended by an independent evolutionary lineage were collapsed, to satisfy that all individuals should be classified and none remained unclassified. To satisfy the exhaustive subdivision criterion, lineage number 4, 5, 8 were collapsed under lineage number 7 which is supported by all 7 genes and combined analysis to recognise the phylogenetic species 1 (PS1).

The PS 2 and PS 3 were recognised based on the support of each single gene trees as distinct sister taxa represented by singletons. Phylogenetic species, PS 4 to PS 11 were recognised based on exhaustive subdivision of the rest of the lineages later assigned to the distinct species based on placement of ex-type and ex-epitype isolates.

The tree generated from the RAxML was used to represent the phylogeny of *D.eres* species complex annotated with host and geographic origin of the each isolate and determination of species (Figure 6.3). The phylogenetic species recognised in above analyses (PS 1-PS 11) were then assigned to the species based on the ex-type and ex-epitype isolates and supported with morphological studies of all available isolates. The species determination was highly similar with the EF1-α phylogenetic tree and the clade credibility values of each of the method increased when compared to the EF1-α phylogenetic tree with a relatively stable tree topology. The limit of the *D. eres* was determined based on the well supported node at lineage number 7 assigned as PS 1 in the combined phylogenetic tree with application of GCPSR criteria. Therefore, a total of 9 phylogenetic species were recognised viz PS 1: *D. eres*, PS 2: *D. pulla*, PS 3: *D. helicis*, PS 4: *D. celastrina*, PS 5: *D. vaccinii*, PS 6: *D. alleghaniensis*, PS 7: *D. bicincta*, PS 8:*D. alnea*, PS 9 = *D. neilliae* within the species complex when using two closely related species, *D. citri* (PS 11) and *D. citrichinenesis* (PS 10) as out-group taxa in the combined analysis (Figure 6.2). Therefore the limit of the *D. eres* species complex was determined corresponding to the node 19 in Figure 6.2, with accepted 9 species, considering *D. citri* and *D. citrichinensis* as basal lineages. These ex-types/reference isolates of these 10 species were used to profile the phylogenetic informativeness of genes (Figure 6.4).

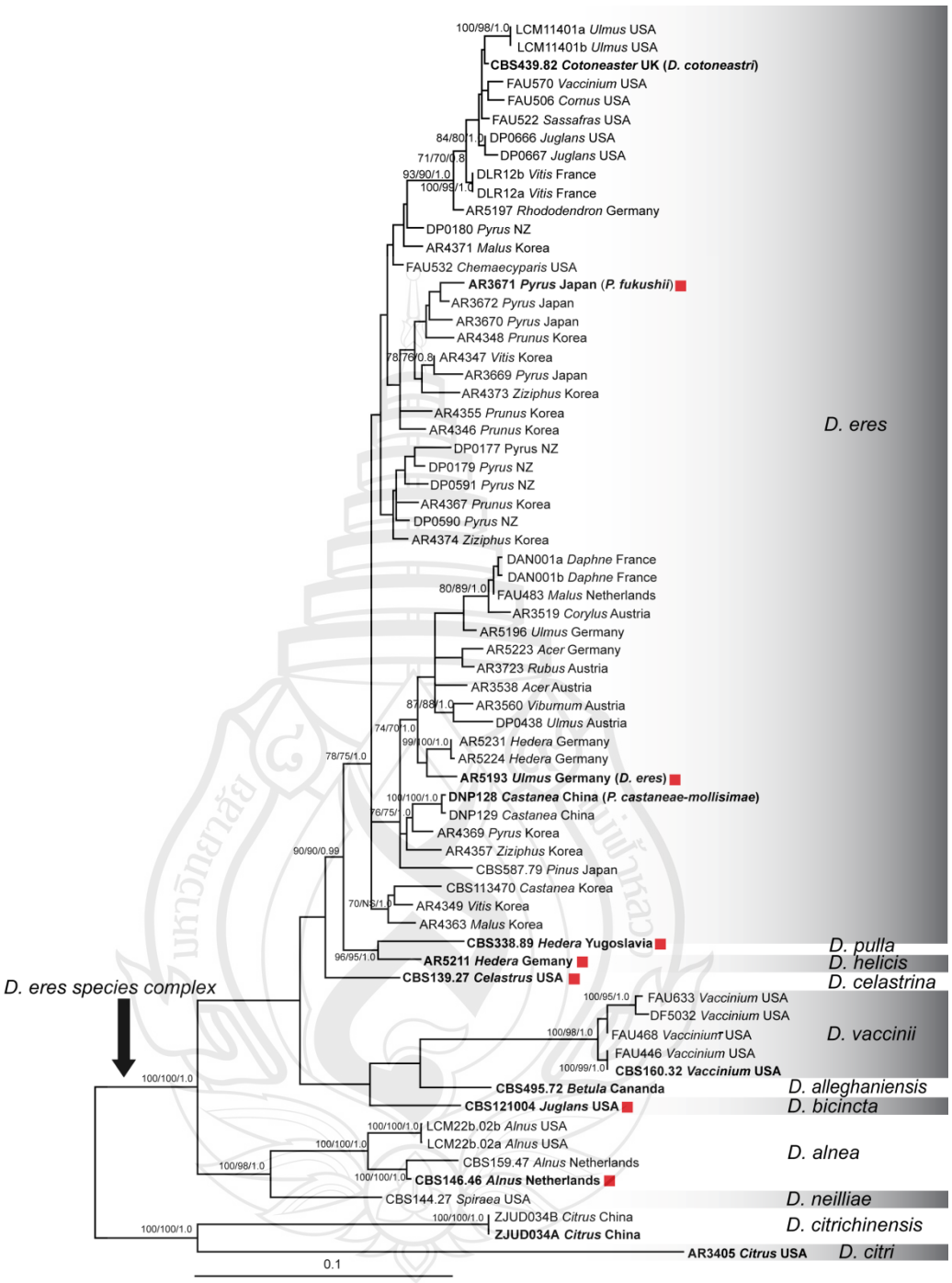
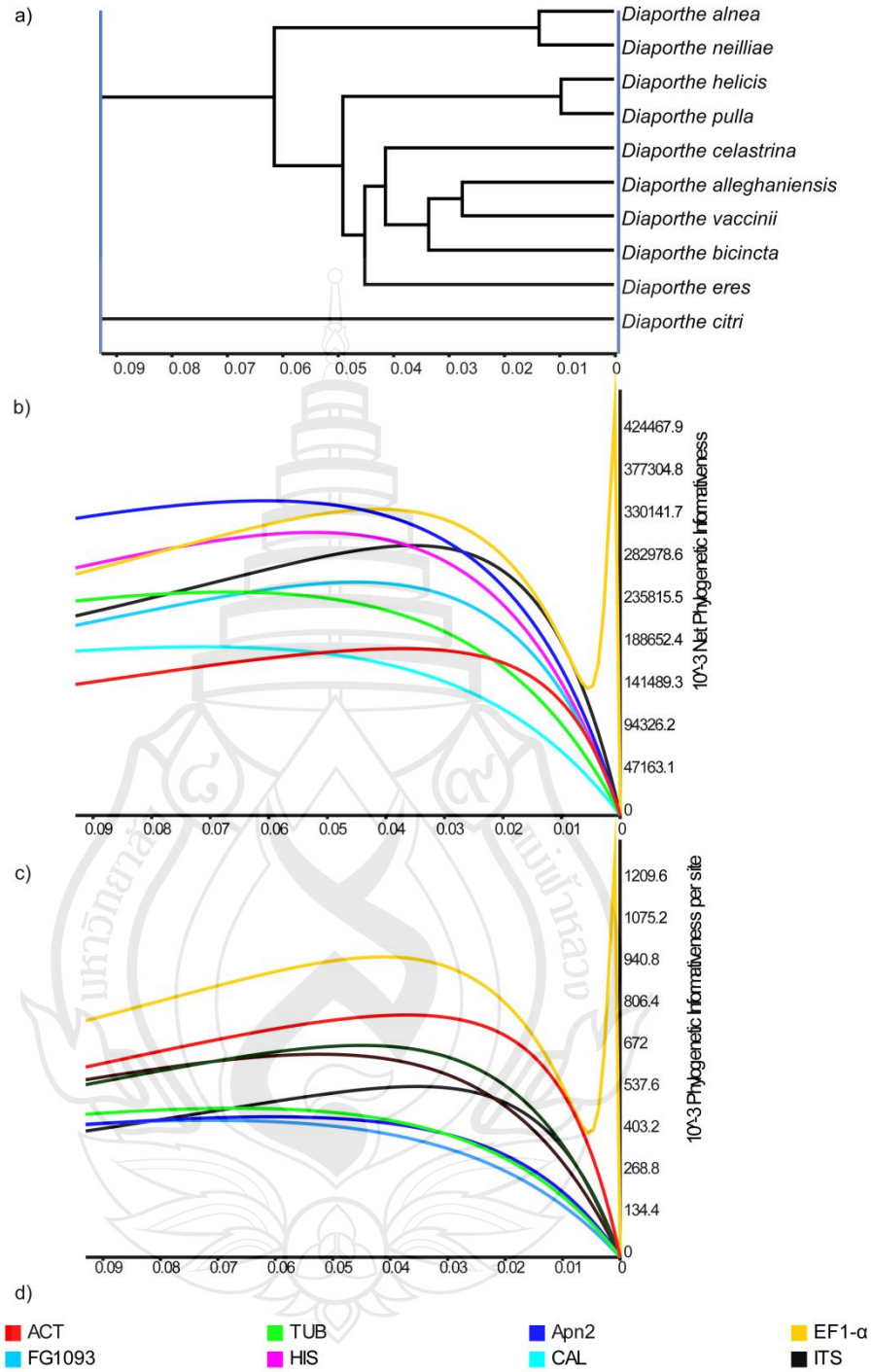


Figure 6.3 The RAxML phylogram based on combined 7 genes. The ML, MP bootstrap values ≥70%, PP≥0.75 are indicated above the branches. The tree is rooted with *Diaporthe citri* (AR3405) and *D. citrichineneis* (ZJUD034A, B). Ex-type and ex-epitype cultures are in bold. Epitypes and neotypes designated in this study are indicated with a red squares.



Notes. a. Ultrametric tree generated from the combined analysis of 8 genes; b. Net Phylogenetic informativeness; c. Phylogenetic informativeness per site; d. key.

Figure 6.4 Profiles of phylogenetic informativeness within the species complex.

Diaporthe pulla (PS 2) and *D. helicis* (PS 3) appeared to be sister taxa and were very closely related each other and with *D. eres* (PS 1). However, based on the comparison of each single gene tree these two species are diverged from the major sub-clade of *Diaporthe eres* and each other to recognise as distinct phylogenetic species.

6.3.4 Phylogenetic informativeness

The informativeness profiles indicated that the EF1-α, Apn2 and HIS genes are the best candidate markers to resolve the phylogenetic species included in this analysis (Figure 6.4). The EF1-α and ACT genes performed the best in terms of phylogenetic informativeness per site. The informativeness ranking predicts the comparative, quantitative measurements to prioritise the utility of genes within this species complex and species recognition based on more informative gene genealogies.

6.3.5 Taxonomy

Based on the phylogenetic analyses, the type species of *Diaporthe*, *D. eres*, is circumscribed along with eleven closely related but phylogenetically distinct lineages, each of which is briefly described and illustrated. If a modern description exists, the species is annotated with host association, distribution and notes on taxonomy and phylogeny. As listed after the descriptions, type and non-type specimens were observed for each species. Epitype specimens were designated for six species. In addition, ex-type, ex-epitype, and non-type cultures were observed, if available.

Diaporthe eres Nitschke, Pyrenomycetes Germanici 2: 245 (1870) , nom. cons. prop. Figure 6.5

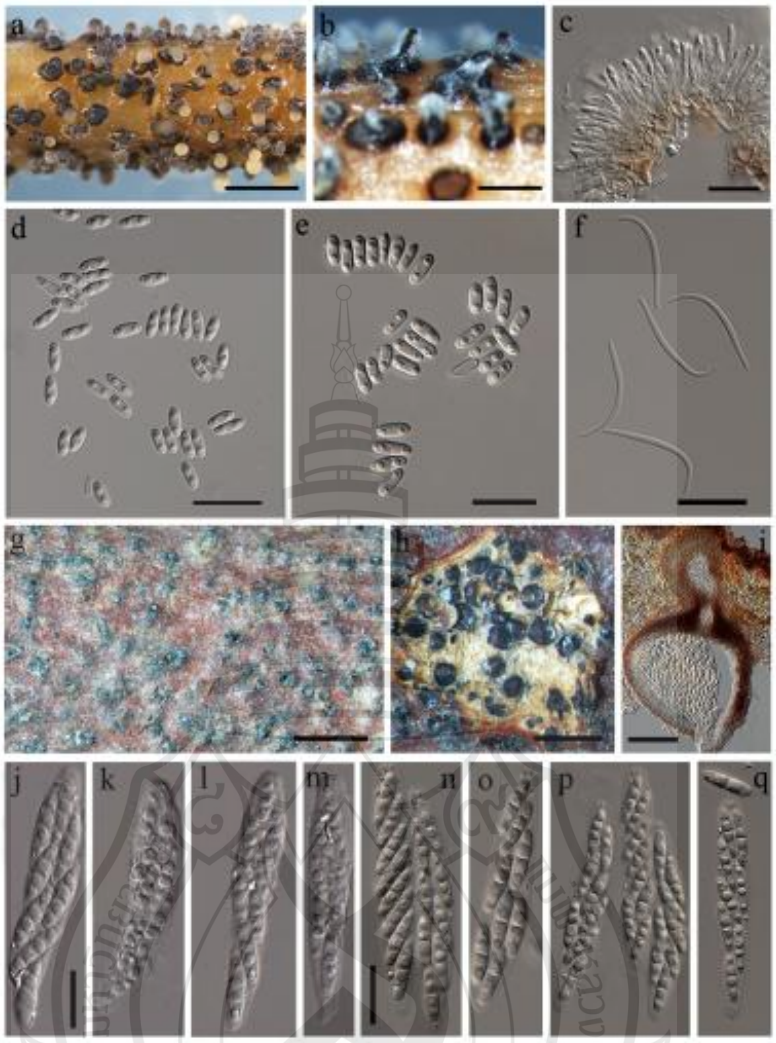
= *Phoma oblonga* Desm., Annls Sci. Nat., Bot., sér. 3, 22: 218. (1853)

≡ *Phomopsis oblonga* (Desm.) Traverso, Fl. ital. crypt., Pars 1: Fungi. Pyrenomycetae. Xylariaceae, Valsaceae, Ceratostomataceae: 248 (1906)

= *Phomopsis cotoneastri* Punith., Trans. Br. mycol. Soc. 60: 157 (1973)

≡ *Diaporthe cotoneastri* (Punith.) Udayanga, Crous & K.D. Hyde, Fungal Diversity 56: 166 (2012)

=*Phomopsis castaneae-mollisimae* S.X. Jiang & H.B. Ma, Mycosystema 29: 467 (2010)



Notes. a. Pycnidia on alfalfa stem on WA; b. pycnidial necks protruding on alfalfa stem; c. conidiophores; d,e. α- conidia; f. β- conidia; g. Ectostroma on the dead twigs of *Ulmus*; h. Perithecia; i. Ascomata in section; j-q. Asci and ascospores. Specimens a-e. AR5193 g-m. B 70 0009145 n-q. epitype specimen (BPI892912), Bars. a=1000 µm, b= 500 µm, c=10 µm, d,e= 15 µm f=10 µm g=1000 µm, h= 500 µm, i= 100 µm, J-q= 15 µm.

Figure 6.5 Morphology of *Diaporthe eres.*

≡ *Diaporthe castaneae-mollisimae* (S.X, Jiang & H.B. Ma) Udayanga, Crous & K.D. Hyde Fungal Diversity 56: 166 (2012)

= *Phomopsis fukushii* Tanaka & S. Endô, in Endô, J. Pl. Prot. Japan 13: [1] (1927)

Perithecia on dead twigs, black, globose, subglobose or irregular, 200–300 µm diam, densely clustered in groups, deeply immersed in host tissue with elongated, tapering necks protruding through substrata, 300–700 µm long. *Asci* unitunicate, 8-spored, sessile, elongate to clavate, (39–)48.5–58.5(–61) µm × (6.5–)7–9 (–11) µm ($\bar{x}$±SD=53±5 × 8±0.7, n= 30). *Ascospores* hyaline, two-celled, often 4-guttulate, with larger guttules at centre and smaller one at the ends, elongated to elliptical, (11–)12.4–14.5(–15.5) × 3–4 µm ($\bar{x}$±SD=13.5±1 × 3.5±0.3, n= 30).

Pycnidia on alfalfa twigs on WA, globose, 200–250 µm diam, embedded in tissue, erumpent at maturity, with an elongated, black neck 200–300 µm high, often with a yellowish, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, straight to sinuous, 10–15 × 2–3 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 µm diam. *Paraphyses* absent. *Alpha conidia* abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ovate to ellipsoidal, often biguttulate, base sub-truncate, (6–)6.5–8.5(–8.9) × 3–4.2 µm ($\bar{x}$±SD =7.7±0.6 × 2.8±0.3, n=30). *Beta conidia* formed in culture and alfalfa stem in some isolates, aseptate, hyaline, smooth, fusiform to hooked, base sub-truncate, (18)22–28(29) × 1–1.5 µm ($\bar{x}$±SD =25±2× 1.3±0.3 , n=30).

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.5±0.2 mm/day (n=8), white, fluffy aerial mycelium, reverse centre dark pigmentation developing in centre; producing abundant, black stroma with elongated necks at maturity.

Host range: *Abutilon* (*Malvaceae*), *Acer* (*Sapindaceae*), *Alliaria* (*Brassicaceae*), *Allium* (*Amaryllidaceae*), *Arctium* (*Asteraceae*), *Castanea* (*Fagaceae*), *Chamaecyparis*, *Juniperus*, (*Cupressaceae*), *Corylus* (*Betulaceae*), *Cornus* (*Cornaceae*), *Cotoneaster*, *Malus*, *Prunus*, *Pyrus*, *Rubus*, *Sorbus* (*Rosaceae*), *Cucumis* (*Cucurbitaceae*), *Daphne* (*Thymelaeaceae*), *Fraxinus*, *Osmanthus* (*Oleaceae*), *Hedera* (*Araliaceae*), *Hordeum* (*Poaceae*), *Ilex* (*Aquifoiaceae*), *Juglans* (*Juglandaceae*), *Laburnum*, *Phaseolus*, *Wisteria*

(*Fabaceae*), *Laurus*, *Sassafras* (*Lauraceae*), *Magnolia* (*Magnoliaceae*), *Opuntia* (*Cactaceae*), *Pice*, *Pinus* (*Pinaceae*), *Rhododendron*, *Vaccinium* (*Ericaceae*), *Rumex* (*Polygonaceae*), *Skimmia* (*Rutaceae*), *Ulmus* (*Ulmaceae*), *Viburnum* (*Adoxaceae*), *Vitis* (*Vitaceae*), *Ziziphus* (*Rhanmnaceae*) (including the host association confirmed with molecular data in Gomes et al. 2013)

Geographic distribution: Austria, China, France, Korea, Germany, Italy, Japan, Latvia, New Zealand, Netherlands, USA, UK

Type material examined: *Diaporthe eres* – GERMANY, Nordrhein-Westfalen, Munsterland, Munster Botanical Gardens, on twigs of *Ulmus* sp., June 1865, Nitschke, (lectotype designated here B 70 0009145, ex herb. Munster, isolectotypes B 70 0009146, B 70 0009147, ex herb. Munster). GERMANY, On *Ulmus laevis*, 5 Jan. 2013 (epitype designated here, BPI892912, ex-epitype culture CBS In Proc. = AR5193). *Phoma oblonga* – FRANCE, on twigs of *Ulmus campestris* (lectotype designated here, bound specimen of Desmazieres, Plantes Cryptogames du Nord de la France, Ed. 2, ser. 2. No. 60 in BPI). GERMANY, Carpinion forest, on dead, attached, corticated twigs of *Ulmus laevis*, 5 Jan. 2013, R. Jarling, comm. R. Schumacher (epitype designated here, BPI892913, culture AR5196); *Phomopsis castaneae-mollisimae* – CHINA, Taian, Shangdong, leaf of *Castanea mollissima*, Apr. 2006, S.X. Jiang (holotype, CLS 0612 not seen, ex-holotype culture BYD1=DNP128 observed, ex-isotype culture BYD4=DNP129). *Diaporthe cotoneastri* – UK, Scotland, Ayr, on *Cotoneaster* sp., May 1982, H. Butin (Isotype CBS-H 7633 not seen, ex-isotype culture, CBS 439.82 observed). *Phomopsis fukushii* – JAPAN, Ibaraki, on *Pyrus pyrifolia*, S. Kanamatsu, August 1994 (neotype designated here BPI In Proc., ex-neotype culture MAFF 625034 = AR3669).

Additional material examined: AUSTRALIA, New South Wales, on *Castanea sativa* (chestnuts in store), 5 July 1999, K.A. Seifert 932 (culture CBS 113470=DAOM 226800). AUSTRIA, Vienna, 21st District, Marchfeldkanalweg, grid square 7764/2, on dead twigs of *Ulmus minor*, 17 Nov. 2002, W. Jaklitsch 2021 (BPI843626, culture DP0438); Vienna, 22nd District. Lobau (Oelhafen), grid square 7865/1, on dead stems of *Acer campestre*, 21 Oct. 2000, W. Jaklitsch 1643 (BPI 748435, culture AR3538); Niederoesterreich, Buschberg, grid square 7464/1, on *Rubus fruticosus*, 11 Aug. 2001, W. Jaklitsch 1771 (BPI843611, culture AR3723);

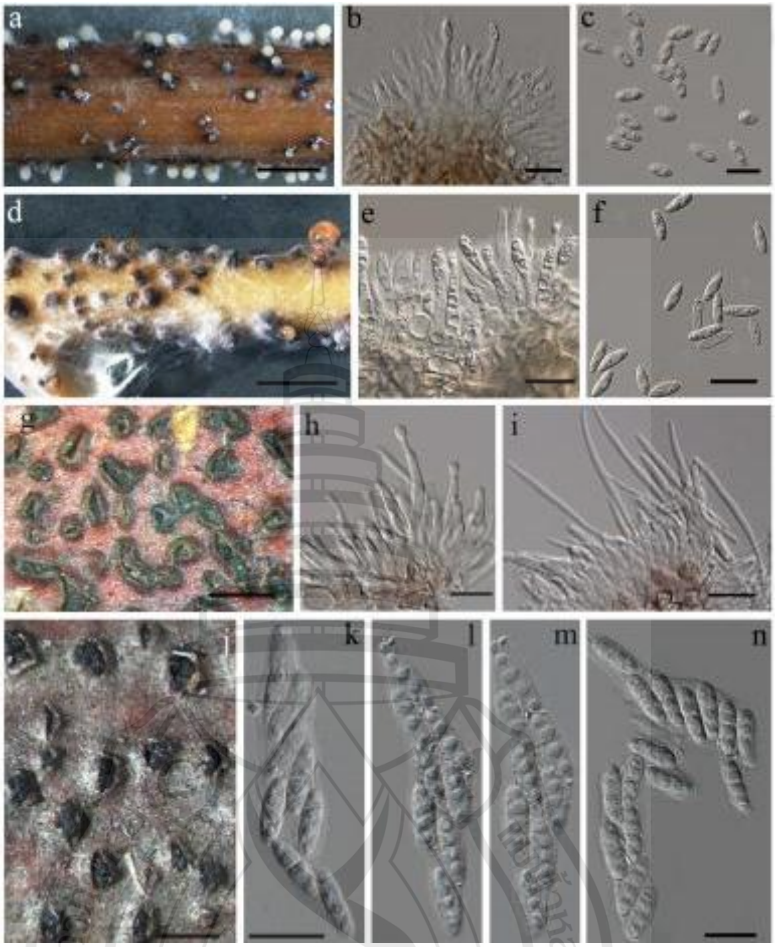
Niederoesterreich, Losenheim, Laerchkogel, on *Corylus avellena*, 30 Sep. 2000, W. Jaklitsch 1605 (BPI747936, culture AR3519=CBS 109497); Wograda, St. Margareten, Kaernten, grid square 9452/3, on *Viburnum lantana*, 27 Oct. 2000, W. Jaklitsch, 1662, (BPI749133, culture AR3560). FRANCE, on *Daphne lauriola* (culture DAN001a=M1115), *ibid*, (DAN001b= M1116), on *Vitis vinifera*, Larignon Phillipe (culture DLR12A=M1117, DLR12B=M1118). GERMANY, mixed forest, on dead twigs of *Acer nugundo*, 13 Apr. 2013, R. Jarling, comm. R. Schumacher (culture AR5223); on dead attached twigs of *Hedera helix*, 26 Mar. 2013, R. Jarling, comm. R. Schumacher (culture AR5224); Planar forest, on attached bud of *Rhododendron* sp., 3 Jan. 2013, comm. R. Schumacher (culture AR5197). JAPAN, Ibaraki, on *Pyrus pyrifolia*, S. Kanematsu, Aug. 1994 (culture AR3670= MAFF 625030, AR3671=MAFF 625033, AR3672=MAFF 625929); on *Pinus pantepella*, G.H. Boerema (culture CBS587.79). KOREA, Eumsnus, on *Prunus persica*, S.K. Hong, Pho 0348 (culture AR4355); Punggi-eup, on *Malus pumila* var. *dulcissima*, S.K. Hong, BD 102 (culture AR4371); Anseong-si, on *Ziziphus jujube*, S.K. Hong, Pho 0345 (culture AR4373), KOREA, Geumsan-gun, on *Ziziphus jujube*, S.K. Hong, Pho 0330 (AR4374); Bubal-eup, on *Prunus mume*, S.K. Hong, BD 173 (culture AR4346); on *Vitis vinifera*, S.K. Hong (culture AR4347); on *Chamaecyparis thyoides*, F. A. Uecker (culture FAU 532); on *Ziziphus jujuba* (culture AR4357); on *Pyrus pyrifolia*, S.K. Hong (culture AR4369) ; on *Vitis* sp., S.K. Hong (culture AR4349); on *Prunus persici*, S.K. Hong (culture AR4348). KOREA, on *Prunus* sp. (culture AR4367); on *Malus* sp., S.K. Hong (culture AR4363). NETHERLANDS, on *Malus* sp. (culture FAU483); on *Pyrus pyrifolia* (culture DP0179). NEW ZEALAND, on *Pyrus pyrifolia* (culture DP0590); on *Pyrus pyrifolia*, W. Kandula (culture DP0591); on *Pyrus pyrifolia* (culture DP0177); on *Pyrus pyrifolia* (culture DP0180). USA, New York, Adirondack Mountains, Buttermilk Falls, on twigs of *Ulmus* sp., 7 June 2007, Louis Mejia (LCM114.01A, B); New Jersey, on *Sassafras albida* (culture FAU522); *Virginia, on Oxydendrum arboreum* (culture FAU570); Maryland, on *Cornus florida* (culture FAU506); North Carolina, Old Fort, on bark of *Juglans cinera*, June 2002, S. Anagnostakis, 5/07 (culture DP0666, DP0667).

Notes: *Diaporthe eres* was designated the type species by Nitschke (1870) and this has been widely accepted in the literature (Wehmeyer et al. 1933, Barr 1978,

Brayford 1990, Rossman et al. 2007). The asexual state of *D. eres* has been known as *Phomopsis oblonga* (basionym: *Phoma oblonga*). Considering the obscurity of the older names listed as synonyms in Wehmeyer (1933) and the difficulty of determining their identity within the genus *Diaporthe*, Rossman et al. (2014) proposed to conserve the name *D. eres* over twenty older synonyms. Originating from the same host and country as the lectotype, an epitype of *D. eres* is designated here from Germany and throughout the temperate regions of the world. The diagnostic morphological features of *Diaporthe eres* are the black stroma, perithecia generally immersed in the host tissue with necks protruding through ruptured host tissue with large asci (48.5–58.5 µm × 7–9 µm) and ascospores (12.4–14.4 × 3–4 µm) compared to other species of *Diaporthe*. Among the cultures used in this study, the majority sporulated on PDA or WA + alfalfa stems producing abundant black pycnidia and conidial masses. Only alpha conidia were observed in some cultures while both alpha and beta conidia were abundant in other cultures. The sexual morph was not observed in culture. Significant morphological differences were not observed in cultures of different ITS types or cultures derived from different hosts. The geo-ecological data for isolates identified here as *D. eres* suggest that this species has a widespread distribution and a broad host range. It has been reported as minor pathogen on fruit trees and herbaceous crops (Kolomiets et al. 2009, Thomidis & Michailides, 2009).

Diaporthe alleghaniensis R.H. Arnold, Can. J. Bot. 45: 787 (1967) Figure 6.6 (a-c)

Pycnidia on alfalfa twigs on WA, globose, 100–200 µm diam, embedded in tissue, erumpent at maturity, with a slightly elongated, black neck 100–180 µm long, often with a yellowish, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, cylindrical to sub-cylindrical, 9–15 × 1–2 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 µm diam. *Paraphyses* absent. *Alpha conidia* abundant in culture and on alfalfa



Notes. a. Pycnidia on alfalfa stem on WA; b. Conidiophores; c. α- conidia; d. Pycnidia on alfalfa stem; e. conidiophores; f. α- conidia; g. infected stem of *Alnus* sp. with ruptures on bark and pycnidia; h. α- conidiophores and conidiognous cells; i. β- conidiophores and conidia; j. Ectostroma on twigs of *Alnus* sp. k-m. Asci. n. Ascospores. Specimens a-c. ex-type culture CBS 495.72, d-f. culture LCM22b.02a, g-h. lectotype specimen Fungi rhenani 1988 in FH, i-n. isolectotype specimen BPI 615718, Bars. a= 800 µm, b,c=10 µm, d = 3000 µm, e,f=12 µm, g= 500 µm, h,i= 12 µm, j= 1000 µm, k-n= 15 µm.

Figure 6.6 Morphology of *Diaporthe alleghaniensis* (a-c) and *D. alnea* (d-n)

twigs, aseptate, hyaline, smooth, ovate to ellipsoidal, biguttulate or multiguttulate, base sub-truncate, 7–9.0 × 3–4 µm ($\bar{x}$±SD=8.1±0.5 × 3.5±0.3, n=30). *Beta conidia* not observed.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.8±0.2 mm/day (n=8), white, aerial mycelium with concentric rings, reverse grey pigmentation developing in centre; stroma not produced in 1wk old cultures.

Type material examined: CANADA. Ontario, Abinger Township, Lennox and Addington Co., Vennacher, P.S.P. 10, on branch of *Betula lenta*, 16 Sep. 1953, R. Horner, J. Newman, A.W. Hill (Holotype DAOM 45776, not seen; ex type culture CBS 495.72 observed).

Host range: On dead branches of *Betula alleghaniensis* (*Betulaceae*).

Geographic distribution: Canada (Ontario), also reported from New Brunswick, Quebec, USA (NH, NY, VT) by Arnold (1967).

Notes: Based on all single gene analyses and the combined phylogeny *Diaporthe alleghaniensis* is clearly distinguished from closely related cryptic taxa. It was recognised as a facultative parasite of yellow birch (*Betula alleghaniensis*) on which it causes an annual bark canker and foliage disease (Arnold 1967). According to the protologue, it is morphologically distinguished from *Diaporthe eres* based on the narrow cylindrical asci each with a truncate apex and the narrow cylindrical-ellipsoid ascospores with a variable position of the single septum. However, the conidia in culture could not be distinguished from those of *Diaporthe eres*.

Diaporthe alnea Fuckel, Jahrb. nassau. Ver. Naturk. 23-24: 207 (1870) Figure 6.6 (d-n)

= *Phomopsis alnea* Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 115: 681 (1906)

Perithecia on dead twigs, black, globose to conical, 200–300 µm diam, scattered evenly on dead twigs, immersed in host tissue with elongated, tapering necks protruding through substrata in clusters, 300–400 µm long. *Asci* unitunicate, 8-spored, sessile, elongate to clavate, 36–46 µm × 6–7 µm ($\bar{x}$±SD=40±5 × 6.5±0.7, n= 30). *Ascospores* hyaline, two-celled, often 4-guttulate, with larger guttules at centre

and smaller ones at ends, elongated to elliptical (11–)12.5–13.5(–14) × 2.5–3 μm ($\bar{x}$±SD=12.7±0.8 × 2.8±0.3, n= 30).

Pycnidia on alfalfa twigs on WA: globose to subglobose, 100–200 μm diam, embedded in tissue, erumpent at maturity, with an elongated, black neck 100–200 μm long, often with a cream colour, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, cylindrical to sub-cylindrical, with larger basal cell 9–16 × 1–2 μm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 μm diam. *Paraphyses* absent. *Alpha conidia* abundant in culture and on alfalfa twigs, aseptate, hyaline, smooth, ellipsoidal, biguttulate or multiguttulate, base subtruncate, 8–10 × 2–3 μm ($\bar{x}$±SD=9±0.6 × 2.8±0.1 , n=30). *Beta conidia* not observed.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 6±0.2 mm/day (n=8), white, aerial mycelium turning grey at edges of plate, reverse yellowish pigmentation developing in centre; stroma not produced in 1wk old culture.

Host range: On species of *Alnus* including *A. glutinosa*, *A. rugosa* and *A. sinuata*

Geographic distribution: Europe (Germany, The Netherlands), USA

Type material examined: GERMANY, on twigs of *Alnus glutinosa*, 1894, L. Fuckel (lectotype designated here, Fungi rhenani 1988 FH); Hesse, Oestrich, *Alnus glutinosa*, 1894, L. Fuckel (isolectotype BPI615718). THE NETHERLANDS, on *Alnus* sp., June 1946, S. Truter 605 (epitype designated here, BPI892917, ex-epitype culture CBS 146.46)

Additional material examined: CZECH REPUBLIC, Moravia, Sternberg, on *Alnus glutinosa*, Feb. 1922, J. Piskor (BPI615717). USA, Idaho, Moscow Mtns., on dead stem of *Alnus sinuata*, 2 July 1898, C.V. Piper (BPI616606); Maine, North New Portland, on twigs of *Alnus rugosa*, 3 Aug. 2006, Luis Mejia (culture LCM 22b.01 = CBS In Proc.); Maryland, Takoma Park, on *Alnus* sp., 1 July 1918, C.H. Kauffman (BPI615716); Michigan, Isle Royale, Rock Harbor, on *Alnus* sp., 15 July 1904, E. T. Harper, Susan A. Harper (BPI616605); New York, Tripoli, Ft. Ann, on *Alnus* sp., 28 June 1914, S.H. Burnham 104 (BPI615284).

Notes: *Diaporthe alnea* is represented here by isolates on *Alnus glutinosa* from Europe and *A. rugosa* in the USA. The geographic origin of CBS the isolates were previously uncertain although the collector's name is known as S. Truter (Gomes et al., 2013). Truter's (1947) doctoral dissertation concerned the die-back of European alder and presumably the collections originated in the Netherlands or close by in Europe. Herein, *D. alnea* is epitypified with one of Truter's isolates based on the historical authenticity and the morphological similarity of this isolate to the type specimen. The name *Diaporthe nivosa* Ellis & Everh. has been applied to a species of *Diaporthe* from *Alnus* in the USA. However, observation of the type specimen of *Diaporthe nivosa* revealed that it is a *Melanconis* sp., having a well developed ectostromata and ascospores characteristic of that genus, thus *D. nivosa* is not synonymous to *D. alnea*.

Type material of *Diaporthe nivosa* examined: USA, Michihgan, Isle Royale, Lake Superior, on dead wood of *Alnus* sp., July 1889, E.W.D. Holway, Ellis & Everhart, North American Fungi Second Series 2535 (lectotype designated here, BPI 616604).

***Diaporthe bicincta* (Cooke & Peck) Sacc.,** Syll. fung. (Abellini) 1: 622 (1882) Figure 6.7 (a-c)

Basionym. *Valsa bicincta* Cooke & Peck, in Peck, Ann. Rep. N.Y. St. Mus. nat. Hist. 29: 64 (1878) [1876]

Pycnidia on alfalfa twigs on WA: globose, 200–300 μm diam, embedded in tissue, erumpent at maturity, well developed, black stroma with a slightly elongated, black neck 50–150 μm high, often with off-white, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, cylindrical to sub-cylindrical, 7–12 × 1–2μm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 μm diam. *Paraphyses* hyaline, smooth, unbranched, elongated with acute apex, 80–100 × 1–2 μm. *Alpha conidia* abundant on alfalfa twigs, aseptate, hyaline, smooth, ovate to ellipsoidal, biguttulate or multiguttulate, base subtruncate, 9–12 × 2–3.5 μm ($\bar{x}$±SD =10±1 × 2.9±0.3 , n=30). *Beta conidia* not observed.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA moderate growth rate, 3.8±0.2 mm/day (n=8), white, aerial mycelium turning to grey at edges of plate, reverse white in centre; stroma produced in 1 wk old culture with abundant conidia.

Host range: On *Juglans cineria* and *Juglans* sp. (*Juglandaceae*)

Geographic distribution: Canada (Ontario); USA (IA, NY, PA, TN).

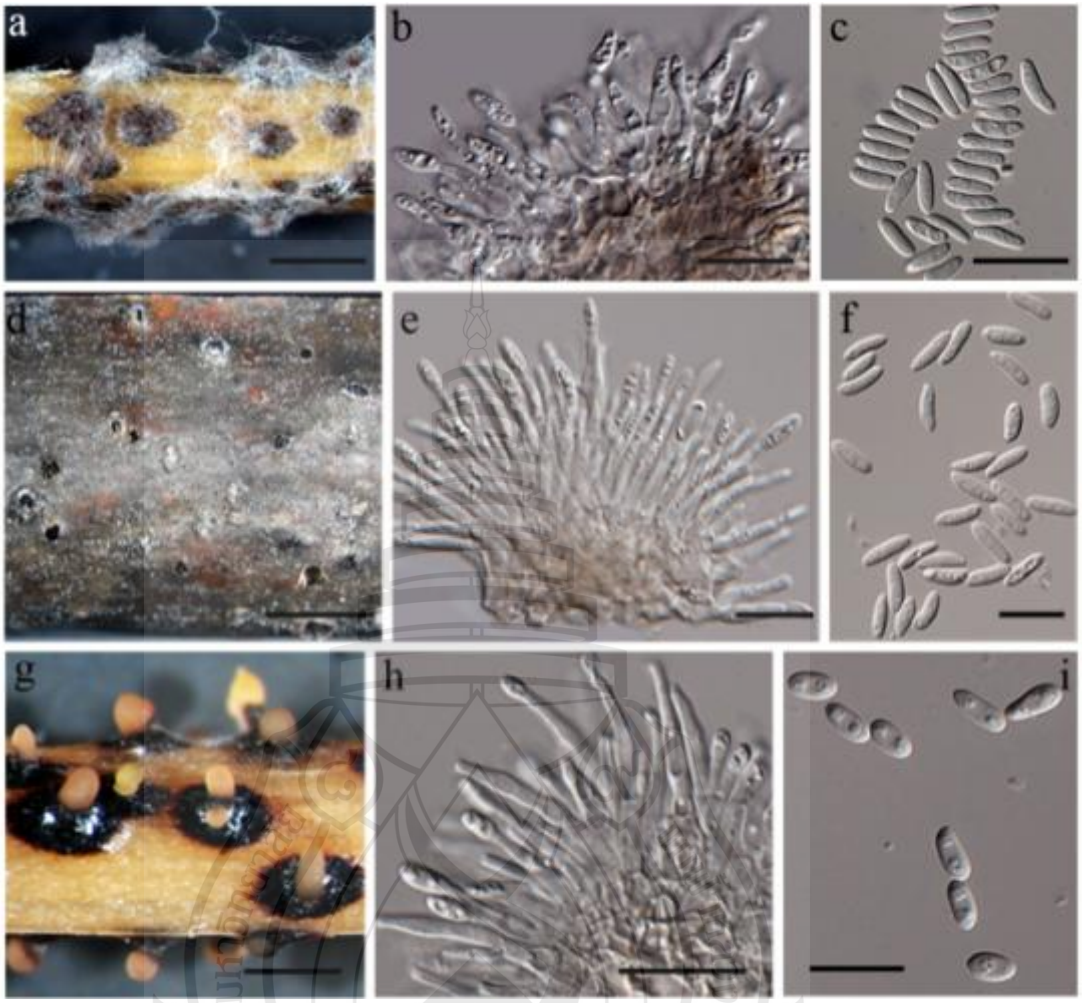
Type material examined: *Valsa bicincta*: USA, New York, Greenbush, on branch of *Juglans cinerea*, (Holotype NYS F 468, not seen). USA, Tennessee, Great Smoky Mts National Park, dead wood of *Juglans* sp., 8 May 2006, L. Vasilyeva (epitype designated here, BPI878472, ex-epitype culture DP0659=CBS 121004).

Additional material examined: CANADA, Ontario, Granton, on dead branches of *Juglans* sp., July 1898, J. Dearness (BPI615762, 615766). USA, Iowa, Decorah, on dead limbs of *Juglans* sp., June 1892, E.W.D. Holway (BPI615761, BPI615765); Pennsylvania, Bethlehem, on twigs of *Juglans cinera*, 9 June 1922, C.L. Shear 4043, det. F. Petrak (BPI615764).

Notes: *Diaporthe bicincta* has long paraphyses and larger conidia (9–12.0× 2–3.5 µm) than *D. juglandina* on *Juglans* in Europe. The isolate CBS 121004 was deposited as *D. juglandina* (Gomes et al., 2013), however, this isolate was originally from the USA (Tennessee) and is here confirmed as *D. bicincta* based on a morphological comparison with the type and non-type specimens.

Diaporthe celastrina Ellis & Barthol., J. Mycol. 8: 173 (1902) Figure 6.7 (d-f)

Pycnidia on host and alfalfa twigs on WA: globose, 200–300 µm diam, embedded in tissue, erumpent at maturity, well developed, black stroma with a slightly elongated, black neck 50–150 µm high, often with an off-white, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, cylindrical, 7–21 × 1–2 µm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 µm diam. *Paraphyses* absent.



Notes. a. Pycnidia on alfalfa stem on WA; b. Conidiophores; c. α-conidia; d. Surface view of infected stem of *Celastrus scandens* with pycnidia; e. conidiophores; f. α- conidia; g. Pycnidia on alfalfa stem on WA; h. conidiophores; i. α-conidia. Specimens a-c. ex-epitype culture CBS 121004, d-f. Holotype BPI615293 g-i. ex-epitype culture AR5211, Bars. a=1000 µm, b,c=15 µm, d=2000 µm, e,f =12µm, g=, 1000 µm, h,i= 10 µm.

Figure 6.7 Morphology of *Diaporthe bicincta* (a-c), *D. celastrina* (d-f), *D. helicis* (g-h).

Alpha conidia abundant on alfalfa twigs, aseptate, hyaline, smooth, ellipsoidal, biguttulate, multiguttulate, or eguttulate, base subtruncate, 9–12.0 × 2–3.5 μm ($\bar{x}$±SD =10.1±0.8 × 2.7±0.3 , n=30). *Beta conidia* not observed.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.8±0.2 mm/day (n=8), white aerial mycelium, reverse white in centre; stroma produced in 1 wk old culture.

Host range: On *Celastrus scandens* (*Celastraceae*).

Geographic distribution: USA (KS, VA).

Type material examined: USA, Kansas, Clyde, *Celastrus scandens*, 18 May 1901, E. Bartholomew *2856* (Holotype, BPI615293). USA, on *Celastrus scandens*, Sep. 1927, L.E. Wehmeyer (Epitype designated here, BPI892915, ex-epitype culture CBS 139.27).

Additional material examined: USA, Virginia, Blacksburg, on *Celastrus scandens.* 13 Oct. 1936, C.L. Shear (BPI615294).

Notes: *Diaporthe celastrina* was originally described from *Celastrus scandens* in the USA (Kansas) and the epitype designated here is collected from the USA on the same host. The host *Celastrus scandens* (American Bittersweet, *Celastraceae*) is native to central and northeastern North America.

Diaporthe helicis Niessl, Verh. Naturforsch. Ver., Brünn 16: 50 (1876). Figure 6.7 (g-h)

[= *Diaporthe nitschkei* J. Kunze, Fungi Selecti Exs. 124. (1877), nom. nud.]

Pycnidia on host and alfalfa twigs on WA, globose, 200–300 μm diam, embedded in tissue, erumpent at maturity, well developed, black stroma with a slightly elongated, black neck 50–150 μm high, often with an off white, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, cylindrical to clavate, (6–) 8–15 (16.5) × 1–2 μm. *Conidiogenous cells* phialidic, cylindrical, terminal, tapering slightly towards apex, 0.5–1 μm diam. *Paraphyses* absent. *Alpha conidia* abundant on alfalfa twigs, aseptate, hyaline, smooth, cylindrical to ellipsoidal, biguttulate or multiguttulate, base subtruncate, (5.5–) 6–8 (9.5) × 2.5–3.5 μm ($\bar{x}$±SD=7.2±0.5 × 3±0.2 , n=30). *Beta conidia* not observed.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.6±0.2 mm/day (n=8), white, aerial mycelium turning to grey, reverse

white, turning to grey in centre; stroma produced in 1 wk old culture with abundant conidia.

Host range: On vines and leaves of *Hedera helix* (*Araliaceae*)

Geographic distribution: Europe (France, Germany)

Type material examined: GERMANY, Saxony, Islebiam, on vines of *Hedera helix*, June 1875, J. Kunze (lectotype designated here, bound collection in BPI Joannes Kunze, Fungi Selecti Exsiccati 124; isolectotypes, BPI1108439; BPI1108445). FRANCE, Veronnes, on vines of *Hedera helix*, 10 Mar. 2011, A. Gardiennet (epitype designated here, BPI892919, ex-epitype culture AR5211 = CBS In Proc.)

Notes: When Niessl (1876) described *Diaporthe helicis*, he referred to the J. Kunze specimen that was distributed as J. Kunze, Fungi Sel. Exsiccati 124 labeled *Diaporthe nitschkei*. Although that exsiccati number was issued in 1875, the label does not include a description and thus that name was not published. The name *D. helicis* published one year later is typified by that same exsiccati number. Based on our collections on *Hedera* in Europe, we recognise several species associated with this host, namely *D. hederae*, *D. helicis*, and *D. pulla*. Observations of the type specimens and additional material from *Hedera* confirmed that the fresh collection from France is *D. helicis* and as does *D. pulla* described below. A comparison of representatives of *D. helicis* and *D. pulla* based the on eight gene alignment and combined analysis revealed genetic differences suggesting that these two species are distinct. The third species on *Hedera*, *D. hederae*, was described from the UK. Observations of a recent collection from *Hedera* in the UK confirmed that it is morphologically differ from *D. helicis* and *D. pulla*. The asexual morph produced by the isolate (M1078, in SMML culture collection, specimen BPI892914), from the UK has longer conidiophores (20–45 × 2–2.4 μm) and the paraphyses are abundant, while *D. helicis* and *D. pulla* have shorter conidiophores (8–15 × 1–2 μm) and paraphyses are absent. The ITS (KM111543) sequence similarity of the above referenced isolate from the UK confirmed that *D. hederae* can be a synonym of *D. rudis* (Udayanga et al. (2014) for description and illustration).

Type material of *Diaporthe hederae* examined: UK, Boxhill, on vines of *Hedera helix*, July 1930, E.W. Mason Detr. L.E. Wehmeyer (BPI1108438).

***Diaporthe neilliae* Peck**, Ann. Rep. N.Y. St. Mus. nat. Hist. 39: 52 (1887) [1886]
Figure 6.8 (a-d)

Perithecia on dead twigs**,** black, globose to conical, 200–300 µm diam, scattered irregularly, immersed in host tissue with elongated, necks protruding through substrata, 300–400 µm long. *Asci* unitunicate, 8-spored, sessile, elongate to clavate, 36–50 µm × 7–10 µm ($\bar{x}$±SD=45±5.0 × 8.5±0.7, n=30). *Ascospores* hyaline, two-celled, often 4-guttulate, with larger guttules at centre and smaller one at ends, elongated to elliptical (11–)12.0–13.5(–14.5) × 3.5–4 µm ($\bar{x}$±SD=12.8±0.8 × 3.8±0.3, n= 30).

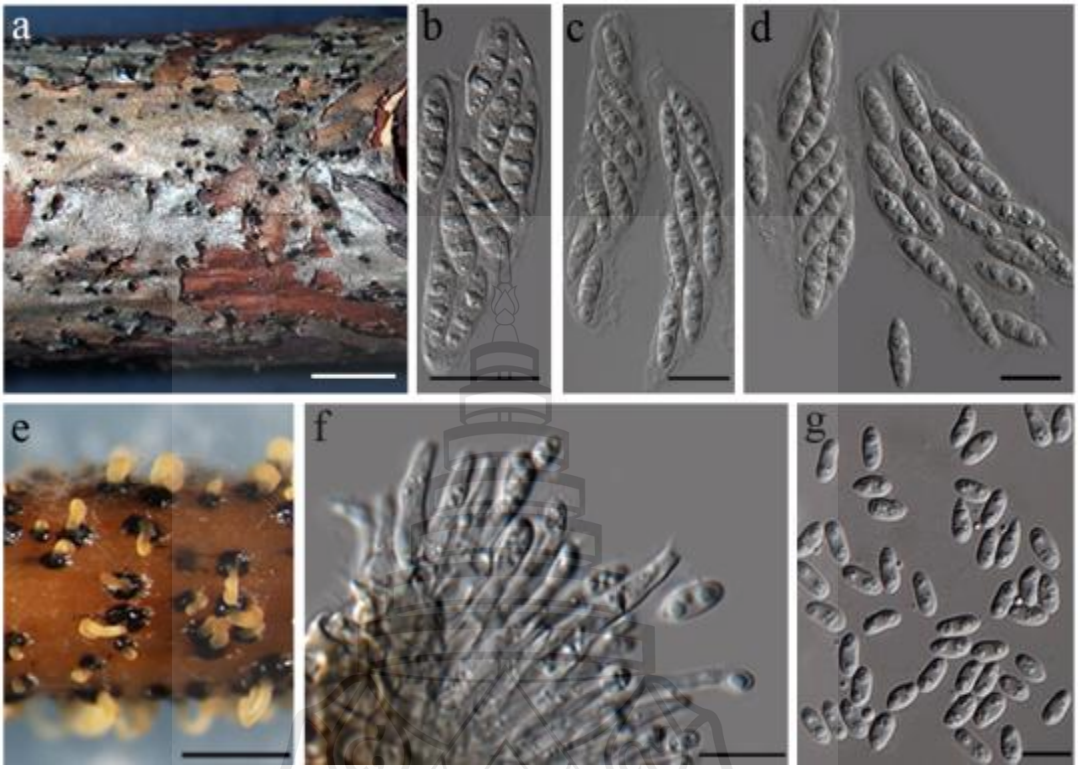
Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA slow growing, 2.6±0.2 mm/day (n=8), white, aerial mycelium, reverse white, turning to grey in centre; no conidia produced.

Host range: On *Physocarpus opulifolius* (*Rosaceae*).

Geographic distribution: USA (NY).

Type material examined: USA, New York, West Albany, on stems of *Physocarpus opulifolius*, Apr, C.H. Peck (Holotype, NYS not examined, isotype, BPI 616581 observed).

Additional material examined: USA, on *Spiraea* sp., Sep. 1927, L.E. Wehmeyer (BPI892921, CBS 144.27).



Notes. a. Ectostoma on dead stem of *Physocarpus opulifolius.* b-c. Asci. d. Asci and ascospores. e. Pycnidia on alfalfa stem on WA. f. conidiophores. g. α- conidia Specimens: a-d. Holotype of *D. neilliae* BPI616581, e-g. ex-epitype culture CBS 338.89 Bars: a= 2000µm, b=15 µm, c,d= 12 µm e= 1800 µm, f= 1 2µm, g= 8 µm.

Figure 6.8 Morphology of *Diaporthe neilliae* (a-d) and *D. pulla* (e-g).

Notes: *Diaporthe neilliae* is known only from the host species *Physocarpus opulifolius,* however, this host has been placed in various genera and has been reported as being on *Neillia opulifolia*, *Opulaster opulifolus* and *Spiraea opulifolia,* all names for the same species. This rosaceous host is native to North America, thus the isolate identified by Wehmeyer is used to represent this taxon; however, due to lack of information about its origin, it is not designated as the epitype.

***Diaporthe pulla* Nitschke**, Pyrenomycetes Germanici 2: 249 (1870)

= *Phoma pulla* Sacc., Michelia 2: 96 (1880)

≡ *Phomopsis pulla* (Sacc.) Traverso, Fl. ital. crypt., Pyrenomycetae (Florence) 2: 244 (1906)

Pycnidia on host and alfalfa twigs on WA: globose, 200–300 μm diam, embedded in tissue, erumpent at maturity, well developed, black stromata with elongated, black necks, 50–300 μm high, often with bright yellow, conidial cirrus extruding from ostiole; walls parenchymatous, consisting of 3–4 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, unbranched, ampulliform, cylindrical to clavate, (10–) 12–20 (–25) × 1–2 μm. *Conidiogenous cells* phialidic, cylindrical, terminal, slightly tapering towards apex, 0.5–1 μm diam. *Paraphyses* absent. *Alpha conidia* abundant on alfalfa twigs, aseptate, hyaline, smooth, cylindrical to ellipsoidal, biguttulate or multiguttulate, base subtruncate, (6.1–) 6.5–7.5 (–8) × (2.5–)2.9–3.4 (–3.5) μm ($\bar{x}$±SD =6.8±0.4 × 3.2±0.2, n=30). *Beta conidia* not observed.

Cultural characteristics: In dark at 25ºC for 1 wk, colonies on PDA fast growing, 5.6±0.2 mm/day (n=8), white aerial mycelium, reverse white, turning to grey in centre; black colour stromata produced in 1 wk with abundant conidia.

Host range: On dead and dying vines and leaves of *Hedera helix* (*Araliaceae*).

Geographic distribution: Europe (Czech Republic, France, Germany, Italy, Serbia)

Type material examined: GERMANY, on vines of *Hedera helix*, (lectotype designated here, Fries Scleromyceti Sueciae No. 307 (BPI Sbarbaro Collection, Bound Centuries III (part) to V. in BPI as *Sphaeria spiculosa*). SERBIA, Belgrade, on vines of *Hedera helix*, July 1989, M. Muntanola-Cvetkovic (epitype designated here, BPI892920, ex-epitype culture CBS 338.89).

Additional material examined: CZECH REPUBLIC (as Czechoslovakia), Maehren, Sternberg, in garden, stems of *Hedera helix*, Oct. 1934, J. Piskor (BPI801639). GERMANY, Schmilka, on stems of *Hedera helix*, Sep. 1903, W. Krieger (BPI1108429); Hesse, Oestrich, on stems of *Hedera* sp., L. Fuckel (BPI 1108479). ITALY, Castel Gandolfo, Rome, on stems of *Hedera helix*, July 1904, D. Saccardo (BPI1108428).

Notes: *Diaporthe pulla* is distinguished from *D. helicis* based primarily on molecular phylogenetic differences. The combined alignment of eight genes that includes the two isolates from *Hedera* as well as the single gene analysis support the distinction of *D. pulla* from *D. helicis*. The other isolates from *Hedera* in Europe were identified as *D. eres* and *D. rudis*. A number of specimens are listed by Nitschke (1870) under the description of *Diaporthe pulla*. The specimens selected here as lectotype was among them and is not the type of *Sphaeria spiculosa* Batsch.

***Diaporthe vaccinii* Shear**, United States Department of Agriculture Technical Bulletin 258: 7(1931)

= *Phomopsis vaccinii* Shear, N.E. Stevens & H.F. Bain, United States Department of Agriculture Technical Bulletin 258: 7 (1931)

For a description ad illustration, see Farr et al. (2002)

Host range: On *V. corymbosum*, *V. macrocarpon*, *V. oxycoccous* (*Ericaceae*) (including the host association confirmed with molecular data in Lombard et al., 2014).

Geographic distribution: USA, Latvia, Lithuania and Netherlands (including the confirmation with molecular data in Lombard et al., 2014).

Type material examined: USA, Oregon, Clatsop, on *Vaccinium macrocarpon*, H. F. Bain, 1924 (holotype, BPI617410).

Additional material examined: USA, Massachusetts, on *Vaccinium macrocarpon*, C.L. Shear (authentic culture, CBS 160.32); Oregon, Seaside, *Vaccinium macrocarpon*, 1923, H.F. Bain, (BPI617405), *ibid*, 02 Sep. 1924, C.L. Shear (BPI617411); Oregon, Carnahan, *Vaccinium macrocarpon*, 20 Sep. 1924, H.F. Bain, det. C.L. Shear (BPI617406); Oregon, Intercepted Seattle Washington #009527, *Vaccinium macrocarpon*, 03 May 1972, coll. W.H. Taussig, det. F.G. Pollack (BPI617407); Oregon, Seaside, *Vaccinium macrocarpon*, 1923, H.F. Bain (BPI617408); Unknown, fruit of *Vaccinium macrocarpon*, 01 Mar. 1929, H.F. Bain (BPI617409).

Notes: The type specimen of *Diaporthe vaccinii* was examined but no useful structures remain as had been noted previously by Wehmeyer (1933) and Farr et al. (2002). The authentic specimen listed in Farr et al. (2002) serves here as the reference material including sequences used in that study. Additional authentic material examined included the conidial state with pycnidial structures and alpha conidia.

Diaporthe vaccinii is known to cause twig blight and fruit rot of *Vaccinium* species and is primarily known from the USA. This is one of relatively host specific pathogens within *Diaporthe* infecting ericaceous hosts.

6.4 Discussion

Fungi are excellent models for studying eukaryotic evolution with many examples of highly diverse species complexes with multiple recently diverged sibling species (Dettman et al. 2003b, 2006; Kohn, 2005; Pringle et al., 2005; Giraud et al., 2008). The genus *Diaporthe* is composed of species varying from relatively host specific to species with broad host ranges. For instance *D. alnea* (*Alnus* spp.), *D. citri* (on *Citrus* spp.), *D. vaccinii* (*Vaccinium* spp.) and *D. ampelina* (formerly known as *Phomopsis viticola* occurring on *Vitis* spp.) are known to be relatively host specific species, are often pathogenic and show a less infraspecific variability (Udayanga et al., 2014). The majority of the host-specific species are generally known as virulent pathogens causing serious diseases on their respective host plants. The occurrence of these host-specific pathogens support for the hypothesis of host switching and specialisation for the speciation within genera in Diaporthales (Sogonov et al., 2008; Mejia, Castlebury, Rossman, Sogonov & White, 2008, Mejia et al., 2011; Walker, Castlebury, Rossman & Struwe, 2014). In contrast, species occurring on a wide range of hosts are mostly opportunistic pathogens or secondary invaders on saprobic host substrata. These species often show high genetic diversity and are sometimes regarded as species complexes by various authors (Gomes et al., 2013). Udayanga et al., (2014), recognised *D. foeniculina* and *D. rudis* as species occurring on an extensive range of hosts similar to *D. eres* as resolved in this study. Definition of the generic type species *D. eres* is essential for a meaningful phylogenetic reappraisal of the genus (Udayanga et al., 2011; Gomes et al., 2013; Rossman et al., 2014). As large numbers of sequences from *Diaporthe* species in public databases have accumulated, subsequent rigorous analyses have shown that the interpretation of the terminals of phylogenetic trees leading to species are subject to much confusion, especially in case of the taxa associated with a broad host ranges (Udayanga et al., 2014). These issues

are not only significant in biodiversity and evolutionary contexts but also in situations where accurate identification of plant pathogenic species is required for quarantine purposes.

The nuclear ribosomal internal transcribed spacer (ITS) region has been proposed as the standard fungal barcode (Schoch et al., 2012) and also it is being used for sequence-based species delimitation in environmental surveys for many groups of fungi (Horton & Bruns, 2001; Begerow et al., 2010). For majority of groups of fungi, ITS is the predominantly available sequence in public databases (Nilsson, Kristiansson, Ryberg, Hallenberg & Larsson, 2008; Kõljalg et al., 2013). Although ITS has been widely used in fungal systematics to delimit species and to understand evolutionary relationships, there are several known issues with the use of this region. On average the variability of the ITS1 exceeds that of ITS2, while the 5.8S fragment embedded between these two regions is highly conserved, and results of phylogenetic analysis of the complete sequence might differ from the analysis of the individual sub-loci (Nilsson et al., 2008; Monard, Gantner & Stenlid, 2013).

ITS has undergone non-concerted patterns of evolution leading to paralogous ITS types within species in some important plant pathogenic genera (O'Donnell & Cigelnik, 1997; Nilsson et al., 2008; Santos et al., 2010) and is considered by some authors to be uninformative due to the lacking of interspecific variation or even misleading the species recognition (Crouch et al., 2009a; Cai et al., 2011; Weir et al., 2012). Although confusion resulting from ITS sequence data in *Diaporthe* has been recognised by several previous authors, it has not been thoroughly examined (Farr et al., 2002a; Murali et al., 2006; Udayanga et al., 2014). The formerly known evidence (Santos et al., 2010), of two ITS types tentatively named as A and B (two groups of isolates resulted from phylogenetic analysis) recovered from the isolates Di-C005/1-10 (from *Hydrangea* in Portugal), derived from 10 individual sibling ascospores from same perithecium were similar to the two clusters observed in our analysis (Figure 6.1-A). However, this study reveals that the unidentified isolates Di-C005/1-10 belong to *Diaprthe eres*, which shows the similar situation with wide environmental sampling of the same species and cluster together as one species in the EF1-α phylogenetic tree. These differences were confined to the ITS1 region and are more extensive than differences often noted among isolates of a single species. Sequence

variability was not noted in the EF1-α and mating type genes for these same sibling isolates and the isolates were fully reproductively compatible (Santos et al., 2010). The same study (Santos et al., 2010) further proved that both ITS types were not found in the genome of the same isolate, indicating that the different ITS types are independently segregated in meiotic events in this species. Comparison of the geographic origins and host associations of the isolates of *D. eres* used in this study with respect to the occurrence of different ITS types revealed that the different ITS sequences can be observed even within the same geographic region and the same host. We detected no evidence of sympatric patterns or host specialisation related to the ITS populations. The discordance of ITS gene trees with other gene trees in this complex in combination with a lack of informative morphological characters to delineate taxa has lead to a confused taxonomy within this species complex.

The EF1-α gene was used initially to provide an estimate of the species boundaries with six additional genes including ACT, Apn2, CAL, FG1093, HIS and TUB genes compared individually and in combinations. The approximately 300 bp complete intron sequence of the translation elongation factor1-α has previously been recognised as a powerful marker within *Diaporthe* to resolve the acute problems of cryptic species by various authors (Castlebury et al., 2001; Santos et al., 2010; Udayanga et al. 2012a; 2012b; Udayanga et al., 2014). The ITS analysis resulted an unresolved phylogenetic tree without definitive bootstrap support at the internodes, with highly discordant with the 7 other genes, therefore not used in the combinable component in this analysis. The seven gene analysis excluding the discordant ITS data resulted in a robust tree congruent with the EF1-α and other single genes. The species boundaries within *D. eres* species complex were resolve in this study by application of criteria of phylogenetic species recognition (Taylor et al., 2000; Dettman et al., 2003a) revealing the cryptic diversity that may be obscured by biological species recognition, morphology and discordance of genes. Several similar conclusions have been made in various other fungal groups with cryptic species diversity, which also may display little or no morphological variations among them (Dettman et al., 2003a, 2006; Walker et al., 2012; Weir et al., 2012).

The structures of the mating type genes and its association with Apn2 genes in *Diaporthe* were illustrated by Kanematsu et al. (2007). DNA-(apurinic or

apyrimidinic site) lyase genes is not traditionally been used as a molecular marker in fungi; however the association with mating type genes of fungi is known in relation to the structure. The Apn2 region has recently been used in conflicting genera like *Colletotrichum* (Crouch & Tomaso-Peterson, 2012; Silva et al., 2012b; Doyle, Oudemans, Rehner & Litt, 2013; Sharma, Kumar, Weir, Hyde & Shenoy, 2013) and the Apn2 and Apn2/MAT-IGS (intergenic spacer between 3′ end of the DNA lyase and mating type locus MAT1-2) genetic markers recommended as a better marker in disentangling the *C. gloeosporioides* species complex (Silva et al., 2012a; 2012b). Mating type genes of *Diaporthe* were amplified in several previous studies and utilised in phylogenetic analyses (Santos et al., 2010, 2011). Portions of the α-1 box in MAT 1-1-1 gene (141 bp) and a portion of HMG domain of MAT 1-2-1 (229bp) regions were shown to have less utility as phylogenetic markers than for screening mating types of isolates (Santos et al., 2010). The MAT phylogenetic trees were strongly correlated with EF1-α phylogenetic trees; however they were less informative regarding the more closely related species clusters. This can be due to the retention of reproductive ability within closely related phylogenetic species, which often can be regarded as one biological species. In our analyses of the available mating type sequences of same taxa with those generated by Santos et al. (2010) (as *Diaporthe cotoneastri* and *D. vaccinii* clade), the species could not be distinguished based on MAT1-1-1 or MAT1-2-1 gene trees (tree not shown). However, in heterothallic species mating type genes may not always be appropriate as phylogenetic markers due to their absence in different strains.

To our knowledge, this study is the first ever utility of Apn2 gene as a phylogenetic markers within the genus *Diaporthe*. The comparison of phylogenetic informativeness revealed that it is a competing marker for EF1-α and HIS genes. The Apn2 region has the advantage of being highly informative and bearing a shorter hypervariable intron region. The genus wide alignments of Apn2 would be more sensible due to the better global alignment instead of the complete intron region in EF1-α region which could be hypervariable in length across the genus, therefore alignment can often contains ambiguous regions. However, the DNA-lyase gene of *Diaporthe* had 97 and 99% infraspecific amino acid identity between *MAT1-1* and *MAT1-2* isolates of W and G types respectively, and 83 and 85% interspecific identity

(Kanematsu et al. 2007). We confirmed that the *Diaporthe* W type (Kanematsu et al., 2003) to be *D. eres* (= *P. fukushii*) and *Diaporthe* G type to be an unidentified species closely related to *D. amygdali* by comparison of ITS and EF-α sequences available in Santos et al. (2010).

Gomes et al. (2013), observed an unresolved sub-clade which they tentatively named as *Diaporthe nobilis* species complex, represented by CBS 587.79, CBS 113470 and some of the isolates used in our analysis including *Phomopsis fukushii* grouped within *D. eres* and only CBS338.89 is recognised as a distinct species. We confirm that this poorly supported non-monophyletic grouping can be observed when the ambiguous ITS sequences are included in the combined analysis misleading the species recognition if not interpreted with care. Therefore, the introduction of *Diaporthe nobilis* species complex (*sensu* Gomes) within *D. eres* is redundant and it should be considered as the same phylogenetic species. Kaliterna et al. (2012), report the association of *Diaporthe eres* with grapevine trunk disease in Croatia with the report of moderate pathogenicity. They suggest that this plurivorous species could play considerable role in aetiology of grapevine trunk disease. Baumgartner et al., (2013) characterise the isolates of *Diaporthe* from north American vineyards and recognised the occurrence of *Diaporthe eres* widely in their collection. Interestingly, they recovered both ITS types of *Diaporthe eres*, one of which named as *Phomopsis fukushii* follow the high similarity with the authentic isolates included in their analysis. However, they did not notice any morphological variability or significance of virulence and pathogenicity within two groups. The weak pathogenicity of *D. eres* has been widely reported associated with ericaceous and rosaceous fruit trees, and grapes (Kanematsu et al., 2007; Kaliterna et al., 2012; Lombard et al., 2014). Additionally the *Phomopsis* sp. 6, reported from South Africa (Niekerk et al., 2005) confirmed to be *Diaporthe eres* also support the common association of *Diaporthe eres* on grapevine as a weak pathogen or opportunistic saprobe.

The phylogenetic informativeness profiles generated based on PhyDesign were used to compare each locus with respect to the species hypothesis inferred based on multi-gene phylogenetic analysis. Apn2, EF 1-α and HIS genes have shown the highest net phylogenetic informativeness, where EF1-α shows the highest informativeness per site. The phylogenetic informativeness per site is useful to

compare relative power of genes regardless of gene length. These profiles are useful to determine the more informative genes facilitating the locus prioritisation increasing the efficiency of sequencing for phylogenetic purposes compared to traditional studies with more laborious and low capacity screening methods (Townsend, 2007). The relatively recent "phantom" spikes in EF1-α phylogenetic informativeness plot arise because the maximum likelihood estimate for the rate of a few sites has its peak at infinity, which has little biological meaning, which probably be avoided in the addition of more taxa (Lopez-Giraldez & Townsend, 2011).

The infraspecific variability of the highly informative genes as well as less informative genes is a factor to be considered in large scale evolutionary reconstruction of the genus to avoid the technical misinterpretation of data and improve the accuracy. However more sampling of each of the species from a wide range of hosts and additional genes to clarify the topological conflicts of single gene analysis would be important. Novel species may be encountered in untouched ecological niches as endophytes, pathogens or saprobes. The utility of standardised reliably annotated, publicly available sequence dataset can often improve the accuracy of identification (Schoch et al., 2012, Kõljalg et al., 2013, and Nilsson et al., 2014).

6.5 Conclusion

The genus *Diaporthe* comprises pathogenic, endophytic and saprobic species with both temperate and tropical distributions. Cryptic diversification, phenotypic plasticity and extensive host associations have long complicated accurate identifications of species in this genus. The delimitation of the generic type species *Diaporthe eres* has been uncertain due to the lack of ex-type cultures. Species limits of *D. eres* and closely related species were evaluated using molecular phylogenetic analysis of eight genes including nuclear ribosomal internal transcribed spacer (ITS), partial sequences of actin (ACT), DNA-lyase (Apn2), translation elongation factor 1-α (EF1-α), beta-tubulin (TUB), calmodulin (CAL), 60s ribosomal protein L37 (FG1093) and histone-3 (HIS). The occurrence of sequence heterogeneity of ITS within *D. eres* was observed, which complicates the analysis and may lead to

overestimation of the species diversity. The strict criteria of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) were applied to resolve species boundaries based on individual and combined analyses of other seven genes except the ITS. We accept nine distinct phylogenetic species including *Diaporthe alleghaniensis*, *D. alnea*, *D. bicincta*, *D. celastrina*, *D. eres*, *D. helicis*, *D. neilliae*, *D. pulla* and *D. vaccinii*. Epitypes are designated for *D. alnea*, *D. bicincta*, *D. celastrina*, *D. eres*, *D. helicis* and *D. pulla*. Modern descriptions and illustrations are provided for these species. Newly designed primers are introduced to amplify and sequence the Apn2 (DNA- lyase) gene in *Diaporthe*. Based on phylogenetic informativeness profiles, EF1-α, Apn2 and HIS genes are recognised as the best markers for defining species in the *D. eres* complex.

CHAPTER 7

A MOLECULAR RE-ASSSESSMENT OF *Colletotrichum* SPECIES ASSOCIATED WITH TROPICAL FRUITS

7.1 Introduction

Colletotrichum Corda, is an important plant pathogenic genus causing anthracnose of a wide range of fruits, vegetables, cereals, grasses and ornamental plants in tropical and temperate regions (Mills, Hodson & Brown, 1992; Johnston & Jones 1997; Freeman, Minz, Maymon & Zveibil, 2001; Chung et al., 2006; Yang et al., 2009; Rojas et al., 2010). Fruit production is mostly affected in both high-value crops and wild fruits in natural habitats. However, *Colletotrichum* species associated with wild fruits are poorly known (Rampersad, 2011; Cannon et al., 2012). *Colletotrichum* spp. were voted as the eighth most important plant pathogens in the world in a recent survey among fungal pathologists, for its perceived scientific and economic importance (Dean et al., 2012). The fruits infected by *Colletotrichum* generally have small, water soaked, sunken, circular spots that may increase in size with age and the centre of an older spot becomes blackish and develops gelatinous pink or orange spore masses (Waller, Lenné & Waller, 2002; Agrios, 2005; Nelson, 2008).

Taxonomic and phylogenetic studies on the genus *Colletotrichum* are in an expansive phase following the epitypification of *C. gloeosporioides* (Penz.) Penz. & Sacc. (Cannon, Buddie & Bridge, 2008), the list of current names in use (Hyde et al., 2009b), and subsequent recommendation for a polyphasic approach to the taxonomy of the genus (Cai et al., 2009). Recent studies have focused on phylogenetic reassessments of species complexes (Damm et al., 2012a; 2012b; Weir et al., 2012) and have determined that what were previously thought to be a single species, comprise

multiple distinct lineages. For example the boninense clade (*Colletotrichum boninense* species complex) now comprises about 18 species (Damm et al., 2012a), while the acutatum clade (*C. acutatum* species complex) now comprises 31 species (Damm et al., 2012b) and the gloeosporioides clade (*C. gloeosporioides* species complex) comprises more than 22 species (Weir et al., 2012). The species numbers in the major clades are likely to rise, unraveling the cryptic taxa based on multigene phylogenetic analyses and incorporating a large number of isolates in worldwide collections (Cannon et al., 2012). In addition to the major species complexes in *Colletotrichum*, several intermediate clades have been studied. These intermediate clades, for example *C. orbiculare*, *C. spaethianum*, *C. destructivum* species complexes comprise comparatively less cryptic species (Crouch, Clarke & Hillman, 2006; Crouch et al., 2009a; Crouch, Beirn, Cortese, Bonos & Clarke 2009b; Choi et al., 2011; Noireung et al., 2012; Liu, Cai, Crous & Damm, 2013). Epitypification of *Colletotrichum gloeosporioides* by Cannon et al., (2008) and subsequent use of multi-gene phylogeny have resulted in this taxon being revealed as a species complex. *Colletotrichum gloeosporioides* was originally described from Citrus in Italy, thus the chosen epitype culture derived from a necrotic spot on leaves of *Citrus sinensis* from same country (Cannon et al., 2008). *Colletotrichum gloeosporioides* was previously thought to be a cosmopolitan species infecting a wide range of plant hosts including tropical fruits (Alahakoon & Brown, 1994; Freeman, Minz, Jurkevitch, Maymon & Shabi, 2000; Abang et al., 2002). Phoulivong et al. (2010) tested this hypothesis by molecular and morphological characterization of *Colletotrichum* strains from anthracnose symptoms on tropical fruits in Laos and Thailand. *Colletotrichum gloeosporioides* sensu stricto was not found from any of the fruit examined in their study, however many strains from various common fruits were not assigned to any known taxa based on the five genes employed.

Recent molecular studies have resulted in the discovery of new species or accurate identification of known taxa as common causal agents of tropical fruit diseases. This includes *C. fructicola* Prihastuti, L. Cai & K.D. Hyde, *C. gloeosporioides, C. kahawae* Waller & Bridge, *C. musae* (Berk. & M.A. Curtis) Arx, *C. theobromicola* Delacroix, *C. siamense* Prihastuti, L. Cai & K.D. Hyde, *C. tropicale* Rojas, S.A. Rehner & G.J. Samuels, and several distinct species within the *C.*

acutatum and *C. boninense* species complexes (Prihastuti et al., 2009; Rojas et al., 2010; Maharaj & Rampersad, 2012). Many species of *Colletotrichum* have wide host ranges while relatively host specific exceptions are *C. musae* (Berk. & M.A. Curtis) Arx, and *C. horii* B. Weir & P.R. Johnst., associated with *Musa* spp. and *Diospyros* sp. respectively. Studies on *Colletotrichum* in tropical Asia have shown that there is a remarkable species diversity found on wide range of hosts. The species of the genus also causes considerable pre and postharvest fungal diseases on fruits, vegetables, ornamentals, other crops and forest trees in India, Indonesia, Laos, Sri Lanka, Thailand, Tropical China and Vietnam (Wijesundera, Bailey, Byrde & Fielding, 1989; Alahakoon & Brown 1994; Galván, Wietsma, Putrasemedja, Permadi & Kik, 1997; Dinh, Chongwungse, Pongam & Sangchote, 2003; Gunawardhana, Seneviratne, Adikaram & Yakandawala, 2009; Phoulivong et al., 2010; Gupta et al., 2010; Mukherjee, Khandker, Islam, Sonia & Shahid, 2011). Tropical Asian countries produce, export and consume a diverse array of edible fruits, and therefore accurate identification of postharvest pathogens of fruits has a significant impact on agriculture, bio security and quarantine (Than et al., 2008a; Adikaram, Karunanayake & Abayasekara, 2010; Ko Ko, Stephenson, Bahkali & Hyde, 2011; Phoulivong, McKenzie & Hyde., 2012; Sharma et al., 2013).

In this study, we focus on the *Colletotrichum* species associated with commercially available, cultivated fruits and wild fruits (from native trees in natural habitats) in Thailand. The aim of the study was to re-assess the *Colletotrichum* species commonly associated with anthracnose of tropical fruits, which is was dominated by the *C. gloeosporioides* complex. Phylogenetic relationships were initially inferred by comparison of ITS sequence data with ex-type sequences. The multi-gene phylogenetic analyses were then applied to infer phylogenetic relationships and boundaries of taxa in the *C. gloeosporioides* species complex. *Colletotrichum siamense sensu lato* was identified as the dominant, most ecologically and genetically diversified clade among the isolates assessed. A new species, *C. syzygicola* associated with anthracnose of *Syzygium samarangense*, is introduced as a novel species with a full description and illustrations.

Notes. a. *Azadirachta indica* (*C. siamense*), b. *Annona retciulata* (*C. siamense*), c. *Ficus racemosa* (*C. siamense*), d. *Cerbera* sp. (*C. siamense*), e. *Caryota urens* (*C. siamense*), f. *Hylocerus undatus* (*C. fructicola*) g. *Mangifera indica* (*C. siamense*), h. *Musa* sp. (*C. musae*) i. *Syzygium samarangense* (*C. syzygicola*) j. *Citrus reticulata* (*C. gloeosporioides sensu stricto*).

Figure 7.1 Anthracnose symptoms on several wild and cultivated tropical fruits.

7.2 Materials and methods

7.2.1 Fungal isolates and morphological studies

A total of 55 *Colletotrichum* strains (Table 7.1) from randomly collected fruits including banana (*Musa* sp.), *Cerbera* sp., custard apple (*Annona reticulata*), coffee (*Coffea arabica*), and pods of *Delonix* sp., dragon fruit/pitaya (*Hylocereus undatus*), fig (*Ficus racemosa*), jujube (*Ziziphus* sp.), lime (*Citrus aurantifolia*, mango (*Mangifera indica*), neem (*Azadirachta indica*), papaya (*Carica papaya*), rose apple (*Syzygium samarangense*), strawberry (*Fragaria* sp.), solitary fishtail palm (*Caryota urens*) and several unidentified wild fruits were obtained for this study (Figure 7.1). Wild fruits (e.g. *Azadirachta indica*, *Cerbera* sp., *Delonix* sp., *Ficus racemosa*) with visible anthracnose symptoms were collected from forest trees and common cultivated fruits (e.g. *Annona reticulata*, *Coffea arabica*, *Hylocereus undatus*, *Musa* sp., *Ziziphus* sp. were collected from home gardens, orchards and local markets in northern Thailand (in Chiang Mai and Chiang Rai Provinces). Isolates from coffee berries were endophytes from healthy fruits collected from an orchard in Chiang Mai Province. Single spore isolates were obtained from symptomatic tissues with visible conidiomata, using the protocol outlined by Udayanga et al. (2012). Endophytic isolates were obtained from the sterile pericarp of coffee berries as described in Photita et al. (2005) and Than et al. (2008a). Additional specimens were collected from some common weeds and several unidentified wild herbaceous hosts. Cultures were initially maintained on potato dextrose agar (PDA), and all the cultures were deposited in MFLUCC (Mae Fah Luang University Culture Collection, Thailand). Colony characters and growth rates were recorded as described in section 3.2.2. Morphology of appressoria, setae, conidiophores and conidia were obtained using a slide culture technique on PCA medium (potato carrot agar) as described in Cai et al. (2009). The morphology of the isolates was recorded and used in species identification where appropriate and for the description of the new species.

Table 7.1 Isolates used and sequences used in this study.

Species	Isolate/culture collection	Host	GenBank Accessions						References
			ACT	TUB2	CAL	GS	GAPDH	ITS	
C. aenigma	ICMP18608	*Persea americana*	JX009443	JX010389	JX009683	JX010078	JX010044	JX010244	Weir et al. (2012)
C. aescynomenes	ICMP17673	*Aeschynomene virginica*	JX009483	JX010392	JX009721	JX010081	JX009930	JX010176	Weir et al. (2012)
C. alienum	ICMP12071	*Malus domestica*	JX009572	JX010411	JX009654	JX010101	JX010028	JX010251	Weir et al. (2012)
C. aotearoa	ICMP 18537	*Coprosma* sp.	JX009564	JX010420	JX009611	JX010113	JX010005	JX010205	Weir et al. (2012)
C. asianum	BMLI3/ MFLUCC 09-0232	*Coffea* sp.	FJ 903188	FJ 907434	FJ 917501	FJ 972586	FJ 972571	FJ 972605	Prihastuti et al. (2009)
	BPDI4/ MFLUCC 09-0233	*Coffea* sp.	FJ 907424	FJ 907439	FJ 917506	FJ 972595	FJ 972576	FJ 972612	Prihastuti et al. (2009)
	BML-I14/ MFLUCC 09-0234	*Coffea* sp.	FJ 907421	FJ 907436	FJ 917503	FJ 972598	FJ 972573	FJ 972615	Prihastuti et al. (2009)
C. clidemiae	ICMP18658	*Clidemia hirta*	JX009537	JX010438	JX009645	JX010129	JX009989	JX010265	Weir et al. (2012)
C. cordylinicola	LC955/ BCC 38864	*Codyline fruticosa*	HM470233	HM47028	HM470236	HM470242	HM470239	HM470245	Pholivong et al. (2010)
	LC856/ BCC 38872	*Codyline fruticosa*	HM470234	HM47029	HM470237	HM470243	HM470240	HM470246	Pholivong et al. (2010)
C. endophytica	DNCL075	wild fruit	KF157827	KF254857	KF254846	KF242154	KF242181	KF242123	This study
	MFLUCC-LC0324	*Pennisetum* sp.	KC692467	-	KC810018	-	KC832854	KC633854	Manamgoda et al. (2013)

Table 7.1 (continued)

Species	Isolate/culture collection	Host	GenBank Accessions						References
			ACT	TUB2	CAL	GS	GAPDH	ITS	
C. fructicola	DNCL013/ MFLUCC 10-0616	*Ziziphus zizyphus*	KF157822	KF254898	KF254874	KF242146	KF242184	KF242115	This study
	DNCL055/ MFLUCC 10-0656	*Hylocerus undatus*	KF157823	KF254898	KF254875	KF242147	KF242185	KF242116	This study
	BPDI16/ MFLUCC 09-0228	*Coffea* sp.	FJ 907426	FJ 907441	FJ 917508	FJ 972593	FJ 972578	FJ 972603	Prihastuti et al. (2009)
	ICMP18646	*Tetragastris panamensis*	JX009581	JX010409	JX009674	JX010099	JX010032	JX010173	Weir et al. (2012)
	BPDI18/ MFLUCC 09-0226	*Coffea* sp.	FJ 907427	FJ 907442	FJ 917509	FJ 972592	FJ 972579	FJ 972602	Prihastuti et al. (2009)
	BPDI12/ MFLUCC 09-0227	*Coffea* sp.	FJ 907425	FJ 907440	FJ 917507	FJ 972594	FJ 972577	FJ 972611	Prihastuti et al. (2009)
C. gloeosporioides	CBS93587	*Citrus sinensis*	FJ 907430	FJ 907445	FJ 917512	FJ 972589	FJ 972582	FJ 972609	Prihastuti et al. (2009)
	CORCG4	Orchid	HM034800	HM03480	HM034802	–	HM034806	HM034808	Phoulivong et al. (2010)
	DNCL053/ MFLUCC 10-0654	*Citrus latifolia*	KF157807	KF254856	KF254852	KF242131	KF242162	KF242100	This study
	DNCL052/ MFLUCC 10-0653	*Citrus latifolia*	KF157806	KF254855	KF254851	KF242130	KF242161	KF242099	This study

Table 7.1 (continued)

Species	Isolate/culture collection	Host	GenBank Accessions						References
			ACT	TUB2	CAL	GS	GAPDH	ITS	
C. gloesporioides	DNCL022/ MFLUCC 10-0625	*Syzygium samarangensis*	KF157804	KF254853	KF254853	KF242128	KF242159	KF242097	This study
	DNCL027/ MFLUCC 10-0629	*Citrus latifolia*	KF157805	KF254854	KF254854	KF242129	KF242159	KF242098	This study
	ICMP12938	*Citrus sinensis*	JX009560	-	JX009732	-	JX009935	JX010147	Weir et al. (2012)
C. horri	ICMP10492	*Diospyros kaki*	JX009438	JX010450	JX009604	JX010137	GQ329681	GQ329690	Weir et al. (2012)
	TSG002	*Diospyros kaki*	GU133379	GU13330	GU133381	GU133382	GQ329680	AY791890	Xie et al. (2010)
C. kahawae subsp. *ciggaro*	ICMP18539	*Olea europaea*	JX009523	JX010434	JX009635	JX010132	JX009966	JX010230	Weir et al. (2012)
= *Glomerella cingulata* var. *migrans*	ICMP17922	*Hypericum perforatu*	JX009450	JX010432	JX009636	JX010120	JX010042	JX010238	Weir et al. (2012)
=*Glomerella rufomaculans* var. *vaccinii*	ICMP19122	*Vaccinium* sp.	JX009536	JX010433	JX009744	JX010134	JX009950	JX010228	Weir et al. (2012)
Vaccinium									
C. kahawae subsp. *kahawae*	IMI363578	*Coffea* sp.	GU133374	GU13335	GU133376	GU133377	GQ329682	AY787483	Prihastuti et al. (2009)
	IMI319418	*Coffea arabica*	JX009452	JX010444	JX009642	JX010130	JX010012	JX010231	Weir et al. (2012)
	ICMP17915	*Coffea arabica*	JX009474	JX010435	JX009638	JX010125	JX010040	JX010234	Weir et al. (2012)

Table 7.1 (continued)

Species	Isolate/culture collection	Host	GenBank Accessions						References
			ACT	TUB2	CAL	GS	GAPDH	ITS	
C. psidii	CBS145.29	*Psidium* sp.	JX009515	JX010443	JX009743	JX010133	JX009967	JX010219	Weir et al. (2012)
C. musae	CBS116870	*Musa* sp.	HQ596284	HQ59620	JX009742	HQ596288	HQ596299	HQ596292	Su et al. (2011)
	DNCL045/ MFLUCC 10-0646	*Musa* sp.	KF157828	KF254902	KF254843	KF242151	KF242178	KF242120	This study
	DNCL039/ MFLUCC 10-0640	*Musa* sp.	KF157829	KF254903	KF254844	KF242152	KF242179	KF242121	This study
	DNCL067/ MFLUCC 10-0668	*Musa* sp.	KF157830	KF254904	KF254845	KF242153	KF242180	KF242122	This study
	BTL32	*Musa* sp.	HQ596285	HQ59621	HQ596296	HQ596289	HQ596300	HQ596293	Su et al. (2011)
	BTL25	*Musa* sp.	HQ596286	HQ59622	HQ596297	HQ596290	HQ596301	HQ596294	Su et al. (2011)
	BTL31	*Musa* sp.	HQ596287	HQ59623	HQ596298	HQ596291	HQ596302	HQ596295	Su et al. (2011)
C. nupharicola	ICMP18187	*Nuphar lutea*	JX009437	JX010398	JX009663	JX010088	JX009972	JX010187	Weir et al. (2012)
C. queenslandicum	ICMP1778	*Carica papaya*	JX009447	JX010414	JX009691	JX010104	JX009934	JX010276	Weir et al. (2012)
C. salsolae	ICMP19051	*Salsola tragus*	JX009562	JX010403	JX009696	JX010093	JX009916	JX010242	Weir et al. (2012)
C. siamense	BPDI12/MFLUCC09-230	*Coffea* sp.	FJ 907423	FJ 907438	FJ 917505	FJ 972596	FJ 972575	FJ 972613	Prihastuti et al. (2009)
	BML-I15/MFLUCC09-231	*Coffea* sp.	FJ 907422	FJ 907437	FJ 917504	FJ 972597	FJ 972574	FJ 972614	Prihastuti et l. (2009)

Table 7.1 (continued)

Species	Isolate/culture collection	Host	GenBank Accessions						References
			ACT	TUB2	CAL	GS	GAPDH	ITS	
C. siamense	DNCL043/ MFLUCC 10-0644	*Ficus* sp.	KF157816	KF254891	KF254870	KF242140	KF242171	KF242109	This study
	COF005/ MFLUCC 10-0681	*Coffea arabica*	KF157808	KF254883	KF254862	KF242132	KF242163	KF242101	This study
	DNCL050/ MFLUCC 10-0651	*Azdirachta indica*	KF157826	KF254901	KF254878	KF242150	KF242177	KF242119	This study
	DNCL072/ MFLUCC 10-0673	*Mangifera indica*	KF157815	KF254890	KF254869	KF242139	KF242170	KF242108	This study
	ICMP18575	*Capsicum annuum*	JX009455	-	JX009717	-	JX010059	JX010256	Weir et al. (2012)
	ICMP18618	*Capsicum annuum*	JX009512	-	JX009718	-	JX009945	JX010257	Weir et al. (2012)
	ICMP19118	*Jasminum sambac*	HM131507	JX010415	JX009713	JX010105	HM131497	HM131511	Weir et al. (2012)
	DNCL054/ MFLUCC 10-0655	*Carica papaya*	KF157817	KF254892	KF254871	KF242141	KF242172	KF242110	This study
	DNCL056/ MFLUCC 10-0657	*Carica papaya*	KF157818	KF254893	KF254872	KF242142	KF242173	KF242111	This study
	DNCL068/ MFLUCC 10-669	*Musa* sp.	KF157819	KF254894	KF254873	KF242143	KF242174	KF242112	This study

Table 7.1 (continued)

Species	Isolate/culture collection	Host	GenBank Accessions						References
			ACT	TUB2	CAL	GS	GAPDH	ITS	
C. siamense	DNCL073a/ MFLUCC 10-0674	*Aeschynanthus radicans*	KF157824	KF254899	KF254876	KF242148	KF242175	KF242117	This study
	DNCL073b/ MFLUCC 10-0674b	*Aeschynanthus radicans*	KF157825	KF254900	KF254877	KF242149	KF242176	KF242118	This study
	DNCL059a/ MFLUCC 10-0660a	*Annona reticulata*	KF157810	KF254885	KF254864	KF242134	KF242165	KF242103	This study
	DNCL059b/ MFLUCC 10-0660b	*Annona reticulata*	KF157809	KF254884	KF254863	KF242133	KF242164	KF242102	This study
	DNCL035a/ MFLUCC 10-0636a	*Cerbera* sp.	KF157812	KF254887	KF254866	KF242136	KF242167	KF242105	This study
	DNCL035b/ MFLUCC 10-0636b	*Cerbera* sp.	KF157813	KF254888	KF254867	KF242137	KF242168	KF242106	This study
	DNCL076/ MFLUCC 10-677	*Ficus racemosa*	KF157811	KF254886	KF254865	KF242135	KF242166	KF242104	This study
	DNCL034/ MFLUCC 10-0635	*Cerbera* sp.	KF157814	KF254889	KF254868	KF242138	KF242169	KF242107	This study
	ICMP18642	*Hymenocallis americana*	GQ856775	JX010410	JX009709	JX010100	JX010019	JX010278	Weir et al. (2012)

Table 7.1 (continued)

Species	Isolate/culture collection	Host	GenBank Accessions						References
			ACT	TUB2	CAL	GS	GAPDH	ITS	
C. syzygicola	DNCL018/ MFLUCC 10-0621	*Citrus reticulata*	KF157800	KF254879	KF254858	KF242124	KF242155	KF242093	This study
	DNCL021/ MFLUCC 10-0624	*Syzygium samarangense*	KF157801	KF254880	KF254859	KF242125	KF242156	KF242094	This study
	DNCL028/ MFLUCC 10-0630	*Syzygium samarangense*	KF157802	KF254881	KF254860	KF242126	KF242157	KF242095	This study
	DNCL051/ MFLUCC 10-0652	*Syzygium samarangense*	KF157803	KF254882	KF254861	KF242127	KF242158	KF242096	This study
C. ti	ICMP4832	*Cordyline* sp.	JX009520	JX010442	JX009649	JX010123	JX009952	JX010269	Weir et al. (2012)
C. tropicale	CBS129949	*Theobroma cacao*	JX009489	JX010407	JX009719	JX010097	JX010007	JX010264	Weir et al. (2012)
	ICMP18651	*Annona muricata*	JX009570	-	JX009720	-	JX010014	JX010277	Weir et al. (2012)
C. simmodsii	BRIP28519	*Carica papaya*	FJ 907428	FJ 907443	FJ 917510	FJ 972591	FJ 972580	FJ 972601	Prihastuti et al. (2009)
	CBS294.67	*Carica papaya*	FJ 907429	FJ 907444	FJ 917511	FJ 972590	FJ 972581	FJ 972610	Prihastuti et al. (2009)

Notes. CBS. Centraalbureau voor Schimmelcultures, ICMP. International Collection of Microorganisms from Plants, Landcare, New Zealand, MFLUCC. Mae Fah Luang University Culture Collection (Thailand), DNCL, COF. Isolate codes of Dhanushka Udayanga housed in MFLUCC, BRIP. Queensland Plant Pathology Herbarium and culture collection (Australia), IMI, CABI Genetic Resource Collection (UK), BCC BIOTEC Culture Collection (Thailand), BML, LC, BTL, BPD, CORCG additional isolates used in the analyses from Prihastuti et al. (2009); Su et al. (2011).

7.2.2 DNA extraction, PCR and sequencing

Isolates were grown on PDA and incubated for 5 days at 25 °C in the dark. DNA was extracted using the protocol as outlined by Udayanga et al. (2012) using the actively growing mycelia from the edge of cultures. The ITS region was sequenced using the forward/reverse primer pair ITS5/ITS4 (White et al., 1990), following the PCR protocols outlined by Udayanga et al. (2012a). A selected set of strains in the *C. gloeosporioides* species complex predetermined based on ITS sequence analysis (gloeosporioides clade in Figure 7.2) were used for the amplification and sequencing of multiple gene regions. Partial actin (ACT): ACT512F/ACT783R (Carbone and Kohn 1999), β-tubulin (TUB2): Bt2a/Bt2b (Glass & Donaldson, 1995), calmodulin (CAL): CL1/CL2A (O'Donnell et al., 2000), glutamine synthetase (GS): GSF1/GSR1 (Stephenson, Green, Manners & Maclean, 1997) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): GDF1/GDR1 (Templeton, Crandall & Sing, 1992) were amplified following the thermal cycles outlined by Prihastuti et al. (2009). All PCR products were visualized on 1 % agarose gel in the presence of Goldview (Geneshun Biotech, China) with D2000 DNA ladder (Realtimes Biotech, Beijing, China). PCR products were then purified and sequenced using the same forward and reverse primers at SinoGenomax Company, Beijing, China.

7.2.3 Sequence alignment, phylogenetic analyses and species recognition

Raw sequences were assembled with Sequencher 4.9 for Windows (Gene Codes Corp., Ann Arbor, Michigan). The assembled consensus sequences were initially aligned with ClustalW, and optimized by online sequence alignment editor MAFFTv.7 in default settings (mafft.cbrc.jp/alignment/server/) and manually where necessary (Katoh & Standley, 2013). Ex-type and authentic sequences were obtained from several contemporary phylogenetic studies (Phoulivong et al., 2010a, b; Su et al., 2011; Cannon et al., 2012; Weir et al., 2012). The ML, MP and Bayesian phylogenetic trees were inferred as described in the section 4.2.4. Phylogenetic trees and data files were viewed in MEGA v. 5 (Tamura et al., 2011), Treeview (Page, 1996) and Fig Tree v1.2.2 (Rambaut & Drummond, 2008). ITS sequence analysis was initially used to place isolates in different clades of *Colletotrichum* (Figure 7. 2). Additional alignments were analyzed individually and in combination to infer

placement of selected isolates in the *C. gloeosporioides* species complex (Figure 7.3). The isolates were identified to species based on multi-gene phylogeny. All the sequences used in multi-gene analyses were deposited in GenBank (Table 7.1) and the additional ITS alignments and trees in TreeBASE (Study ID = 14511: www.Treebase.org).

7.3 Results

7.3.1 Phylogenetic placement based on ITS analysis

The ITS sequence alignment comprised 91 sequences including the outgroup taxon and 509 characters were included in the analysis. Parsimony analysis revealed that 353 characters were constant, 69 characters were parsimony informative, while 87 variable characters were parsimony uninformative. The analysis of the alignment yielded 463 equally parsimonious trees and the first tree is presented here (Figure 7. 2) (TL=211, CI=0.844, RI=0.961, RC=0.811, HI=0.156). MP/BI and RAxML trees were identical. This tree provides a preliminary guide for identification of isolates used in this study. The ITS analysis demonstrated that the 55 isolates of *Colletotrichum* from fruits in Thailand belonged to the acutatum, boninense, gloeosporioides, and truncatum species complexes in the genus. Forty-one isolates (75 % of total isolates) clustered in the gloeosporioides clade and were isolated from the fruits of *Annona reticulata, Azadirachta indica, Carica papaya, Cerbera* sp., *Citrus aurantifolia, Coffea* sp., *Ficus racemosa, Hylocerus undatus, Mangifera indica, Musa* sp., *Syzygium samarangense, Ziziphus* sp. and several unidentified wild fruits. The isolates that clustered in the acutatum clade were from *Fragaria* sp., *Mangifera* sp., *Musa* sp. and *Ziziphus* sp. and may represent different phylogenetic species known in the clade. The two isolates in the boninense clade were from coffee berries, and one isolate from a *Delonix* pod clustered in the truncatum clade. The other isolates in truncatum clade were from a stem of *Amaranthus* sp., and leaf spot of *Piper* sp. (wild pepper). These results illustrate the diversity of *Colletotrichum* species associated with tropical fruits in northern Thailand with the dominated group belonging to the *C. gloeosporioides* species complex.

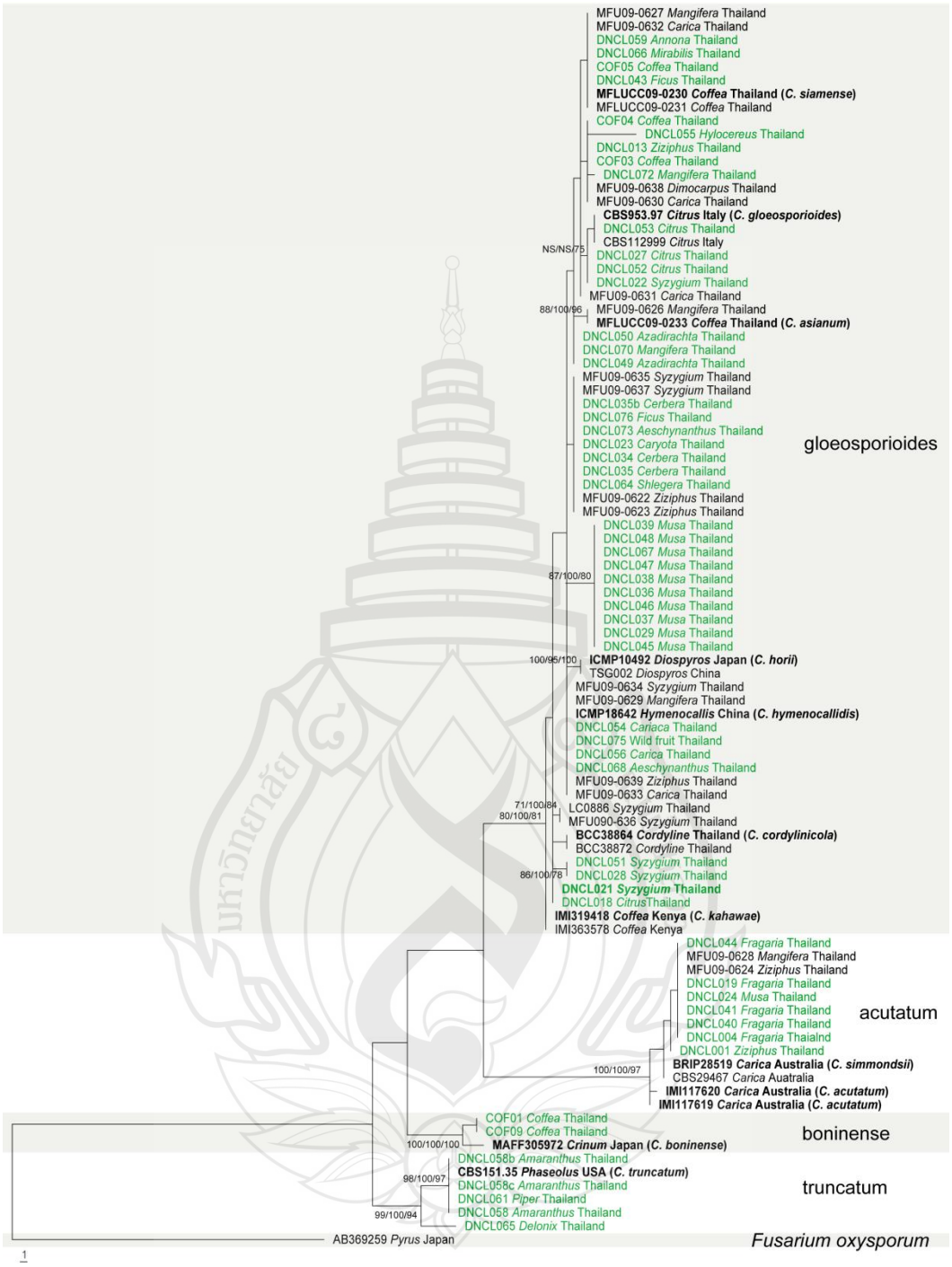


Figure 7.2 Phylogram inferred from parsimony analysis of ITS of *Colletotrichum* spp. MP/ Bayesian posterior probabilities/ RAxML bootstrap values ≥70 % are displayed. Ex-type and ex-epitype cultures isolates are in bold. The tree is rooted with *Fusarium oxysporum* (AB369259).

7.3.2 Multi-gene phylogeny and species recognition

The combined aligned data matrix contains 77 sequences including the outgroup and 3,275 characters (including gaps), 148 characters were excluded in the parsimony analysis. The parsimony analysis revealed that 1961 characters were constant, 987 characters were parsimony informative, while 184 variable characters were parsimony uninformative. The parsimony analysis of the alignment yielded 48 equally parsimonious trees and the first tree is presented here (Figure 7. 3) which enables identification of the isolates to species level (TL=1903, CI=0.762, RI=0.898, RC=0.684, HI=0.238). MP/BI and RAxML trees were identical. In combined analysis of six loci of *C. gloeosporioides* complex, the 33 isolates grouped into seven subclades including *C. endophytica* Manamgoda, D. Udayanga, K.D. Hyde, *C. fructicola*, *C. gloeosporioides* sensu stricto, *C. musae*, *C. siamense* sensu lato, *C. syzygicola* and an undetermined phylogenetic species closely related to *C. fructicola* represented by two nonsporulating endophytes from coffee berries collected in Thailand (*Colletotrichum* sp. indet. 6). Most of the isolates from wild fruits clustered in the *C. siamense* sensu lato clade, and one isolate was *C. endophytica* (see Manamgoda et al. (2013) for descriptions and illustrations). The other isolates from cultivated fruits were *C. fructicola*, *C. gloeosporioides*, *C. musae* and several distinct lineages within *C. siamense* clade.

Colletotrichum siamense sensu lato was recognized based on the strong bootstrap support (99 %) in the combined phylogenetic tree. Isolates from *Azadirachta* (DNCL050), *Coffea* (COF005), *Ficus* (DNCL043) and *Mangifera* (DNCL072) clustered with the ex- type isolate of *C. siamense* (MFLUCC 09-030) which are recognized to be the same species. The other isolates did not cluster with *C. siamense*, *C. jasmine-sambac* or *C. hymenocallidis* were assigned to the distinct lineages of *Colletotrichum* sp. indet. 1-5. We restrain from defining new species within the *C. siamense* clade until the phylogeny is revisited with all recently described cryptic taxa.

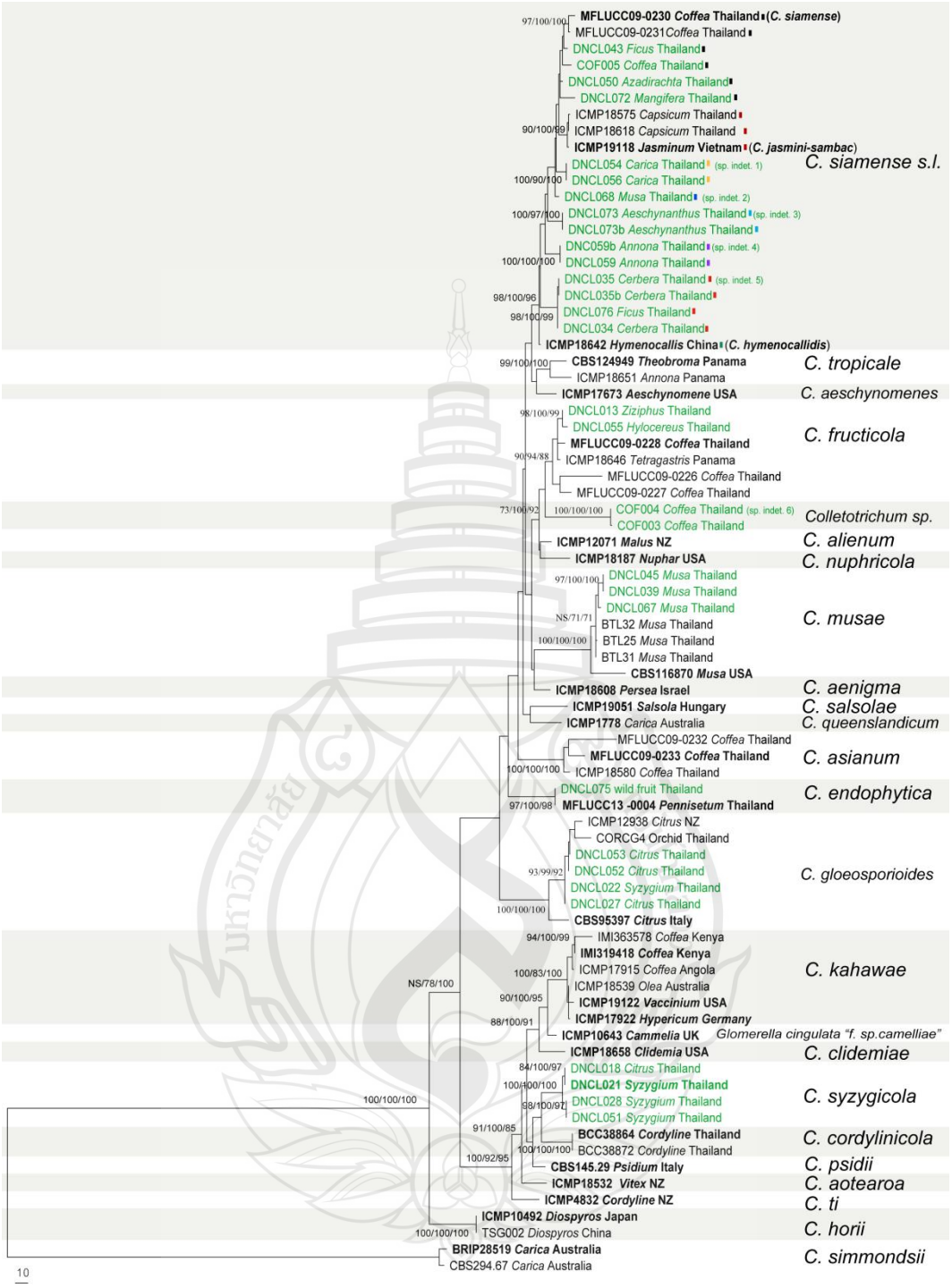


Figure 7.3 Phylogram inferred from the parsimony analysis of the *C. gloeosporioides* species complex. MP/ Bayesian posterior probabilities/ RAxML bootstrap values ≥70 % are displayed. Ex-type and ex-epitype cultures isolates are in bold. The tree is rooted with *C. simmondsii* (BRIP 28519).

7.3.3 Taxonomy

Isolates associated with fruit anthracnose of *Syzygium samarangense* (*Myrtaceae*) and one isolate associated with a fruit of *Citrus aurantifolia* (Rutaceae) clustered in a unique sub-clade in *Colletotrichum gloeosporioides* species complex in multi-gene phylogeny inferred by six gene combined alignment (Figure 7.3). The individual gene genealogies were compared with each other and with the combined analysis. The new species, *C. syzygicola* (Figure 7.4) described here, is closely related to *C. cordylinicola* Phoulivong, L. Cai & K.D. Hyde, originally described from *Cordyline fruticosa* (an ornamental plant belongs to *Asparagaceae*) in Thailand (Phoulivong et al., 2010).

Colletotrichum syzygicola Udayanga, Manamgoda, K. D. Hyde, sp. nov.

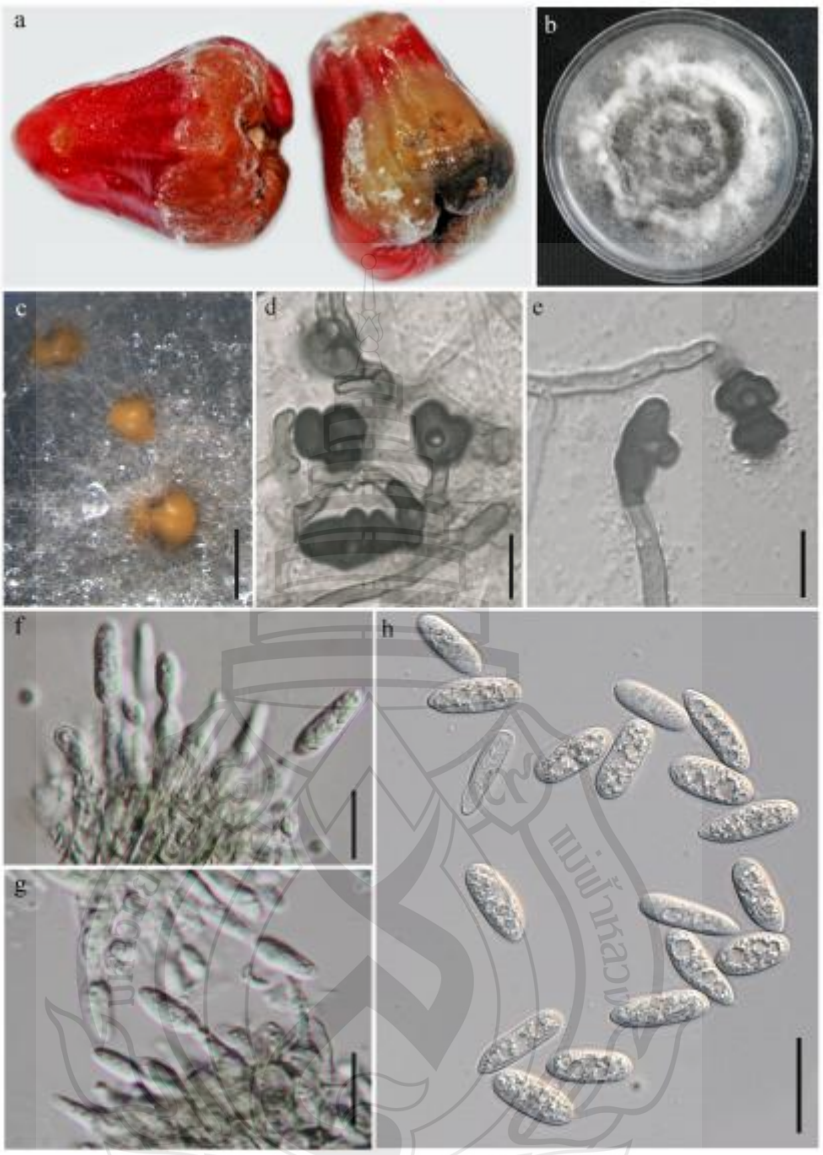
MycoBank: MB803938

Etymology: primarily associated with fruit anthracnose of *Syzygium samarangense*

Colonies on PDA attaining 72 mmdiam., in 7 days at 25 °C, growth rate 5.5±0.5 mm/day (n=6), white to grey, reverse grey at the centre, aerial mycelium, dense and raised, with orange conidial masses, sclerotia present. Setae absent in culture on PDA or slide cultures in PCA. Conidiophores (10.5–) 12–16 (–21)×4–5 μm (mean ± SD=14±2×4.5±0.5 μm, n=50), cylindrical or clavate, wide at the base, hyaline, unbranched, occurring in densely arranged clusters. Conidiogenous cells enteroblastic, hyaline, cylindrical to clavate. Conidia (12–) 13–15 (–18)×5.5–6.5 μm (mean ± SD=14.7±0.9×6± 0.5 μm), unicellular, hyaline, ovoid to cylindrical or clavate, with rounded apices. Appressoria formed in slide culture (18–) 19–22 (–24) μm×7–8 (mean ± SD=20±1.5× 7.6±0.5 μm, n=50), formed frombranchedmycelia, terminal, brown to dark brown, variable in shape, irregular or knobbed. Sexual state not observed in culture.

Known Hosts: On fruits of *Syzygium samarangense* and *Citrus aurantifolia* associated with anthracnose symptoms

Known distribution: Northern Thailand



Notes. a. Infected fruits of *S. samarangense*; b. Culture on PDA (2 wk old); c. Conidiomata with spore masses on PDA; d,e. Appressoria; f, g. Conidiophores; h. Conidia. Bars. c=1000 µm d, e= 20 µm, f, g, h=15 µm.

Figure 7.4 Morphology of *Colletotrichum syzygicola*.

Type material: THAILAND, Chiang Rai Province, Nang Lae, Fah-Thai market, on fruits of *Syzygium samarangense*, 18 April 2010, Dhanushka Udayanga (Holotype, MFLU12-2476, dried sporulating culture on PDA; ex –holotype culture DNCL021= MFLUCC 10-0624) ; Isotype, *ibid*. (MFLU 12-2477: dried culture; ex-isotype culture DNCL028=MFLUCC 10- 0630).

Additional material examined: THAILAND, Chiang Rai Province, Nang Lae, Fah-Thai market, on fruits of *Citrus aurantifolia*, 27 February 2010, Phongeun Sysouphanthong (DNCL018, living culture MFLUCC10-0621); *ibid*., Chiang-Kong city market, on fruits of *Syzygium samarangense*, 10 April 2010, Dhanushka Udayanga (DNCL051, living culture MFLUCC 10-0652).

Notes: Isolates of *Colletotrichum syzygicola* primarily from *Syzygium samarangense* formed a divergent lineage from species of the kahawae clade (i.e. *C. aotoreae* B. Weir & P.R. Johnst., *C. clidemia* B. Weir & P.R. Johnst, *C. cordylinicola*, *C. psidii* Curzi, *C. kahawae*, *C. kahawae* subsp. *ciggaro* B. Weir & P.R. Johnst., *C. ti* B. Weir & P.R. Johnst. and *Glomerella cingulata* f. sp. *camelliae*) in the combined phylogenetic analysis (Weir et al., 2012),. Isolates from *Syzygium* sp. in Phoulivong et al. (2010; 2012) were named as *C. cordylinicola*, however they stated that these isolates represent a different pathotype infecting tropical fruits, whereas *C. cordylinicola* originally isolated from *Cordyline* does not infect fruits. The isolate BCC38864 from *Syzygium* sp. which forms a distinct lineage (Phoulivong et al., 2011) was regarded as *C. cordylinicola* in their multi-gene phylogenetic tree, could be due to insufficient sampling to recognize an unequivocally distinct lineage. To our knowledge, there are no any distinct morphological or phylogenetic species originally described within *C. gloeosporioides* complex from *Syzygium* sp., which is the common host of *C. syzygicola*. The other recently defined species associated with ericaceous hosts in north America (*Colletotrichum fructivorum* V. Doyle, P.V. Oudem. & S.A. Rehner, *Colletotrichum temperatum* V. Doyle, P.V. Oudem. & S.A. Rehner and epitype of *Colletotrichum rhexiae* Ellis & Everh.), which are closely related to *C. kahawae* were regarded as distinct from *C. syzygicola* by comparison of available single genes and relative phylogenetic affinity.

7.4 Discussion

Molecular phylogenetic studies have revolutionized the understanding of species diversity and their ecological and evolutionary significance in *Colletotrichum* (Hyde et al., 2009a; Damm, Woudenberg, Cannon & Crous, 2009; Damm et al., 2010; O'Connell et al., 2004, 2012; Silva et al., 2012b). Within the C. gloeosporioides species complex, 90 % of the phylogenetically distinct taxa (e.g. *C. aenigma*, *C. alienum*, *C. asianum*, *C. fructicola*, *C. fructivorum*, *C. gloeosporioides* sensu stricto, *C. musae*, *C. kahawae*, *C. psidii*, and *C. queenslandicum*, *C. siamense*, *C. syzygicola*, *C. tropicale*, *C. temperatum* and *C. viniferum*) are originally described from fruits causing anthracnose or similar symptoms. In this study, we recognized that the dominant anthracnose pathogens in tropical Asia comprise several species in C. gloeosporioides complex, although C. *gloeosporioides* sensu stricto has a comparatively limited range of hosts. We identified *C. gloeosporioides* only from *Citrus aurantifolia* and *Syzygium* in northern Thailand, while Peng, Yang, Hyde, Bahkali and Liu (2012) and Huang et al. (2013b) found this species to be the most common on Citrus leaves and fruits in China.

Colletotrichum tropicale is a common species associated with *Annona* and *Theobroma* in Panama, and *C. theobromicola* with *Annona* and *Theobroma cacao* in Panama and Mexico are not found from the fruits obtained from Thailand in this study. *Colletotrichum endophytica*, a common endophyte in tropical grasses (Manamgoda, Udayanga, Cai, Chukeatirote & Hyde, 2013), was also found from an unidentified wild fruit in this study. *Colletotrichum syzygicola* is a common pathogen infecting *Syzygium* fruits and less frequently on Citrus obtained from local markets in Thailand.

Colletotrichum fructicola (from coffee and dragon fruit), *C. musae* (from banana), C. *siamense* sensu lato (a wide range of hosts) were also dominant species on tropical fruits sampled. In the ITS analysis (Figure 7.2) the isolates in acutatum clade were commonly associated with *Fragaria* (strawberry) and *Ziziphus* (jujube). Many species within the *C. acutatum* species complex have also been reported as common causative agents of the fruit diseases of tropical and temperate hosts in different

studies (Than et al. 2008a; 2008b; Shivas & Tan, 2009). A multi-gene phylogenetic analysis is required for the accurate identification of species within *C. acutatum* complex from *Fragaria* since *C. fioriniae* (Marcelino & Gouli) R.G. Shivas & Y.P. Tan, *C. godetiae* Neerg, *C. nymphaeae* (Pass.) Aa, *C. salicis* (Fuckel) Damm, P.F. Cannon & Crous and *C. simmondsii* R.G. Shivas & Y.P. Tan (Damm et al., 2012a) have been reported from this host. Weir et al. (2012) indicate the occurrence of *C. fructicola, C. theobromicola* (syn. *C. fragariae* A.N. Brooks) and *C. siamense* associated with *Fragria* in United States and Cananda. The species within the *Colletotrichum boninense* and *C. truncatum* species complexes were less frequently found in this study but can also be considered as important species associated with tropical fruits. *Colletotrichum siamense* was originally described as a pathogen associated with anthracnose of coffee berries in northern Thailand (Prihastuti et al., 2009). In the present study, we identified *C. siamense* sensu stricto as an endophyte in pericarp of healthy coffee berries and as a pathogenic species causing anthracnose on fruits of *Ficus racemosa, Azadirachta india* and *Mangifera indica*. The conventional six gene analysis of *C. gloeosporioides* complex results a strong monophyly of a broad phylogenetic group of *C. siamense* (Weir et al., 2012), however it is less reliable to resolve the species relationships and boundaries within the clade. Considerable genetic diversity was observed in the clade recognized as *C. siamense* sensu lato from sampling within a limited geographic region including six collection sites in northern Thailand (Figure 7.3). The distinct lineages strictly correlated with host association in case of isolates fruits of *Annona, Cerbera* and leaves of *Aeschynanthus*. The close inspection of six gene combine alignment and single gene analyses reveal that the *C. hymenocallidis*, *C. jasmini- sambac* and *C. siamense* can be considered as recently diverged species with minimum phylogenetic distinction to establish their strong monophyly. However, the single gene phylogenetic analyses of ITS, GPDH and GS (trees not shown), support the distinction of the above three taxa from the other groups (sp. indet. 1-5) not assigned to a known species. Therefore, we recognize *C. siamense* as a species complex which urgently needs phylogenetic reappraisal. Doyle et al. (2013) and Sharma et al. (2013) also showed *C. siamense* to be a species complex based onAPN2 (DNA lyase) and APN2/MAT-IGS (intergenic spacer between 3′ end of the DNA lyase and mating type locus MAT1-2) genetic

markers. *Colletotrichum siamense* sensu lato was also reported as the most common endophyte associated with two tropical grass species in Thailand (Manamgoda et al., 2013). In our analysis we observed that the GS gene in the *C. siamense* sensu lato clade has a higher variability as compared to that of other *Colletotrichum* species. This was also noted by Silva et al. (2012a) and considered as discordance of gene-trees within C. *siamense* which they recognized as a common issue in resolving evolutionary relationships in the *C. gloeosporioides* complex. Although we observed several distinct lineages within in *C. siamense* species complex, novel species were not introduced in this study until it is revisited in a future comprehensive assessment.

Therefore, this study lays the groundwork to refine the understanding of the extent of cryptic diversification within the *C. siamense* clade in future. It has been common desirable practice to link existing species names of *Colletotrichum* with new collections via epitypification (Hyde et al., 2009b; Weir et al., 2012; Rossman, 2013). The general unwritten rules of epitypification require that an epitype should be collected from the same host and host organ, and same country or region as the protologue and have the same characteristics (Zhang & Hyde, 2008). In genera such as *Colletotrichum*, species have wide host ranges and large numbers of names exist in the literature. Previous authors have linked newly resolved species with old scientific names and have designated epitypes for these earlier names. For example, *Colletotrichum musae* from *Musa* sp., *C. rhexiae* Ellis and Everh. from *Rhexia virginica* are recently epitypified based on fresh collections (Su et al., 2011; Doyle et al., 2013). Some authors have introduced novel species only when the taxonomic entity cannot be reliably linked to earlier names (Crouch & Tamaso-Peterson, 2012; Lima et al., 2013; Peng et al., 2013). However, the description of novel taxa should be well supported by adequate molecular datasets and state- of- the-art analysis with the inclusion of frequent and abundant fresh collections (Hyde et al., 2013; Sharma et al., 2013).

Tropical Asia is an economically and physiographically rich region with a unique, hyperdiverse flora and fungi (Hyde, 2003; Hawksworth, 2004; Mueller & Schmit 2007; Karunarathna et al., 2012). Post-harvest management of fruit and vegetables in most developing countries in the Asian region is, however, far from satisfactory (Report of International Centre for Science and High Technology, United

Nations Industrial Development Organization, 2012). Major constraints include inefficient handling and transportation and loss due to fungal and bacterial diseases due to lack of implementation of postharvest technologies (Adikaram, 1986; Aidoo, 1993; Choudhury, 2006). Cultivated fruits in Southeast Asia suffer from great yield losses due to factors including fungal disease encouraged by favourable environmental factors (Li, 1970; Yaacob & Subhadrabandhu, 1995; Rolle, 2006). Phytosanitary standards maintained by some developed countries limit the exports of fruits from developing countries which often impact on agricultural trade (Romberg & Roberts, 2008). The species associated with the diseases of commercial fruits are critical to re- regulating movement of pathogens and establish effective disease control measures. Therefore, it is important to integrate the dynamic changes taking place in classification and nomenclature of pathogenic fungi with the broad applications in biosecurity, quarantine and disease control (Rossman & Palm-Hernández, 2008; Cai et al., 2011). Incorporation of molecular data derived from the isolates in regional and global sampling of *Colletotrichum* species, improves the knowledge of diversity, population structure, extent of host and geographic distribution.

7.5 Conclusion

Species of *Colletotrichum* are associated with anthracnose of a wide range of host plants including cultivated and wild tropical fruits. The genetic and ecological diversity of species associated with wild fruits are poorly explored, as compared to those associated with pre and postharvest diseases of cultivated fruits. In the present study, isolates of *Colletotrichum* were obtained from commercially available cultivated fruits, wild fruits (from native trees in natural habitats) and a few herbaceous hosts collected in northern Thailand. These isolates were initially characterized based on analysis of complete sequences of nuclear ribosomal internal transcribed spacer (ITS), into the genetically defined species complexes of *Colletotrichum gloeosporioides*, *C. acutatum*, *C. boninense* and *C. truncatum*. The isolates were primarily identified in the C. gloeosporioides species complex, based on

a strongly supported clade within the ITS gene tree and were further characterized using multi-gene phylogenetic analyses and morphology. Phylogenetic analyses of ITS, partial sequences of actin (ACT), calmodulin (CAL), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamine synthetase (GS) and β-tubulin (TUB2) genetic markers were performed individually and in combination.

A new species, *Colletotrichum syzygicola*, associated with *Syzygium samarangense* in Thailand, is introduced with descriptions and illustrations. This study highlights the need to re-assess the evolutionary relationships of *Colletotrichum* species occurring on cultivated and wild fruits with emphasis on their ecology and cryptic diversification including sampling at regional and global scales.

CHAPTER 8

OVERALL CONCLUSIONS

8.1 Current status of molecular phylogeny and taxonomy of *Diaporthe*

Taxonomy and phylogeny of *Diaporthe* has been confused in the last few decades due to the lack of knowledge on important phytopathogenic species, their host association, distribution and reliable molecular data (Udayanga et al., 2011). The incorporation and rigorous analyses of molecular data in systematics have improved the current understanding of the species limits of the genus.

At the beginning of this study, only the ITS, EF1-α, and mating type genes had been used by various authors in the phylogenetic analyses of *Diaporthe* for a limited number of studies (Rehner & Uecker, 1994, Niekerk et al., 2005, Santos & Phillips 2009, Santos et al., 2010). We used up to eight gene regions (ACT, Apn2, CAL, EF1-α, TUB, HIS, FG1093 and ITS) depending on the complexity of the group considered. In addition, alternative and new primers were designed to amplify, calmodulin, actin and Apn2 genes (Figure 8.1).

The status of the taxonomy and phylogeny by the time of this project was initiated is comprehensively reviewed in the first chapter. The first ITS backbone tree was inferred (Figure 1.2), and the tree only consists of 45 isolates and most of them were authentic or otherwise the cultures were not available. The genus wide analysis was conducted with combined phylogeny of four genes and (Figure 2.3), subsequently used for definition of new species and resolving the cryptic species complexes. The combined phylogenetic trees were successfully used in thr last two years (2012, 2013) to describe new species and designate epitypes for the different other authors in their analyses.

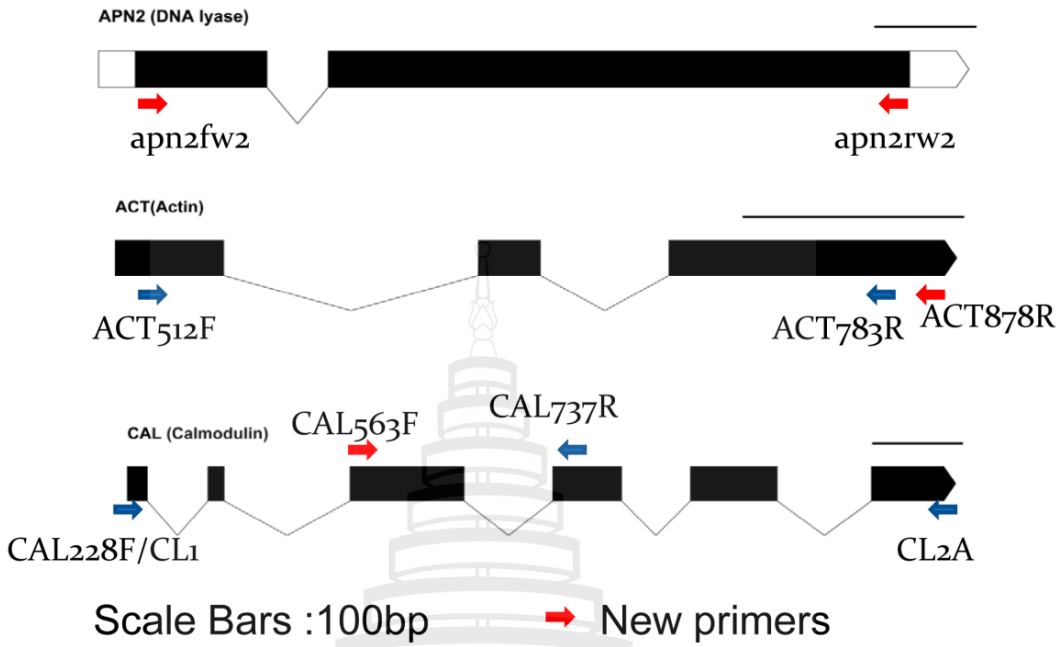


Figure 8.1 Summary of the new primer sets for Apn2, ACT and CAL genes. The new primers designed in this study are indicated in red arrows.

A backbone tree based on up to date published data provided by Hyde et al. (2014), Udayanga et al. (2014c) can be used as a quick reference source, which need to be updated in future studies with the availability of new sequence data. Herein, we also provide simple six step guidelines for future studies in the genus *Diaporthe* for the accurate identification of known species and describing novel taxa using molecular data (Figure 8. 2). We conclude that, it is the best to perform phylogenetic analysis with a careful selection of isolates in pilot analyses considering their evolutionary relatedness rather than direct genus wide analysis which can often obscure the species relationships, due to the problems of discordance of some genes. ITS region often can mislead the species limits within difficult species complexes in *Diaporthe*. Therefore, it is recommended to exclude from the analysis based on justifiable grounds regarding the sampling used and the result of congruence analyses.

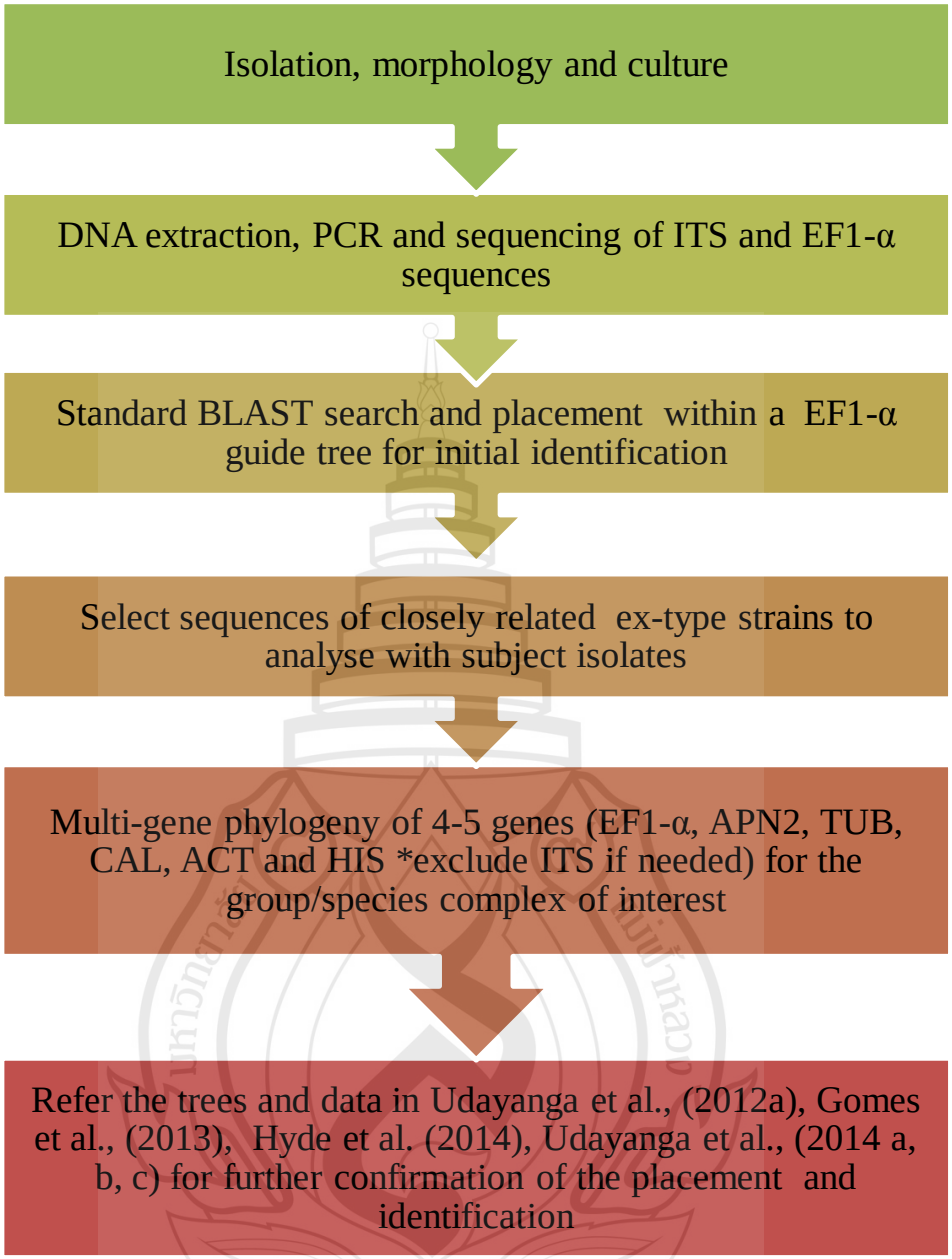


Figure 8.2 Simple six step guidelines for the identification of *Diaporthe* species and description of novel taxa.

8.2 Species and species complexes – Insights in to the genus *Diaporthe*

The confusion in the interpretation of ITS sequence data within species complexes and gene tree discordance of several other gene regions were often discussed by various authors worked on *Diaporthe*. Application of GCPSR based on multiple gene genealogies allowed us to delimit the confusing clades like *Diaporthe eres*, *D. foeniculina*, *D. rudis* and *D. sojae* species complexes. The difficulties to resolve highly similar phylogenetic species likely to be due to the gene flow among species and recombination. We observed that more genetic variability and conflicts occur in species delimitation within the minor or opportunistic pathogens which infect an extensive range of hosts in wide geographic distribution (e.g. *Diaporthe eres*, *D. rudis*, *D. foeniculina*). In contrast, relatively host specific species (e.g. *Diaporthe vaccnii*, *D. ampelina*, *D. citri*) are generally serious pathogens and clearly distinguished by gene genealogies therefore it is relatively easy to delimit the species.

The strict application of GCPSR criterion in the species complexes resolved the species limits in *Diaporthe eres complex* and designation of epitypes for well resolved taxa. The discordance of gene trees and heterogeneity of ITS within a species are observed. Recombination is the general hypothesis for the discordance of gene trees (Taylor et al., 2000) however, the hidden paralogy of the genes like TUB can also result the conflict among gene trees in recently evolving taxa (Udayanga et al. 2014a). We practiced the GCPSR which uses both genealogical concordance and non-discordance to recognize species boundaries with broad taxonomic sampling to improve the phylogenetic accuracy to provide the insights in to the phylogeny and speciation of in *Diaporthe*.

This study also revealed an abundance of previously unrecognized genetic diversity in the genus, extent of host association, species complexes and insights in to interspecific evolutionary relationships. Many of the taxonomic and phylogenetic confusions of the important phylogenetic taxa are resolved with broadening the understanding of the genus.

8.3 Molecular phylogeny of anthracnose pathogens of tropical fruits – current status

The current re-evaluation of the *Colletotrichum* species associated with tropical fruits revealed the occurrence of high species diversity associated with anthracnose symptoms. *Colletotrichum gloesporioides* has previously been thought to be a ubiquitous species occurring on various fruits and other trees. However in this study, the species was only found from a few hosts including on *Citrus*, of which it was originally described. A novel species *C. syzygicola* was described from *Syzygium samarangense* in Thailand, which is closely related to *C. cordylinicola*. According to the current knowledge about 30 phylogenetic species are now known within the *C. gloesporioides* species complex (Weir et al., 2012, Hyde et al., 2014). Regional and global scale sampling form a wide range of hosts will be useful in future studies in order to reveal the extent of diversity and distribution of anthracnose pathogens. Accurate identification of anthracnose pathogens in fruits in tropical Asia is critical to update the quarantine regulations and effective phytosanitation in export agriculture.

8.4 Significance and contribution of this thesis for the advancement of Science

This study provides a comprehensive understanding of the plant pathogenic genus *Diaporthe* with insights in to the cryptic species complexes and their phylogenetic relationships. The principles of GCPSR were applied to the overall genus and to resolve the specific clades within the genus broadening the understanding of prevalent phytopathogenic species. In addition, the phylogeny of *Colletotrichum gloeosporioides* species complex occurring on tropical fruits is revisited with reference to the isolates from tropical Asia from common domestic fruits and uncommon wild hosts.

Five species were introduced new to science (*Diaporthe pterocarpicola*, *D. siamensis*, *D. thunbergii*, *D. ueckerae* & *Colletotrichum syzygicola*) with clarification of taxonomy and phylogeny of about 17 significant phytopathogenic species

(*Diaporthe citri*, *D. cytosporella*, *D. foeniculina* (= *Phomopsis califonica*), *D. rudis* (=*D. medusaea*), *D. eres (=Phomopsis fukushii*), *D. alnea*, *D. pulla*, *D. helicis*, *D. bicincta*, *D. celastrina*, *D. phaseolorum*, *D. longicolla*, *D. batats*, *D. cucurbitae*, *D. sojae*, *D. arctii* and *D. pterocarpi*) with epitypes designated based on fresh collections otherwise ex-type cultures were sequenced providing multiple DNA sequence data and clarification of nomenclature.

Pathogens associated with *Citrus* and soybean was resolved with global collections and re-definition causative agents of major diseases. The re-definition of these major phytopathogens is significant in agriculture, plant health, quarantine and various applications in phytopathology including development of disease resistant varieties. The data will be highly useful for quick identification of potentially harmful phytopathogens for high value crops like soybean, sunflower, grapes and *Citrus*.

The generic type species *Diaporthe eres* is re-defined, with epitype designated and resolving the species complex, which provide insights in to taxonomy of the genus and evolutionary relationships of cryptic species. The phenomenon of occurrence of two different ITS types within one species of *Diaporthe* is confirmed with reference to *D. eres* species complex. The epitypification of generic type is also useful for the robust definition of the generic concept, as well as ex-type cultures is now available for genetic and genomic studies.

The new primers to amplify ACT, CAL and Apn2 genes were designed and tested and made available to use for fungal taxonomist's toolbox. This study report the first ever utility of 8 gene regions in *Diaporthe* with the recommendation of EF1-α and Apn2 markers to be used in future phylogenetic studies of cryptic species complexes, with the confirmation of phylogenetic informativeness profiles.

In total 1100 gene sequences were generated and deposited in GenBank with complete feature annotations of coding regions and geo-ecological meta data. In parallel ITS sequences of all the ex-types were re-annotated providing the ex-type status and further verifications in UNITE platform (Nilsson et al., 2014) and GenBank reference sequence project (Schoch et al., 2014). About 1000 fresh specimens, 300 herbarium specimens in worldwide herbaria, 75 type specimens were observed. The annotations for all the specimens examined were returned to herbaria with updates of taxonomy and nomenclature, and made available in public databases and publications.

The overall project design and outcome will provide a successful experimental model study to resolve highly diversified genera in fungi with more effective and promising approach. Significant findings revealed in this study focusing on evolutionary relationships of phytopathogens have broaden the understanding of process of speciation, evolutionary ecology of phytopathogens, agriculture, biotechnology and in other basic and applied contexts of advancing life sciences.

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APPENDICES

APPENDIX A

MEDIA COMPOSITION AND PREPARATION

WATER AGAR WITH ALFALFA STEM SPORULATION MEDIA for *Diaporthe*

Agar……15g

The media were prepared with 15g agar/ 1 litre distilled water and sterilised by autoclaving at 121°C for 15 min. A sterile alfalfa stem was placed on the setting agar plate and used to inoculate fungi.

POTATO CARROT AGAR (PCA) FOR SPORULATION OF *Colletotrichum*

Potatoes……	20.0 g
Carrots……..	20.0 g
Agar…….	15.0 g
Distilled water……	1.0 L

Dice potatoes and carrots and cook in water for 1/2 hour. Strain through cheesecloth and add 15.0 g agar to the filtrate. Autoclave at 121 ℃ for 15 minutes.

APPENDIX B

PCR PROTOCOLS

Generalised PCR protocol for all molecular markers used in this study

95 ℃	5.0 min
95 ℃	0.30 min
55 ℃ *	0.30 min
72 ℃	1.0 min
72 ℃	7.0 min
10 ℃ / 4 ℃	hold

X 40 cycles

* Annealing temperatures are varied for each gene regions 55 ℃ (ITS, CAL, HIS, ACT), 58 ℃ (TUB, EF 1-α), 54 ℃ (APN2)

Touchdown PCR condition for FG1093 gene

95 ℃…….	2 min	
95 ℃…….	1 min	X 10 cycles
65-55 ℃ decreasing by 1 in each cycle	30 sec	
72 ℃……	1 min	
95 ℃…….	1 min	X 35 cycles
59 ℃…….	30 sec	
72 ℃…….	1 min	
4 ℃……..	hold	

APPENDIX C

CTAB METHOD FOR DNA EXTRACTION

1. Turn on water bath/incubator and set to 65 °C.

2. Check solutions: 2% CTAB buffer, Phenol, Chloroform: Isoamyl alcohol 24:1, Isoporoponol, 70% ethanol, TE buffer/distilled/ de-ionized water.

3. Harvest mycelia from the leading edge of growing plate (PDA)/ 2-5 days old depending on growth rates (fresh and actively growing mycelia) 0.1-0.2g.

4. Add ½ volume of PVP (optional), and Quartz sand (needed).

5. Add 200 µl of CTAB buffer, and to eppendorf, and break the tissue by grinding with sterile glass pestle, 10-15 min will be needed OR use a garnet bead in a cryovial and use Fast-prep to break the cells.

6. Add 400 µl CTAB buffer and incubate in water bath for 40 min to 1hr.

7. Centrifuge 12,000 rpm for 10 min, transfer the aqueous phase to a new tube.

8. Add ½ volume of (300 µl) of phenol and ½ volume of chloroform:isoamyl alcohol and mix gently ; can repeat the extraction for better results.

9. Transfer the aqueous phase in to new tube and add 600 µl of chloroform; isoamyl alcohol.

10. Centrifuge 12,000 rpm for 10 min, transfer the aqueous phase in to new tube.

11. Add equal volume of Ice cold, isopropanol, and keep in 4 °C 1 hour OR -20 for 10-20 min.

12. Centrifuge 12,000 rpm for 10 min, and discard the aqueous phase.

13. Add 500 µl of 70% ethanol and wash the precipitate gently, centrifuge again and recover the precipitate. Wash with 20% ethanol when more times impurities detected.

14. Air dry the precipitate and dissolve 50 µl of TE buffer/ DDwater, add 2 µl of RNAse, leave in room temp for few minutes and store in -20 refrigerator.

APPENDIX D

COMPOSITION OF SOLUTIONS

CTAB Buffer

100 ml 1 M Tris HCl pH 8.0

280 ml 5 M NaCl

40 ml of 0.5 M EDTA

20 g of CTAB (cetyltrimethyl ammonium bromide)

Bring total volume to 1 L with ddH_2O.

TE Buffer

10 ml 1 M Tris HCl pH 8.0

2 ml 0.5 M EDTA

Bring total volume to 1 L with ddH_2O.

1 M Tris HCl pH 8.0

121.1 g Tris

Dissolve in about 700 ml of H_2O.

Bring pH down to 8.0 by adding concentrated HCl (you'll need about 50 ml).

Bring total volume to 1 L with ddH_2O.

0.5 M EDTA

186.12 g EDTA

Add about 700 ml H_2O

16-18 g of NaOH pellets

Adjust pH to 8.0 by with a few more pellets, EDTA won't dissolve until the pH is near 8.0

Bring total volume to 1 L with ddH_2

5 M NaCl

292.2 g of NaCl

700 ml H_2O

Dissolve (don't add NaCl all at once, it will never go into solution) and bring to 1 L.

Chloroform/Isoamyl Alcohol (24:1)

Chloroform 96 ml

Isoamyl alcohol 4 ml

Prepare in fume hood.

Proteinase K (20 µg/µl)

Proteinase K 500 mg

ddi H_2O (or equivalent) 25 ml

Aliquot immediately into convenient size volumes and freeze. Remix after thawing.

TAE buffer (1L of 10x solution)

48.5 g Tris

11.4 mL glacial acetic acid

20 mL 0.5M EDTA (pH 8.0)

1. Dissolve Tris in about 800 mL of deionized water.
2. Add acetic acid and EDTA.
3. Add deionized water to 1L.
4. Store at room temperature.

Dilute stock solution 10:1 to make a 1x working solution.

1x buffer will contain 40 mM Tris, 20 mM acetic acid and 1 mM EDTA

APPENDIX E

PCR PRODUCT PURIFICATION AND SEQUENCING

EXO-SAP (Exonuclease plus Shrimp Alkaline Phosphatase) PCR clean-up Protocol

PCR product purification kit: ExoSAP-IT (USB Corp., Cleveland, OH, USA)

1. Mix 8 µl PCR product with 3 µl ExoSap working solution

2. Pipette ExoSap working solution onto PCR product. Dispense the ExoSap working solution on the side of the tube above the PCR product. It will mix by convection in the thermocycler.

3. Cycling conditions
   1. 37ºC for 30 min (Let ExoSap work)
   2. 80ºC for 15 min (inactivate ExoSap)
   3. 10ºC for 1 min (cool down block)

Composition of Big dye Sequencing Reaction Mixture (for one reaction)

BigDye® Terminator v3.1 Ready Reaction Mix (1 µl)
5X Sequencing Buffer Primer (3 µl)
Sequencing Primer/1mM (1 µl)
Purified PCR product (1 µl)
ddH_2O (4 µl)

Thermal cycles for Big Dye sequencing reaction

96 °C	1.0 min
95 °C	0.1 min
50 °C	0.05 min
60 °C	4.0 min
4°C	hold

X 35 cycles

APPENDIX F

PUBLICATIONS

Fungal Diversity (2011) 50:189–225
DOI 10.1007/s13225-011-0126-9

The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens

Dhanushka Udayanga · Xingzhong Liu · Eric H. C. McKenzie · Ekachai Chukeatirote · Ali H. A. Bahkali · Kevin D. Hyde

Received: 21 June 2011 / Accepted: 22 July 2011 / Published online: 6 September 2011

Abstract The genus *Phomopsis* (teleomorph *Diaporthe*) comprises phytopathologically important microfungi with diverse host associations and a worldwide distribution. Species concepts in *Phomopsis* have been based historically on morphology, cultural characteristics and host affiliation. This paper serves to provide an overview of the current status of the taxonomy in *Phomopsis* with special reference to biology, applications of various species, species concepts, future research perspectives and names of common pathogens, the latter being given taxonomic reappraisal. Accurate species identification is critical to understanding disease epidemiology and in developing effective control measures for plant diseases. Difficulties in accurate species identification using morphology have led to the application of alternative approaches to differentiate species, including virulence and pathogenicity, biochemistry, metabolites, physiology, antagonism, molecular phylogenetics and mating experiments. Redefinition of *Phomopsis/Diaporthe* species has been ongoing, and some species have been redefined based on a combination of molecular, morphological, cultural, phytopathological and mating type data. Rapid progress in molecular identification has in particular revolutionized taxonomic studies, providing persuasive genetic evidence to define the species boundaries. A backbone ITS based phylogenetic tree is here in generated using the sequences derived from 46 type, epitype cultures, and vouchers and is presented as a rough and quick identification guide for species of *Phomopsis*. The need for epitypification of taxonomic entities and the need to use multiple loci in phylogenies that better reflect species limits are suggested. The account of names of phytopathogens currently in use are listed alphabetically and annotated with a taxonomic entry, teleomorph, associated hosts and disease symptoms, including brief summaries of taxonomic and phylogenetic research. Available type culture information and details of gene sequences derived from type cultures are also summarized and tabulated.

Keywords Anamorph · Antagonism · Biocontrol · Canker · Chemotype · Endophyte · Epitypification · Genetic transformation · Mating type · Molecular phylogeny · Pathogen · Morphology · Mycotoxins · Quarantine

D. Udayanga · X. Liu
State Key Laboratory of Mycology, Institute of Microbiology,
Chinese Academy of Sciences,
No 3 1st West Beichen Road, Chaoyang District,
Beijing 100101, People's Republic of China

D. Udayanga · E. Chukeatirote · K. D. Hyde (✉)
School of Science, Mae Fah Luang University,
Thasud,
Chiang Rai 57100, Thailand
e-mail: kdhyde3@gmail.com

E. H. C. McKenzie
Landcare Research,
Private Bag 92170,
Auckland, New Zealand

A. H. A. Bahkali · K. D. Hyde
College of Science, Botany and Microbiology Department,
King Saud University,
Riyadh, Saudi Arabia

Introduction

Phomopsis (Sacc.) Bubák is an important phytopathogenic genus in urgent need of taxonomic reappraisal (Rehner and Uecker 1994; Farr et al. 2002a, b; Cristescu 2003; Murali et

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DOI 10.1007/s13225-012-0190-9

A multi-locus phylogenetic evaluation of *Diaporthe* (*Phomopsis*)

Dhanushka Udayanga · Xingzhong Liu · Pedro W. Crous · Eric H. C. McKenzie · Ekachai Chukeatirote · Kevin D. Hyde

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Abstract The genus *Diaporthe* (*Phomopsis*) includes important plant pathogenic fungi with wide host ranges and geographic distributions. In the present study, phylogenetic species recognition in *Diaporthe* is re-evaluated using a multi-locus phylogeny based on a combined data matrix of rDNA ITS, and partial sequences from the translation elongation factor 1-α (EF 1-α), β tubulin (TUB) and calmodulin (CAL) molecular markers. DNA sequences of available ex-type cultures have been included, providing a multi-locus backbone tree for future studies on *Diaporthe*. Four utilizable loci were analyzed individually and in combination, and ITS, EF 1-α and multi-locus phylogenetic trees are presented. The phylogenetic tree inferred by combined analysis of four loci provided the best resolution for species as compared to single gene analysis. Notes are provided for nine species previously known in *Phomopsis* that are transferred to *Diaporthe* in the present study. The unraveling of cryptic species complexes of *Diaporthe* based on Genealogical Concordance Phylogenetic Species Recognition (GCPSR) is emphasized.

Keywords Ex-type culture · Host diversity · Mating types · Molecular systematics · New combination · Phytopathogen · Species recognition · Taxonomy

D. Udayanga · E. Chukeatirote · K. D. Hyde
Institute of Excellence in Fungal Research,
Mae Fah Luang University,
Chiang Rai 57100, Thailand

D. Udayanga (✉) · E. Chukeatirote · K. D. Hyde
School of Science, Mae Fah Luang University,
Chiang Rai 57100, Thailand
e-mail: udayasun55@yahoo.com

D. Udayanga · X. Liu (✉)
State Key Laboratory of Mycology, Institute of Microbiology,
Chinese Academy of Sciences,
No 3 1st West Beichen Road, Chaoyang District,
Beijing 100101, People's Republic of China
e-mail: liuxz@im.ac.cn

P. W. Crous
CBS-KNAW Fungal Biodiversity Centre,
Uppsalalaan 8,
3584 CT, Utrecht, The Netherlands

E. H. C. McKenzie
Landcare Research,
Private Bag 92170,
Auckland, New Zealand

Introduction

The genus *Diaporthe* Nitschke (anamorph *Phomopsis* (Sacc.) Bubák) includes phytopathologically important taxa with wide host ranges and geographic distributions (Uecker 1988; Crous and Groenewald 2005; Rossman et al. 2007). *Diaporthe* species have also been reported as endophytes in healthy leaves and stems, saprobes on decaying wood and leaf litter, and even parasites in humans and other mammals (van Warmelo et al. 1970; Sutton et al. 1999; Garcia-Reyne et al. 2011; Iriart et al. 2011; Botella & Diez 2011; Sun et al. 2011; Rocha et al. 2011). The host specificity and geographic distributions of most phyopathogenic species of *Diaporthe* are unknown, hindering the international exchange of agricultural commodities (Udayanga et al. 2011; Cowley et al. 2012; Sun et al. 2012). Studies on phytopathogenic *Diaporthe* species are therefore particularly important to plant pathologists working on wide range of crop diseases (e.g. grapes, sunflower, soybean and various diseases associated with fruit and ornamental trees). DNA sequence comparisons have made it possible to reliably connect sexual and asexual states of the species of pleomorphic genus *Diaporthe*. Being the older name, *Diaporthe* has priority over *Phomopsis* and should be the generic name adopted for these taxa in future studies (Santos et al. 2010,

Cryptogamie, Mycologie, 2012, 33 (3) Numéro spécial Coelomycetes*: 295-309*

Multi-locus phylogeny reveals three new species of *Diaporthe* from Thailand

Dhanushka UDAYANGA[a,b,c], Xingzhong LIU[a], Eric H.C. MCKENZIE[d], Ekachai CHUKEATIROTE[b,c] & Kevin D. HYDE[b,c]*

[a]Institute of Excellence in Fungal Research, [b]School of Science,57100, Mae Fah Luang University, Chiang Rai, Thailand; email: kdhyde3@gmail.com

[c]State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, No 3 1stWest Beichen Road, Chaoyang District, Beijing 100101, P.R. China; email: liuxz@im.ac.cn.

[d]Landcare Research, Private Bag 92170, Auckland, New Zealand

Abstract – Species of *Diaporthe* are important phytopathogens with wide host ranges and a global distribution. In the present study multi-locus phylogeny based on combined sequences of rDNA ITS, and partial sequences from the translation elongation factor 1-α (EF 1-α), β tubulin (TUB) and calmodulin (CAL) genes, reveal three new species from fresh collections made in northern Thailand. The new species *Diaporthe siamensis*, *D. thunbergii* and *D. pterocarpicola* are introduced in this paper with full descriptions and comparison with similar taxa. *Phomopsis pterocarpi* which is epitypified and synonymised under *Diaporthe pterocarpi* is described based on a collection from northern Thailand.

Endophyte / Host diversity / New combination / *Phomopsis* / Phytopathogen / Taxonomy

INTRODUCTION

Diaporthe Nitschke (asexual state *Phomopsis* (Sacc.) Bubák) comprises important phytopathogens, often with wide host ranges and distributions (Uecker, 1988; Rossman *et al*., 2007; Crous, 2005; Udayanga *et al*., 2011, 2012, Cowley *et al*., 2012). Species recognition criteria in *Diaporthe* have historically been based on morphology, culture characteristics and host affiliation (Rehner and Uecker, 1994; Mostert *et al*., 2001; van Niekerk *et al*., 2005; Murali *et al*., 2006; Santos & Phillips, 2009). The current state of taxonomic knowledge of *Diaporthe* effectively means that strains can be identified to species level only if molecular techniques are employed (Santos *et al*., 2010). Udayanga *et al*. (2012) provided a multi-locus phylogenetic study of *Diaporthe* using combined sequences of rDNA ITS, and partial sequences of EF1-α, TUB and CAL genes.

We are restudying and carrying out inventories of the plant pathogens of northern Thailand and China based on morphology and molecular sequence analysis (KoKo *et al.*, 2011) and are discovering many new cryptic species in

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What are the common anthracnose pathogens of tropical fruits?

Dhanushka Udayanga · Dimuthu S. Manamgoda · Xingzhong Liu · Ekachai Chukeatirote · Kevin D. Hyde

Received: 16 April 2013 / Accepted: 7 July 2013

Abstract Species of *Colletotrichum* are associated with anthracnose of a wide range of host plants including cultivated and wild tropical fruits. The genetic and ecological diversity of species associated with wild fruits are poorly explored, as compared to those associated with pre and postharvest diseases of cultivated fruits. In the present study, isolates of *Colletotrichum* were obtained from commercially available cultivated fruits, wild fruits (from native trees in natural habitats) and a few herbaceous hosts collected in northern Thailand. These isolates were initially characterized based on analysis of complete sequences of nuclear ribosomal internal transcribed spacer (ITS), into the genetically defined species complexes of *Colletotrichum gloeosporioides*, *C. acutatum*, *C. boninense* and *C. truncatum*. The isolates were primarily identified in the *C. gloeosporioides* species complex, based on a strongly supported clade within the ITS gene tree and were further characterized using multi-gene phylogenetic analyses and morphology. Phylogenetic analyses of ITS, partial sequences of actin (ACT), calmodulin (CAL), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamine synthetase (GS) and β-tubulin (TUB2) genetic markers were performed individually and in combination. *Colletotrichum gloeosporioides sensu stricto* was identified from lime (*Citrus aurantifolia*) and rose apple (*Syzygium samarangense*). *Colletotrichum fructicola* was isolated from dragon fruit (*Hylocerous undatus*) and jujube (*Ziziphus* sp.). *Colletotrichum endophytica* was found only from an unknown wild fruit. We observed a considerable genetic and host diversity of species occurring on tropical fruits within the clade previously known as *Colletotrichum siamense sensu lato*. The clade consists of isolates identified as pre and postharvest pathogens on a wide range of fruits, including coffee (*Coffea arabica*), custard apple (*Annona reticulata*), *Cerbera* sp., figs (*Ficus racemosa*) mango (*Mangifera indica*), neem (*Azadirachta indica*) and papaya (*Carica papaya*) and was the dominant group of species among most wild fruits studied. With the exception of one isolate from banana, which grouped in the *C. siamense* clade, all the other isolates were identified as *Colletotrichum musae*. A new species, *Colletotrichum syzygicola*, associated with *Syzygium samarangense* in Thailand, is introduced with descriptions and illustrations. This study highlights the need to re-assess the evolutionary relationships of *Colletotrichum* species occurring on cultivated and wild fruits with emphasis on their ecology and cryptic diversification including sampling at regional and global scales.

Keywords *Colletotrichum gloeosporioides* · Multi-gene phylogeny · Postharvest diseases · Quarantine · Systematics · Species complex · Tropical Asia · Wild fruits

D. Udayanga · D. S. Manamgoda · X. Liu (✉)
State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, No 3 1st West Beichen Road, Chaoyang District, Beijing 100101, People's Republic of China
e-mail: liuxz@im.ac.cn

D. Udayanga · D. S. Manamgoda · E. Chukeatirote · K. D. Hyde
Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand

D. Udayanga · D. S. Manamgoda · E. Chukeatirote · K. D. Hyde
School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand

Introduction

Colletotrichum Corda, is an important plant pathogenic genus causing anthracnose of a wide range of fruits, vegetables, cereals, grasses and ornamental plants in tropical and temperate regions (Mills et al. 1992; Johnston and Jones 1997; Freeman et al. 2001; Chung et al. 2006; Yang et al. 2009; Rojas et al. 2010). Fruit production is mostly affected in both high-value crops and wild fruits in natural habitats. However,

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RESEARCH ARTICLE

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Species limits in *Diaporthe*: molecular re-assessment of *D. citri*, *D. cytosporella*, *D. foeniculina* and *D. rudis*

D. Udayanga[1,2,3], L.A. Castlebury[1], A.Y. Rossman[1], K.D. Hyde[2,3]

Key words

epitypification
genealogical sorting index
melanose
multi-gene phylogeny
new primers
Phomopsis
species recognition
stem end rot
systematics

Abstract Species of *Diaporthe* are important plant pathogens of a wide range of hosts worldwide. In the present study the species causing melanose and stem end rot diseases of *Citrus* spp. are revised. Three species of *Diaporthe* occurring on *Citrus* are characterised, including *D. citri*, *D. cytosporella* and *D. foeniculina*. Morphology and phylogenetic analyses of the complete nuclear ribosomal internal transcribed spacer regions and partial sequences of actin, beta-tubulin, calmodulin and translation elongation factor 1-α were used to resolve species on *Citrus* and related *Diaporthe* species. *Diaporthe citri* occurs on *Citrus* throughout the *Citrus*-growing regions of the world. *Diaporthe cytosporella* is found on *Citrus* in Europe and California (USA). *Diaporthe foeniculina*, including the synonym *D. neotheicola*, is recognised as a species with an extensive host range including *Citrus*. *Diaporthe medusaea*, a name widely used for *D. citri*, was determined to be a synonym of *D. rudis*, a species with a broad host range. *Diaporthe citri* is delimited based on molecular phylogenetic analysis with the inclusion of the conserved ex-type and additional collections from different geographic locations worldwide. *Diaporthe cytosporella*, *D. foeniculina* and *D. rudis* are epitypified, fully described and illustrated with a review of all synonyms based on molecular data and morphological studies. Newly designed primers are introduced to optimise the amplification and sequencing of calmodulin and actin genes in *Diaporthe*. A discussion is provided of the utility of genes and the need for multi-gene phylogenies when distinguishing species of *Diaporthe* or describing new species.

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INTRODUCTION

The genus *Diaporthe* is an economically important group of plant pathogenic fungi causing diseases on a wide range of crops, ornamentals and forest trees (Farr et al. 2002a, b, Crous 2005, Udayanga et al. 2011). Accurate species identification is vital for controlling the diseases caused by these fungi as well as for implementing quarantine regulations (Rossman & Palm-Hernández 2008, Cai et al. 2011, Shivas & Cai 2012). Until recently, species of *Diaporthe* have been defined based on morphology and host association. However, patterns of host association and speciation have yet to be fully understood within *Diaporthe*. Multiple species of *Diaporthe* can often be found on a single host and a single species of *Diaporthe* can be associated with many different hosts (Crous 2005, van Niekerk et al. 2005, Santos & Phillips 2009, Diogo et al. 2010, Gomes et al. 2013). Using molecular data, much progress has been made towards identifying and characterising emerging pathogens, prevalent endophytes and saprobes in the genus *Diaporthe* (Santos & Phillips 2009, Diogo et al. 2010, Luongo et al. 2011, Udayanga et al. 2012a, b, Thomidis et al. 2013).

Modern systematic accounts of *Diaporthe* have used DNA sequence data as the most accurate means to circumscribe species within this genus (Rehner & Uecker 1994, Castlebury et al. 2003, van Rensburg et al. 2006). Markers used in contemporary phylogenetic revisions include the complete nuclear ribosomal internal transcribed spacer regions (ITS) and more recently partial sequences of actin (ACT), beta-tubulin (TUB), calmodulin (CAL), histone H3 (HIS), mating type genes (MAT 1-1-1 and MAT 1-2-1) and translation elongation factor 1-alpha (EF1-α) (van Niekerk et al. 2005, Diogo et al. 2010, Santos et al. 2010, Udayanga et al. 2012a, b, Gomes et al. 2013). Multi-gene phylogenetic species delineation has become the most effective tool for taxonomic studies of fungi compared to traditional mating experiments and morphology (Taylor et al. 2000, Dettman et al. 2003). Although the ITS region is often useful for identification of *Diaporthe* species, multi-gene phylogenetic analyses are required for accurate reconstruction of species boundaries and relationships (Udayanga et al. 2012a, Gomes et al. 2013). Intraspecific variation observed in ITS sequences in several species of *Diaporthe* can cause confusion in species recognition when used alone (Farr et al. 2002a, b, Santos et al. 2010).

Diaporthe citri is a pathogen that causes melanose and stem end rot disease of *Citrus* spp. throughout the world (Whiteside & Timmer 2000a, Mondal et al. 2007). Melanose disease can affect young leaves and fruits of different species and varieties of *Citrus* causing black blemishes on fruit rind and small, black, raised lesions often surrounded by yellow necrotic halos (Timmer & Kucharek 2001). Symptoms of the disease may vary with host variety, geographic location, seasonal occurrence, ecophysiological factors and severity of infection (Timmer & Fucik 1976, Whiteside 1977, Kucharek et al. 1983). The range of symptoms varies from small spots, scab lesions and mudcake to star melanose on different tissues of *Citrus* spp. (Timmer 2000, Whiteside & Timmer 2000a, Agostini et al. 2003). Perithecia and pycnidia are only produced on dead and dying twigs and on fruit affected by stem end rot. Because perithecia are rarely formed, conidia produced by pycnidia are the primary source of inoculum (Bach & Wolf 1928, Kuhara 1999).

[1] *Systematic Mycology and Microbiology Laboratory, United States Department of Agriculture, Agricultural Research Service, Beltsville, MD, 20705 USA; corresponding author e-mail: Lisa.Castlebury@ars.usda.gov.
[2] Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand.
[3] School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand.

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Insights into the genus *Diaporthe*: phylogenetic species delimitation in the *D. eres* species complex

Dhanushka Udayanga · Lisa A. Castlebury · Amy Y. Rossman · Ekachai Chukeatirote · Kevin D. Hyde

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Abstract The genus *Diaporthe* comprises pathogenic, endophytic and saprobic species with both temperate and tropical distributions. Cryptic diversification, phenotypic plasticity and extensive host associations have long complicated accurate identifications of species in this genus. The delimitation of the generic type species *Diaporthe eres* has been uncertain due to the lack of ex-type cultures. Species limits of *D. eres* and closely related species were evaluated using molecular phylogenetic analysis of eight genes including nuclear ribosomal internal transcribed spacer (ITS), partial sequences of actin (ACT), DNA-lyase (Apn2), translation elongation factor 1- α (EF1-α), beta-tubulin (TUB), calmodulin (CAL), 60s ribosomal protein L37 (FG1093) and histone-3 (HIS). The occurrence of sequence heterogeneity of ITS within *D. eres* is observed, which complicates the analysis and may lead to overestimation of the species diversity. The strict criteria of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) were applied to resolve species boundaries based on individual and combined analyses of other seven genes except the ITS. We accept nine distinct phylogenetic species including *Diaporthe alleghaniensis*, *D. alnea*, *D. bicincta*, *D. celastrina*, *D. eres*, *D. helicis*, *D. neilliae*, *D. pulla* and *D. vaccinii*. Epitypes are designated for *D. alnea*, *D. bicincta*, *D. celastrina*, *D. eres*, *D. helicis* and *D. pulla*. Modern descriptions and illustrations are provided for these species. Newly designed primers are introduced to amplify and sequence the Apn2 (DNA- lyase) gene in *Diaporthe*. Based on phylogenetic informativeness profiles, EF1-α, Apn2 and HIS genes are recognised as the best markers for defining species in the *D. eres* complex.

Keywords Diaporthaceae · DNA-lyase · Epitypification · Gene discordance · Generic type · Molecular phylogeny · New primers · Phylogenetic informativeness · Speciation

Electronic supplementary material The online version of this article (doi:10.1007/s13225-014-0297-2) contains supplementary material, which is available to authorized users.

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D. Udayanga · K. D. Hyde (✉)
Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China
e-mail: kdhyde3@gmail.com

D. Udayanga · K. D. Hyde
World Agroforestry Centre, East and Central Asia, Kunming 650201, China

D. Udayanga · E. Chukeatirote · K. D. Hyde
Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand

D. Udayanga · E. Chukeatirote · K. D. Hyde
School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand

D. Udayanga · L. A. Castlebury · A. Y. Rossman
Systematic Mycology and Microbiology Laboratory, United States Department of Agriculture- Agricultural Research Service, Beltsville, MD 20705, USA

Introduction

In the last two decades much progress has been made in the ability to define fungal species through the use of molecular data (Hibbett and Taylor 2013; Hyde et al. 2013). Circumscribing species within cryptic species complexes that have complicated life histories is essential for determining patterns of speciation and potential hyperdiversity within a genus (Bickford et al. 2007; Silva et al. 2012a; Fekete et al. 2012; O'Donnell et al. 2013). Genealogical Concordance Phylogenetic Species Recognition (GCPSR) as an approach

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www.sbfito.com.br

REVIEW / *REVISÃO*

The need to carry out re-inventory of plant pathogenic fungi

Lei Cai[1], Dhanushka Udayanga[1,2], Dimuthu S. Manamgoda[1,2], Sajeewa S.N. Maharachchikumbura[1,2], Eric H.C. McKenzie[3], Liang Dong Guo[1], Xing Zhong Liu[1], Ali Bahkali[4] & Kevin D. Hyde[2,4]

[1]Key Laboratory of Systematic Mycology & Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100190, P.R. China; [2]School of Science, Mae Fah Luang University, Thasud, Chiang Rai 57100, Thailand; [3]Landcare Research, Private Bag 92170, Auckland, New Zealand; [4]King Saud University, College of Science, Botany and Microbiology Department, P.O. Box: 2455, Riyadh 1145, Saudi Arabia

Author for correspondence: Lei Cai, e-mail: mrcailei@gmail.com

OUTLINE

Plant pathogenic fungi have long been documented through concerted efforts of mycologists and plant pathologists; these records have served as the basis for regional and countrywide checklists which have since been put into databases listing hosts and associated fungi. They are used by governments and scientists to formulate trade quarantine policies and determine research funding, such as in plant breeding programs and disease control. With the ability to use molecular characters to study the systematics of fungi it is clear that morphologically defined species are often large complexes comprised of genetically and biologically distinct species. Use of molecular techniques to examine species complexes has revealed cryptic species in many important plant pathogenic genera, e.g. *Botryosphaeria*, *Colletotrichum*, *Fusarium*, and *Mycosphaerella*. It has occurred to such an extent that existing checklists and databases need updating. It is important that the data from these studies, including changes in taxonomy and nomenclature, be incorporated into the databases of plant pathogenic fungi to support accurate plant quarantine decisions. In addition, epitypifying fungi by re-collecting material from type habitats and isolating the organism into pure culture will provide essential materials for systematics studies to further clarify the taxonomy and phylogeny of plant pathogenic fungi. Overall, we conclude that disease lists are likely to be highly outdated and advocate the need for countrywide re-inventory of plant pathogens. As a result of these studies, tools can be developed that use morphological or molecular characters, or both, to promote accurate identification of plant pathogenic fungi.

Key words: *Cochliobolus, Colletotrichum*, *Diaporthe, Fusarium, Phomopsis,* cryptic species, disease associated fungi, quarantine.

Introduction

There have been worldwide concerted efforts by generations of mycologists and plant pathologists to document plant disease-associated fungi (Roger, 1951-1954; Holliday, 1980; Kohler et al., 1996), although the disease causal agents are probably better known in temperate than tropical regions (Hofmann et al., 2010). Surveys of plant pathogens have resulted in the publication of numerous checklists (Peregrine & Kassim, 1982; Dingley et al., 1981; McKenzie & Jackson, 1986; Hyde & Alcorn, 1993) and more recently many of these have been incorporated into databases such as: USDA, http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm; Database of Plant Disease Names in Japan, www.gene.affrc.go.jp/databases-micro_pl_diseases_en.php; New Zealand Fungi, http://nzfungi.landcareresearch.co.nz; Fungos relatados em plantas no Brasil - http://pragawall.cenargen.embrapa.br/aiqweb/michtml/micbanco01a.asp. These lists of plant pathogenic fungi have been extremely important in formulating quarantine policies and thus have an immense impact on trade and global biosecurity.

Traditionally, researchers primarily relied on morphology to identify the plant-associated fungi. Usually surveys would be carried out, disease material collected, dried and returned to the laboratory where the associated fungi were later identified (Hyde & Alcorn, 1993). Molecular data have rapidly advanced the understanding of species boundaries and relationships in several important plant pathogenic genera, revealing numerous cryptic species (Cai et al., 2009; Kvas et al., 2009; Summerell et al., 2010). Databases usually continue to cite fungal taxa recorded in dated publications, but as our knowledge of disease-associated fungi has increased, the checklists and databases have become more and more inaccurate. This has occurred to such an extent that existing checklists (e.g. Anonymous, 1960; Guba, 1961; Raabe, 1966) and databases need updating. It is therefore imperative to re-collect many of these pathogens in species complexes and re-identify them based on modern protocols and updated species concepts. For example, Phoulivong et al. (2010a) have demonstrated that the previously believed axiom that "most of the fruit rot diseases in tropics are caused by *Colletotrichum gloeosporioides*" was wrong. Mycologists therefore need to re-identify all these diseases and give them names that reflect their natural relationships.

In a recent paper Hyde et al. (2010) were of the opinion that plant pathologists and mycologists should re-inventory Australian plant pathogens in order to make quarantine measures more effective. The purpose of this

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(2141) Proposal to conserve the name *Phomopsis citri* H.S. Fawc. (*Diaporthe citri*), with a conserved type, against *Phomopsis citri* (Sacc.) Traverso & Spessa (*Ascomycota*: *Diaporthales*: *Diaporthaceae*)

Amy Rossman,[1] Dhanushka Udayanga,[1,2] Lisa A. Castlebury[1] & Kevin D. Hyde[2]

1 *Systematic Mycology and Microbiology Laboratory, United States Department of Agriculture – Agriculture Research Services, Beltsville, Maryland 20705 U.S.A.*

2 *Institute of Excellence in Fungal Research, School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand*

Author for correspondence: *Amy Rossman, Amy.Rossman@ars.usda.gov*

(2141) ***Phomopsis citri*** H.S. Fawc. in Phytopathology 2: 109. Jun 1912, nom. cons. prop.

Typus: United States, Florida, Lake Alfred, Ana, "On twigs of *Citrus* sp.", 26 Apr 2000, *Timmer* (BPI No. 892456 [dried culture of AR 3405]), typ. cons. prop.

(H) *Phomopsis citri* (Sacc.) Traverso & Spessa in Bol. Soc. Brot. 25: 123. 1910 (*Phoma citri* Sacc. in Nuovo Giorn. Bot. Ital. 8: 200. Aug 1876).

Lectotypus (hic designatus): Italy, Treviso, a Vittorio, "on branch of *Citrus limon*", "*Diplodia citri*", Oct 1875, *Saccardo* in Mycotheca Veneto No. 332 (FH).

The name *Diaporthe citri* applies to a fungus that causes a disease on *Citrus* known as melanose or stem end rot of mature fruit after harvest and occurs widely in North America and Asia (Mondal & al. in Pl. Dis. 91: 387–392. 2007; Fischer & al. in Sci. Agric. (Piracicaba) 66: 210–217. 2009). Initially described as the illegitimate *Phomopsis citri* H.S. Fawc. 1912, non *P. citri* (Sacc.) Traverso & Spessa 1910 (≡ *Phoma citri* Sacc. 1876), the Fawcett name was placed in *Diaporthe* by Wolf (in J. Agric. Res. 33: 625. 1926); thus, as *Diaporthe citri* F.A. Wolf, the name currently has priority only from 1926.

Plant pathologists have referred to this fungus as *Diaporthe citri* or *Phomopsis citri* for the past one hundred years (Timmer & Kucharek, Melanose, Plant Pathology Factsheet PP-150, Inst. Food Agric. Sci. Univ. Florida, 2001: http://edis.ifas.ufl.edu/pdffiles/CH/CH01900.pdf; Nozaki & al. in Fitopatol. Brasil. 29: 429–432. 2004; Mondal, l.c.; Fischer, l.c.). The genus *Phomopsis* Sacc. & Roum. (1884) is a synonym of *Diaporthe* Nitschke (1870). In addition the name *Diaporthe medusaea* Nitschke (Pyrenomyc. Germ. 2: 251. 1870) has also been used for this disease following Wehmeyer (in Univ. Michigan Stud., Sci. Ser. 9: 1–349. 1933) who regarded *D. citri* as a synonym of *D. medusaea*.

Recent research has clarified the relationship of *D. citri* with *D. medusaea* and these two species are now considered distinct (Udayanga & al. in Persoonia [accepted]). In the course of that research two earlier names were discovered for *D. citri*, namely *Diaporthe citrincola* Rehm (in Leafl. Philipp. Bot. 6: 2269. 1914) and *Phomopsis caribaea* W.T. Horne (in Phytopathology 12: 417. 1922). Neither of these two names has been used to any extent. Because the name *Diaporthe citri* has been applied to melanose and stem rot disease of *Citrus* for decades and Fawcett (l.c.) explicitly stated that he was unaware of any previous *Diaporthe* or *Phomopsis* on *Citrus*, it is proposed that *Phomopsis citri* H.S. Fawc. be conserved against its earlier homonym *P. citri* (Sacc.) Traverso & Spessa. This will make the former name available as a basionym for *Diaporthe citri* (H.S. Fawc.) F.A. Wolf, giving this name priority over what would otherwise be the earlier taxonomic synonyms *D. citrincola* and *Phomopsis caribaea*, both of which predate the currently first legitimate publication as *Diaporthe citri* F.A. Wolf.

As no type material of *P. citri* was found at either BPI or FLAS, leaving only an illustration (Fawcett, l.c.) as a potential, but unsatisfactory, iconotype, a recent specimen from diseased *Citrus* sp. is proposed as a conserved type for *Phomopsis citri*.

(2304) Proposal to conserve the name *Diaporthe eres* against twenty-one competing names (*Ascomycota*: *Diaporthales*: *Diaporthaceae*)

Amy Rossman,[1] Dhanushka Udayanga,[1,2] Lisa A. Castlebury[1] & Kevin D. Hyde[2]

1 *Systematic Mycology and Microbiology Laboratory, United States Department of Agriculture – Agricultural Research Service, Beltsville, Maryland 20705, U.S.A.*

2 *Institute of Excellence in Fungal Research, School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand*

Author for correspondence: *Amy Rossman, Amy.Rossman@ARS.USDA.GOV*

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(2304) ***Diaporthe eres*** Nitschke, Pyrenomyc. Germ.: 245. 1870, nom. cons. prop.
Lectotypus (hic designatus): Germany, Nordrhein-Westfalen, Münsterland, Botanischer Garten Münster. On twigs of *Ulmus* sp., Jun 1865, *Nitschke* ex Herb. Münster (B barcode B 70 0009145; isolectotypi: B barcodes B 70 0009146 & B 70 0009147).

(=) *Sphaeria velata* Pers., Syn. Meth. Fung. 1: 32. 31 Dec 1801.
Lectotypus (hic designatus): on *Tilia*, ex Herb. Persoon; Shear Study Coll. Types & Rarities Ser. 1 (BPI No. 801267).

(=) *Phoma spiraeae* Desm. in Mém. Soc. Roy. Sci. Lille 1830: 13. 1830.
Lectotypus (hic designatus): France, on dead stems of *Spiraea aruncus*, Desmazières, Pl. Crypt. France, Ser. I: No. 481 (BPI[bound]).

(=) *Sphaeria tenella* Schwein. in Trans. Amer. Philos. Soc., ser. 2, 4: 218. 1832.
Lectotypus (hic designatus): U.S.A., Pennsylvania, Bethlehem, on *Hibiscus*, ex Herb. Schweinitz No. 1658, Shear Study Coll. Types & Rarities Ser. 1 (BPI No. 801136).

(=) *Sphaeria concresens* Schwein. in Trans. Amer. Philos. Soc., ser. 2, 4: 198. 1832.
Lectotypus (hic designatus): U.S.A., Pennsylvania, Bethlehem, on *Ribes aureum*, ex Herb. Schweinitz, Shear Study Coll. Types & Rarities Ser. 1 (BPI No. 799375)

(=) *Sphaeria discutiens* Berk. in Smith, Engl. Fl., ed. 2, 5(2): 245. 1836.
Lectotypus (vide Wehmeyer in Univ. Michigan Stud., Sci. Ser. 9: 99. 1933), on *Ulmus*, near Edinburgh, *Greville* (K).

(=) *Sphaeria controversa* Desm. in Ann. Sci. Nat. Bot., ser. 2, 17: 102. 1842.
Lectotypus (hic designatus): France, Caen, on *Fraxinus*, *Roberge*, ex Herb. Michener, Shear Study Coll. Types & Rarities Ser. 1 (BPI No. 799411).

(=) *Sphaeropsis depressa* Lév. in Ann. Sci. Nat. Bot., ser. 3, 5: 295. 1846.
Lectotypus (hic designatus): France, Arduennes, on *Syringa*, Shear Study Coll. Types & Rarities Ser. 1 (BPI No. 799557).

(=) *Sphaeria conorum* Desm. in Ann. Sci. Nat. Bot., ser. 3, 6: 76. 1846.

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Lectotypus (hic designatus): France, Caen, on *Pinus sylvestris*, *Roberge*, ex Herb. Michener, Shear Study Coll. Types & Rarieties Ser. 1 (BPI No. 799399).

(=) *Cytospora buxi* Desm. in Ann. Sci. Nat. Bot., ser. 3, 10: 355. 1848.
Lectotypus (hic designatus): France, Normandy, near Caen, on branches of *Buxus sempervirens*, *Roberge* (BPI No. 364561).

(=) *Phoma occulta* Desm. in Ann. Sci. Nat. Bot., ser. 3, 11: 283. 1849.
Lectotypus (hic designatus): France, inside the dry stems of *Phragmites australis* (as *Arundo phragmites*), autumn, Desmazières, Pl. Crypt. France, Ser. I: No. 1868 (BPI[bound]).

(=) *Diatype verrucella* Fr., Summa Veg. Scand.: 385. 1849.
Lectotypus (vide Wehmeyer in Univ. Michigan Stud., Sci. Ser. 9: 66. 1933): on *Alnus incana*, ex Herb. Fries (UPS).

(=) *Phoma oblonga* Desm. in Ann. Sci. Nat. Bot., ser. 3, 20: 218. 1853.
Lectotypus (hic designatus): on dried branch of *Ulmus*, Desmazières, Pl. Crypt. France, Ser. 2: No. 60 (BPI[bound]).

(=) *Phoma coronillae* Westend., Pl. Crypt. Exs. 19–20: No. 966. 1857.
Typus: non designatus.

(=) *Sphaeria nucleata* Curr. in Trans. Linn. Soc. London 22: 270. 1858.
Holotypus: Weybridge, on *Ulex* "furze", Jan 1856, ex Herb. Currey (K).

(=) *Sphaeria badhamii* Curr. in Trans. Linn. Soc. London 22: 270. 1858.
Lectotypus (vide Wehmeyer in Univ. Michigan Stud., Sci. Ser. 9: 100. 1933): Dec 1854 (K).

(=) *Sphaeria quadrinucleata* Curr. in Trans. Linn. Soc. London 22: 325. 1859.
Lectotypus (vide Wehmeyer in Univ. Michigan Stud., Sci. Ser. 9: 100. 1933): Weybridge, Surrey, on stick, 7 Sep 1856, ex Herb. Currey (K).

(=) *Sphaeria ciliaris* Curr. in Quart. J. Microscop. Sci. 7: 231. 1859.
Holotypus: Weybridge, on *Fraxinus*, Oct 1857, ex Herb. Currey (K).

(=) *Diatrype incarcerata* Berk. & Broome, Ann. Mag. Nat. Hist., ser. 3, 3: 365. 1859.
Lectotypus (vide Wehmeyer in Univ. Michigan Stud., Sci. Ser. 9: 94. 1933): on stems of *Rosa*, ex Herb. Berkeley (K).

(=) *Sphaeria ryckholtii* Westend. in Bull. Acad. Roy. Sci. Belgique, ser. 2, 7: 82. 1859.
Lectotypus (hic designatus): Belgium, Termonde, on branches of *Symphoricarpos racemosa*, in the garden of Mme van Landeghem (GENT).

(=) *Sphaeria landeghemiae* Westend. in Bull. Acad. Roy. Sci. Belgique, ser. 2, 7: 83. 1859.
Lectotypus (hic designatus): Termonde, on dead branches of *Philadelphus coronarius*, in the garden of Mme van Landeghem-Anne (BR).

(=) *Valsa occulta* Fuckel, Fungi Rhenani Exs.: No. 622. 1863.
Lectotypus (hic designatus): Germany, Hessen, in Hostrich forest, on *Pinus abies*, summer (FH).

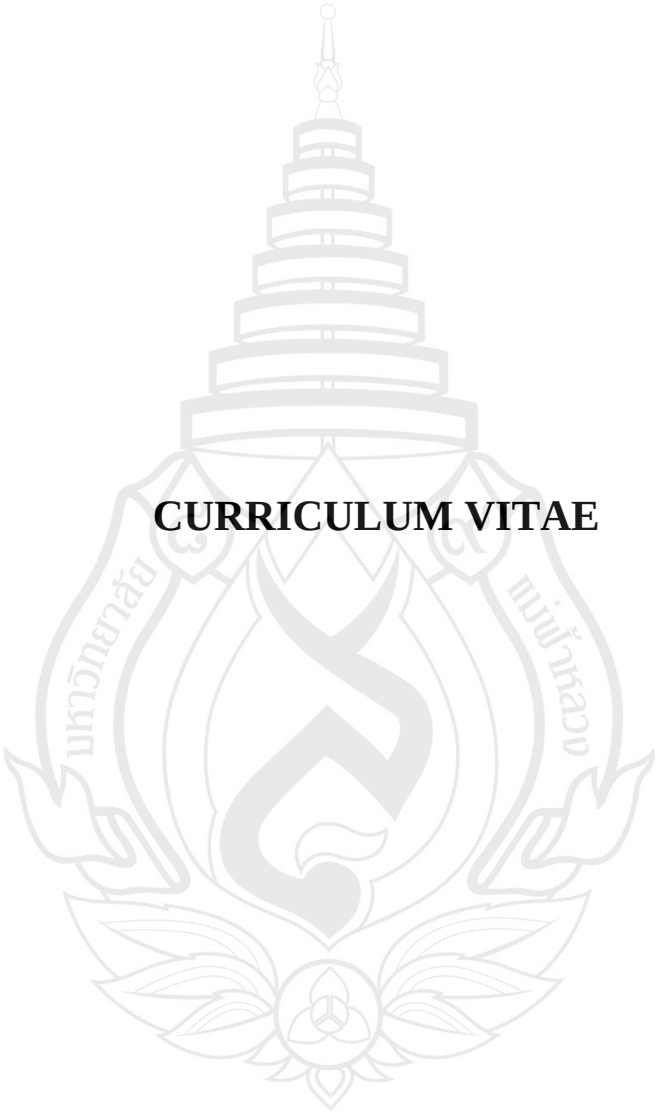
With the change to one scientific name for pleomorphic fungi in the *Melbourne Code* (McNeill & al. in Regnum Veg. 154. 2012) based on relative priority, *Diaporthe* Nitschke (Pyrenomyc. Germ.: 245. 1870) represents the generic name that is older than the synonym *Phomopsis* (Sacc.) Bubak (in Osterr. Bot. Z. 55: 78. 1905), nom. cons. At present *Diaporthe* includes over 800 names while the number of names described in *Phomopsis* exceeds 1000, thus merging these two genera is a significant task. Given the almost equal numbers of names in each genus and equal use of each generic name, recent workers have decided it is preferable to allow priority to operate, thus retaining *Diaporthe* and regarding *Phomopsis* as a synonym (Gomes & al. in Persoonia 31: 1. 2013; Udayanga & al. in Persoonia 31: 83. 2014).

Until recently species in these genera were assumed to be host-specific, thus when a fungus was encountered on a host for which a name did not exist, a new name was described. Starting with Rehner & Uecker (in Canad. J. Bot. 72: 1666. 1994) in which molecular sequence data were used to define species, it is now known that species of *Diaporthe* are not host-specific; i.e., one species may occur on many different host genera; similarly one host may harbor several different species of *Diaporthe*. The definition of species within the genus *Diaporthe* is still evolving with a trend toward use of multigene phylogenies (Gomes & al., l.c.; Udayanga & al., l.c.). However, the application of existing names in *Diaporthe* and *Phomopsis* to species clades has proven difficult because of the lack of morphological characteristics. In order to maintain stability, it seems expedient to maintain commonly used names such as those in Wehmeyer (in Univ. Michigan Stud., Sci. Ser. 9: 1–349. 1933) rather than attempt to define the many names that existed prior to that publication.

The type of *Diaporthe* has been accepted as originally designated by Nitschke (l.c.: 245–246) as *D. eres* by both Clements & Shear (Genera Fung.: 264. 1931) and Wehmeyer (l.c.: 64) along with many others (Brayford in Mycol. Res. 94: 691. 1990; Castlebury & al. in Mycologia 94: 1017. 2002, in Mycoscience 44: 203. 2003; Rossman & al. in Mycoscience 48: 135. 2007; Gomes & al., l.c.; AFTOL ID: 935). The asexual state of *D. eres* has been known by the older name *Phomopsis oblonga* (Desm.) Traverso (Fl. Ital. Crypt. 1: 248. 1906), based on *Phoma oblonga* Desm. (Ann. Sci. Nat., Bot., sér. 3, 20: 6. 1853), since Wehmeyer (l.c.: 99). This name also provides an earlier epithet for *D. eres*. Under either the sexual or asexual state name, *Diaporthe eres* is known to cause diseases of trees such as butternut and woody crop plants such as grapes and peaches (Anagnostakis in Pl. Dis. 91: 1198. 2007; Thomidis & Michailides in Pl. Dis. 93: 1293. 2009; Baumgartner & al. in Pl. Dis. 97: 912. 2013).

In the only monograph of *Diaporthe* in existence published by Wehmeyer (l.c.), *D. eres* is included with synonymous names listed under each host of which twenty-one were published prior to 1870. In addition, a number of names published prior to 1870 are listed as synonyms of *D. eres* in *Species Fungorum* (http://www.speciesfungorum.org/). All of these names are absent from the recent literature. It is not known for certain if these names are indeed synonyms of *D. eres* and it would be difficult to determine this. No living type cultures exist for any of these names.

Given the widespread acceptance of the name *D. eres* for the type of *Diaporthe* and the need to circumscribe the species in *Diaporthe* using the type as a reference point, the conservation of *D. eres* over the twenty-one older names listed above is proposed.



CURRICULUM VITAE

CURRICULUM VITAE

NAME Mr. Dhanushka Udayanga

DATE OF BIRTH 26th October 1984

ADDRESS E 16/1,
Makura, Hettimulla, Kegalle,
Sri Lanka, 71210.

EDUCATIONAL BACKGROUND

2009	B.Sc. (Molecular Biology and Biotechnology) Faculty of Science, University of Peradeniya, Sri Lanka, Second class upper division honors (GPA 3.64/4.00)

PROFESSIONAL EXPERIENCE

2009	Temporary Instructor, Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Sri Lanka
2009	Temporary Instructor, Postgraduate Institute of Science, Faculty of Science, University of Peradeniya, Sri Lanka
2010-2014	Postgraduate Research student, Mae Fah Luang University, Chiang Rai, Thailand
2010- 2011	Visiting Research Student, State Key Lab of Mycology, Chinese Academy of Sciences, Beijing, China

2013-2013	Visiting Research Student: Systematic Mycology and Microbiology Laboratory USDA-ARS, Beltsville, MD, USA
2011-present	Ad-Hoc reviewer of Fungal Diversity, Fungal Biology, Journal of Crop Protection, Sydowia, Persoonia, European Journal of Plant Pathology, Phytotaxa, Plant Disease and several other international journals in Mycology

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ABSTRACTS AND PROCEEDINGS

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