

**Molecular studies on the taxonomy, host-associations  
and viruses of the *Diplodia*-like anamorphs of the  
Botryosphaeriaceae**

By

**Juanita de Wet**

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Department of Microbiology and Plant Pathology  
University of Pretoria  
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**Supervisor: Prof. M.J. Wingfield**  
**Co-supervisors: Prof. B.D. Wingfield**  
**Dr. O. Preisig**



## DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Philosophiae Doctor (PhD) to the University of Pretoria contains my own independent work and has hitherto not been submitted for any degree at any other University.

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Juanita de Wet

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## PREFACE

The Botryosphaeriaceae represents a family of fungi that includes important pathogens of agricultural and forestry crops. *Diplodia pinea* is one of the best-known members of this family causing serious die-back and canker diseases mainly on *Pinus* spp. but also other conifers. The *D. pinea* species complex includes three morphologically similar forms that have been referred to as the A, B and C morphotypes of the fungus. Management of *Diplodia*-associated diseases is difficult as various biotic and abiotic factors influence the initiation and severity of the diseases. Hypovirulence (attenuation of virulence)-inducing dsRNA elements could provide an alternative mode of control against this fungus in a manner that has been shown for the chestnut blight fungus, *Cryphonectria parasitica*. An overall theme during studies conducted as part of this dissertation was to consider the taxonomy, host associations and viruses in isolates of the *D. pinea* species complex. Associated with this objective, I also considered the phylogenetic relationships of this fungus with other *Diplodia*-like anamorphs of the Botryosphaeriaceae. The first chapter represents a literature review with a particular focus on members of the *D. pinea* species complex and their associated viruses. It reflects on various aspects of the taxonomy, pathogen biology, population genetics and disease management of members of this species complex. The potential of using dsRNA elements, which occur naturally in members of this species complex as biocontrol agents, is also considered. Detailed knowledge of members of the *D. pinea* species complex, closely related species, and their associated mycoviruses form the foundation for the research questions addressed in this dissertation.

The second chapter deals with the phylogenetic relationships between the morphotypes of *D. pinea*. The A, B and C morphotypes of the fungus had previously been shown to be

distinguishable based on morphology, RAPD (randomly amplified polymorphic DNA) banding profiles and SSR (short sequence repeats) markers. They also differ with regards to their distribution, population genetic structure and virulence. The aim of the study was to generate a multiple gene genealogy for isolates representing these morphotypes and closely related species from which more accurate phylogenetic inferences could be drawn. This was achieved using partial sequences of five protein-coding gene regions and microsatellite markers.

In the third chapter of this dissertation, *Diplodia*-like isolates from hosts other than *Pinus* spp. are characterized based on morphology and DNA sequences. These isolates all have conidia that are similar in size and shape, they are thick-walled and often become pigmented with age. For this reason some of these isolates have been treated as synonyms. Host association has also been used to provide an indication of identity. Like *D. pinea* f.sp. *cupressi* causing a canker disease of *Cupressus sempervirens* similar to that of *D. pinea* on *Pinus* spp. In this study, I characterized a set of *Diplodia*-like isolates by combining phylogenetic analysis of DNA sequences with morphological characteristics in an attempt to reveal their phylogenetic status as part of the Botryosphaeriaceae.

In the fourth chapter, I conducted a phylogenetic study to resolve relationships between morphologically similar species of the *Diplodia*-like anamorphs of the Botryosphaeriaceae (*Diplodia*, *Lasiodiplodia* and *Dothiorella*). The availability of sequence data for most genera of the Botryosphaeriaceae made it possible to extend the phylogeny and to explore host association patterns. The hope was that knowledge of these host association patterns and factors driving them would contribute to a better understanding of the evolution of the Botryosphaeriaceae, their co-evolution with their hosts and also help in the prediction of new diseases.

The fifth chapter of this dissertation treats the distribution and frequency of multiple virus infections in a collection of isolates belonging to the *D. pinea* species complex. Various dsRNA elements have previously been reported from the *D. pinea* species complex. Two of these, isolated from a South African *D. pinea* isolate were characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2). A third dsRNA element was found to be more commonly associated with the B morphotype of *D. pinea*. Using Real-time PCR and three virus-specific primers, virus infections were genotyped to assess their frequency and distribution patterns in isolates of the *D. pinea* species complex.

In chapter six, the previously identified, undescribed dsRNA element most commonly associated with the B morphotype of *D. pinea* was characterized and its full nucleotide sequence determined. The genome was assembled by overlapping contigs obtained through RT-PCR and virus-specific primers. The open reading frames (ORFs) were analyzed for homologies to other viruses and phylogenetic relationships with other virus families were assessed.

All studies presented in this dissertation concern the *D. pinea* species complex and associated viruses. They were conducted independently and have been written as separate publishable units. Some repetition between chapters may, therefore, occur as it represents a progression of knowledge obtained over a relatively long period of time. I, nonetheless hope these studies will contribute to a deeper understanding of the *D. pinea* species complex, viruses associated with them and their interaction.



## SUMMARY

The Botryosphaeriaceae is a family of fungi that includes many species, which are well-known as pathogens, saprophytes and endophytes of plants and especially of trees. As a result of their pathogenic nature and potential threat to plantations and agricultural crops, much research has been devoted to their identification. The main focus of studies that make up this thesis has been on the fungal complex referred to as *Diplodia pinea sensu lato*. These fungi are members of the Botryosphaeriaceae and studies have specifically concentrated on their taxonomy, host associations and mycovirus infections associated with them.

*Diplodia pinea sensu lato* represents a species complex of highly similar morphological types that mainly infect *Pinus* spp., world-wide. The species complex includes what have in the past been known as the A, B and C morphological types of *D. pinea*. Multiple gene genealogies based on sequences of partial protein-coding genes and microsatellite markers were used to resolve the species complex into two genera, *D. pinea* and *D. scrobiculata* (= B morphotype).

Diplodia-like isolates from Australia, Greece and Cyprus were characterized using both morphological and molecular characteristics. Morphologically, these isolates all have dark, thick-walled conidia (Diplodia-like) but phylogenetically, they could belong to three distinct genera of the Botryosphaeriaceae namely *Diplodia*, *Lasiodiplodia* and *Dothiorella*. Results of this study led to the description of *Dothiorella casuarini* from *Casuarina* spp. in Australia and they highlight the fact that similar morphological characteristics and disease etiology does not necessarily provide a true reflection of the evolutionary history of a pathogen.

Phylogenetic studies on species of the Botryosphaeriaceae with Diplodia-like anamorphs revealed intriguing host association patterns. The availability of sequence data for many species of the Botryosphaeriaceae made it possible to extend the phylogeny to include six of the ten lineages as previously described for the Botryosphaeriaceae. Angiosperms appeared to be the most common, and possibly ancestral, host group of the Botryosphaeriaceae, with the exception of *Macrophomina*, *Guignardia*, *Saccharata* and “*Botryosphaeria*” *quercuum*. Infection of gymnosperms most likely occurred more recently, only in specific groups (*Diplodia* and *Lasiodiplodia*) via host shifts.

Three distinct viruses have now been characterized from isolates of *D. pinea sensu lato*. Two of these were previously characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2). The third dsRNA element more commonly found in association with *D. scrobiculata* was characterized in this dissertation and named *Diplodia scrobiculata* RNA virus 1 (DsRV1). It has a genome of 5018 bp with a unique genome organization characterized by two open reading frames (ORFs). One ORF codes for a putative polypeptide similar to proteins of the vacuolar protein-sorting (VPS) machinery and the other one for a RNA dependent RNA polymerase (RdRp). The hypothetical protein probably has a role in transport or protection of this unencapsulated virus into membranous vesicles. Phylogenetically, DsRV1 groups closest to a dsRNA element from *Phlebiopsis gigantea* (PgV2) and they both group separately from other families in which fungal viruses have been classified.

The frequency and distribution of DsRV1, SsRV1 and SsRV2 were determined in a collection of *D. pinea* and *D. scrobiculata* isolates using Real-time PCR. Infections with SsRV1 and SsRV2 occurred in both *D. pinea* and *D. scrobiculata*, while DsRV1

was mainly found in *D. scrobiculata*. DsRV1 was also found to always occur in combination with SsRV1 and/or SsRV2. Therefore, DsRV1 probably selected against a coat protein as the result of a fitness trade-off. Although earlier studies indicated that these viruses have no effect on the phenotype or virulence of *D. pinea* and *D. scrobiculata* isolates, the presence of specific viruses in their host populations serve as a useful marker in studying movement of fungal pathogens.

The ultimate aim of studies making up this dissertation was to expand the base of knowledge regarding species in the *D. pinea* species complex. This was justified by the fact that *D. pinea* is one of the most important tree pathogens in South Africa and that an expanded knowledge might contribute to reducing diseases caused by it. Clearly understanding the identity of the fungus must clearly underpin many elements of a management strategy and this was one of the aims of the suite of studies conducted. Furthermore, I attempted to augment the knowledge base regarding dsRNA elements in *D. pinea sensu lato*. These studies were of a basic nature and relatively far removed from the practical application level. Nonetheless, it is my hope that they have pushed ahead knowledge barriers and that in some way they will contribute to reducing the impact of *Diplodia*-associated diseases in the future.



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**CHAPTER 1**

***DIPLODIA PINEA SENSU LATO* AS PART OF THE  
BOTRYOSPHAERIACEAE AND ASSOCIATED  
MYCOVIRUSES**

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## 1. INTRODUCTION

Most species belonging to the Botryosphaeriaceae are well-known pathogens causing disease symptoms such as die-back and cankers on numerous woody and non-woody hosts (Eldridge 1961; Buchanan 1967; Punithalingam & Waterston 1970). Some of these well-recognized species are *Diplodia pinea* (Desm.) Kickx. (= *Sphaeropsis sapinea* (Fr.) Dyko & Sutton), the conifer pathogen (Swart *et al.* 1985), *Botryosphaeria dothidea* (Moug. Fr.) Ces. & De Not. and *N. eucalyptorum* Crous, H. Smith & M.J. Wingf., pathogens of *Eucalyptus* L'Hér. (Smith *et al.* 1994, 2001) and *D. seriata* De Not. (= "*Botryosphaeria*" *obtusa*) and *D. mutila* (Fr.) Mont., fruit tree pathogens (Phillips *et al.* 2007; Slippers *et al.* 2007).

*Diplodia pinea* is an asexual fungus but is clearly recognized as a species of the Botryosphaeriaceae. Together with other species of *Diplodia* and *Lasiodiplodia*, it forms one of the ten lineages of the Botryosphaeriaceae as described by Crous *et al.* 2006. The taxonomy of the fungus has been complex and confused mainly due to the lack of a clear distinction between *Diplodia* and *Sphaeropsis*. The description of four distinct forms of *D. pinea* (A, B, C and I morphotypes) has furthermore, confused the taxonomic status of this fungus (Wang *et al.* 1985; Palmer *et al.* 1987; Hausner *et al.* 1999; De Wet *et al.* 2000). For the purpose of this chapter, the term *D. pinea sensu lato* has been applied to refer to the A, B and C morphotypes of this fungus. Members of the *D. pinea sensu lato* complex are able to infect various *Pinus* spp. as well as, species of *Larix*, *Cedrus*, *Picea* and *Pseudotsuga* belonging to different sub-families of the Pinaceae (Stanosz *et al.* 1999; Zhou & Stanosz 2001). The different morphotypes of *D. pinea* have different host ranges, but they can overlap. The host association patterns of *D. pinea sensu lato* and other species of the Botryosphaeriaceae might reveal some interesting observations with regards to the driving forces of evolution in this group.



*Diplodia pinea* can persist in a latent form in healthy pine tissue (Smith *et al.* 1996; Stanosz *et al.* 1997; Flowers *et al.* 2001, 2003) but in association with unfavorable environmental conditions or harsh physical factors, it gives rise to many different disease symptoms (Laughton 1937; Buchanan 1967; Swart *et al.* 1987a; Stanosz *et al.* 2001). The most common of these symptoms are die-back, whorl cankers, crown wilt, a root disease and blue stain of timber or logs (Laughton 1937; Da Costa 1955; Gilmour 1964; Punithalingham & Waterston 1970; Wingfield & Knox-Davies 1980a; Chou 1984; Swart & Wingfield 1991b). The morphotypes of *D. pinea sensu lato* differ in their virulence. Isolates of the A morphotype are more virulent than those of the B morphotype (Palmer *et al.* 1987) while the C morphotype is considered to be the most virulent (De Wet *et al.* 2002).

The genetic structure of *D. pinea* populations plays an essential role when considering optimal management and quarantine strategies for the fungus. As an asexual fungus, populations are expected to be almost clonal due to a lack of recombination. The fungus is, however, seed-borne (Rees & Webber 1988; Fraedrich & Miller 1995; Vujanovic *et al.* 2000) and multiple introductions of seed from different sources can result in a complex genetic structure (Burgess *et al.* 2001a). The different morphotypes of *D. pinea* also have different population structures (Burgess *et al.* 2004a, 2004b). Populations of the A morphotype have limited gene and genotypic diversities (Burgess *et al.* 2004a), while those of the B morphotype have high allelic diversity with considerable genetic distance between populations (Burgess *et al.* 2004b).

Current management strategies for *Diplodia*-induced disease symptoms are based on planting of resistant host species and the implementation of optimal management strategies and silvicultural practises (Lückhoff 1964; Swart *et al.* 1985). Chemical control is only viable in nurseries and against blue stain. Therefore, great economic losses due to *D. pinea* infections are still incurred

especially in plantations of non-native pine species in the southern hemisphere (Laughton 1937; Lückhoff 1964; Zwolinski *et al.* 1990a, 1990b). Integration of biological control strategies with conventional management strategies, are emerging as a potentially feasible management of fungal pathogens such as *D. pinea*.

Biological control using dsRNA-mediated hypovirulence has been implemented against the chestnut blight pathogen, *Cryphonectria parasitica* (Murrill) M. E. Barr. (Anagnostakis 1988). Hypovirulence refers to a condition where cytoplasmic determinants such as dsRNA elements that occur naturally in fungi attenuate virulence (Anagnostakis 1988; McCabe & Van Alfen 2002; Nuss 2005). Most dsRNA elements in fungi are, however, latent but their biology and simple genomic structure make them ideal candidates for genetic manipulation.

In the *D. pinea sensu lato* species complex, several dsRNA elements have been isolated (Wu *et al.* 1989; Preisig *et al.* 1998; Steenkamp *et al.* 1998; De Wet *et al.* 2001; Adams *et al.* 2002). Of these two have been characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (Preisig *et al.* 1998). The characterization of a third dsRNA element mainly associated with the B morphotype of the fungus is addressed as part of this dissertation. Although none of the dsRNA elements in *D. pinea* have been shown to confer hypovirulence (Wu *et al.* 1989; Preisig *et al.* 1998; Steenkamp *et al.* 1998; De Wet *et al.* 2001; Adams *et al.* 2002), the distribution patterns and interaction between multiple infections needs to be assessed and their role ascertained.

This literature review and the chapters of the dissertation that follow it, deal mainly with two issues pertaining to *D. pinea sensu lato*. One relates to the appropriate identification of *D. pinea* morphotypes and other *Diplodia*-like isolates encountered during this study and their phylogenetic relationship with other genera of the Botryosphaeriaceae are considered. The

second subject concerns the identification and characterization of a novel dsRNA element and the distribution of these dsRNA elements in members of the *D. pinea sensu lato* species complex.

## 2. TAXONOMY OF THE *DIPLODIA PINEA SENSU LATO* SPECIES COMPLEX

### 2.1 Taxonomic history

In 1842, a pathogen was isolated from *P. sylvestris* L. trees in France and described as *Sphaeria pinea* Desm. by Desmazières (Waterman 1943). In 1867, the fungus was transferred to *Diplodia* by Kickx as *Diplodia pinea* (Desm.) Kickx (Waterman 1943). Some years later, Petrak & Sydow (1927) proposed the name *Macrophoma pinea* (Desm.) Petrak & Syd., as an earlier epithet of *S. pinea*. Petrak (1961), however, found *M. pinea* to be a later homonym of *M. pinea* Pass. (= *Dothiorella pinea* (Pass.) and renamed the fungus *M. sapinea* (Fr.) Petrak. In the CMI descriptions of pathogenic fungi and bacteria, Punithalingam & Waterston (1970) presented *D. pinea* as a synonym of *S. pinea*, *M. pinea* and *M. sapinea*.

*Diplodia pinea* was transferred to *Sphaeropsis* Sacc. as *S. sapinea* (Sutton 1980) and the relevance of *Macrophoma* for the fungus best known as *D. pinea* was reconsidered. This change in name was supported by the percurrent proliferation of the conidiogenous cells of the fungus that were considered to be more typical of *Sphaeropsis* than of *Diplodia* (Sutton 1980). Phillips (2002), however, showed that percurrent proliferation is also common in the conidiogenous cells of *Diplodia* spp. Sutton (1980) also used septation to separate species of *Sphaeropsis* and *Diplodia*. He considered conidia of *Sphaeropsis* as aseptate with a faint septum developing prior to germination, while his interpretation was that conidia of *Diplodia* become euseptate as they mature. The distinction between *Sphaeropsis* and *Diplodia* was thus, never clearly defined resulting in considerable controversy with regards to the taxonomy of this fungus.

*Sphaeropsis* and *Diplodia* Fr., as well as, *Fusicoccum* Corda and *Lasiodiplodia* Ellis & Everh. were all considered as anamorph genera of the Botryosphaeriaceae based on morphology. Combining, morphology with DNA sequence data, later resulted in only two anamorph genera being recognized namely *Diplodia* and *Fusicoccum* (Jacobs & Rehner 1998; Denman *et al.* 2000, Zhou & Stanosz 2001). Species with dark, wide conidia were thus shown to reside in the *Diplodia*-group and those with light coloured, narrow conidia in the *Fusicoccum*-group. These two groups were also referred to as section *Brunnea* and section *Hyala* (Zhou & Stanosz 2001). A teleomorph state has never been observed for *S. sapinea* but it has been shown using ribosomal DNA (rDNA) and protein-coding gene sequence data that this fungus consistently groups with *Diplodia*-anamorphs of Botryosphaeriaceae (Jacobs & Rehner 1998; Denman *et al.* 2000; Zhou & Stanosz 2001; Zhou *et al.* 2001). The recommendation was, therefore, made to revert to the name *D. pinea* (Denman *et al.* 2000; Chapter 2 of this thesis).

The two anamorph system proposed for anamorphs of Botryosphaeriaceae (Jacobs & Rehner 1998; Denman *et al.* 2000; Zhou & Stanosz 2001; Zhou *et al.* 2001) was simplistic and clearly not a representation of the natural classification of this group. A recent phylogenetic study has changed the taxonomy of the Botryosphaeriaceae markedly (Crous *et al.* 2006). Ten lineages have been recognized (Fig. 1) namely *Diplodial/Lasiodiplodia/Tiarosporella* (no designated teleomorph), *Botryosphaeria* (*Fusicoccum* anamorphs), *Macrophomina* (teleomorph unknown), *Neoscytalidium* (teleomorph unknown), *Dothidotthia* (*Dothiorella* anamorphs), *Neofusicoccum* (*Botryosphaeria*-like teleomorphs, *Dichomera*-like synanamorphs), *Pseudofusicoccum* (teleomorph unknown), “*Botryosphaeria*” *quercuum* (*Diplodia*-like anamorph), *Saccharata* (*Fusicoccum*-like teleomorph, *Diplodia*- and *Fusicoccum*-like synanamorphs) and *Guignardia* (*Phyllosticta* anamorphs). In the above mentioned study, the *Diplodial/Lasiodiplodia* clade was

unresolved and accommodates all *Diplodia*-like isolates with dark, >10 µm broad, thick-walled conidia. The *Dothiorella* clade also include species with *Diplodia*-like conidia but these conidia are dark and single-septate early in development unlike those of *Diplodia* and *Lasiodiplodia* turning dark and multi-septated over time. The *Botryosphaeria* clade accommodates species like *B. dothidea* that has *Botryosphaeria* teleomorphs and *Fusicoccum*-like anamorphs with light, <10 µm broad and thin-walled conidia and no synanamorphs. The *Neofusicoccum* clade also accommodates species with *Botryosphaeria*-like teleomorphs but has *Fusicoccum*-like anamorphs and *Dichomera*-like synanamorphs. The *Pseudofusicoccum* clade accommodates the *Fusicoccum*-like anamorphs with unusually large conidiomata, conidia that are thick-walled and surrounded by a mucous sheath. The other clades all have their unique features that easily distinguish them from the rest (Crous *et al.* 2006). The Botryosphaeriaceae was consequently reclassified under a new order the Botryosphaeriales (Schoch *et al.* 2006), better suited for this group than the Dothideales (Miller 1928; VonArx & Müller 1975). The *D. pinea sensu lato* species complex is well placed in this group.

## **2.2 Taxonomy of *Diplodia pinea* and its morphotypes**

*Diplodia pinea* is an asexual fungus belonging to the Coelomycetes. Asexual spores or conidia are produced in pycnidia (Haddow & Newman 1942; Waterman 1943). Conidia are oblong to clavate with blunt basal ends, rounded apices and develop monoblastically via percurrent proliferation of the conidiogenous cells (Sutton 1980). A range of conidial sizes, 30-45 x 10-16 µm, have been reported for *D. pinea* (Punithalingam & Waterston 1970; Sutton 1980). The huge range of conidial sizes is apparently influenced by the age of the conidia and the existence of different morphotypes for *D. pinea*. Juvenile conidia are hyaline, thick-walled and non-septate, while mature conidia are dark brown with up to three septa. Spermatia and spermatiphores

have been reported in the fungus (Wingfield & Knox-Davies 1980b). They are hyaline, independent of age, and smaller than conidia. It could be an indication of *D. pinea* having a reduced or lost sexual state.

Four morphotypes have been described for *D. pinea*. These have been defined based on morphological characteristics, particularly of the conidia and cultural characteristics. These four morphotypes have been referred to as the A, B, C and I morphotypes (Wang *et al.* 1985; Palmer *et al.* 1987; Hausner *et al.* 1999; De Wet *et al.* 2000, 2002). Isolates of the A morphotype are characterized by fluffy, aerial mycelium, conidia (34  $\mu\text{m}$  x 16  $\mu\text{m}$ ) longer than those of the B morphotype but shorter than those of the C morphotype, smooth conidial walls and usually no or only one septum (Wang *et al.* 1985, 1986; Palmer *et al.* 1987). Isolates of the B morphotype are characterized by appressed mycelium, growing close to the surface or in the agar, conidia (32  $\mu\text{m}$  x 15  $\mu\text{m}$ ) shorter than those of the other morphotypes, pitted conidial walls and up to three septa (Wang *et al.* 1985, 1986; Palmer *et al.* 1987). Isolates of the C morphotype are closely related to those of the A morphotype also having fluffy, aerial mycelium, smooth conidial walls, none or only one septum but with conidia (37  $\mu\text{m}$  x 15  $\mu\text{m}$ ) longer than both those of the A and B morphotypes (De Wet *et al.* 2000, 2002). Isolates of the I morphotype were described having characteristics of both the A and B morphotypes with neither fluffy nor appressed mycelium and smooth or pitted conidial walls (Hausner *et al.* 1999).

Various techniques have been used to authenticate the existence of the morphotypes of *D. pinea*. Isozyme profiles were initially used to distinguish between the A and B morphotypes of the fungus (Palmer *et al.* 1987; Swart *et al.* 1991). Random amplified polymorphic markers (RAPDs) were later applied to this question and resulted in the distinction of the A, B and C morphotypes (Smith & Stanosz 1995; Stanosz *et al.* 1996; De Wet *et al.* 2000). Restriction

fragment length polymorphisms (RFLPs) resulted in distinction of the A, B and I morphotypes (Hausner *et al.* 1999). More recently, short sequence repeats (SSRs) have made it possible to distinguish among the A, B and C morphotypes of *D. pinea* and have shown that isolates of the I morphotype represent the anamorph of “*Botryosphaeria*” *obtusa* (d.i. *D. seriata*) (Burgess *et al.* 2001b). The above mentioned techniques could distinguish between the morphotypes of *D. pinea* only after pure cultures were obtained.

Overcoming the constraints of first having to produce pure *Diplodia* cultures, followed by DNA extractions and DNA sequence comparisons to determine the identity of the *Diplodia* morphotype or closely related species have resulted in the development of various rapid identification assays. Fingerprinting profiles generated through MSP-PCR (microsatellite-primed polymerase chain reaction) and rep-PCR (repetitive-sequence-based polymerase chain reaction) are able to distinguish between the morphotypes of *Diplodia*, as well as, 25 other species belonging to the Botryosphaeriaceae (Alves *et al.* 2007). Some other techniques for the quick differentiation of morphologically similar species of the Botryosphaeriaceae include ISSR (inter simple or short sequence repeats fingerprinting (Zhou *et al.* 2001) and ARDRA (amplified ribosomal DNA restriction analysis) (Alves *et al.* 2005).

As part of this dissertation, isolates representing the B morphotype of *D. pinea* were shown to represent a distinct taxon, which has been provided the name *Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf. This review precedes the scientific study and thus refers to the fungus as the B morphotype of the *D. pinea sensu lato* species complex.

### 3. PATHOGEN BIOLOGY

#### 3.1 Distribution and host range

*Diplodia pinea sensu lato* has a worldwide distribution and a host range that includes various *Pinus* spp. and some other conifers (Fisher 1912; Waterman 1943; Eldridge 1961; Buchanan 1967; Punithalingam & Waterston 1970; Gibson 1979; Swart *et al.* 1985; Stanosz *et al.* 1996, 1999). Isolates of the different morphotypes of the fungus differ in their host specificity and distribution (Wang *et al.* 1985; Smith & Stanosz 1995). The A morphotype of *D. pinea* is the most common form, occurring in all conifer-growing regions of the world and on a wide range of *Pinus* and other conifer species (Stanosz *et al.* 1999). Until recently, isolates of the B morphotype were thought to be restricted to *P. banksiana* Lamb. and *P. resinosa* Sol. ex Aiton in the north central United States (Palmer *et al.* 1987), but have since been reported from other hosts and from Europe (Smith & Stanosz 1995; Stanosz *et al.* 1999; Zhou & Stanosz 2001). The C morphotype of *D. pinea*, has been reported only from Indonesia on *P. patula* Schiede ex Schlthdl. & Cham. (De Wet *et al.* 2000). This type appears not to have moved out of South East Asia and extensive sampling worldwide make it reasonably certain to say it does not occur in other pine-growing countries.

#### 3.2 Disease symptoms

*Diplodia pinea* is an opportunistic pathogen and the most common disease symptoms associated with it are die-back and shoot blight (Fisher 1912; Nicholls 1977; Peterson 1977), whorl cankers and crown wilt (Gilmour 1964; Marks & Minko 1969; Nicholls 1977; Chou 1984, 1987; Swart *et al.* 1985; Palmer 1991), collar rot (Punithalingam & Waterston 1970; Swart *et al.* 1985), a root disease (Wingfield & Knox-Davies 1980a) and blue stain (Laughton 1937; Da Costa 1955; Eldridge 1961). Disease symptoms normally appear four to ten days after initiation of infection



providing that conditions are favorable for germination of conidia and proliferation of the fungus (Brookhouser & Peterson 1971; Peterson 1977; Chou 1984; Swart *et al.* 1987a, 1987b).

### **3.2.1 Die-back and shoot blight**

Die-back and shoot blight due to *D. pinea* infection result in the loss of normal growth of terminal shoots. The terminal shoots become brittle, stunted and resin is exuded (Fisher 1912; Eldridge 1961; Nicholls 1977; Peterson 1977). The needles turn brown and are shed. Internally, the woody tissue discolors, pycnidia are formed and the tissue becomes necrotic (Swart *et al.* 1985).

### **3.2.2 Whorl cankers and crown wilt**

*Diplodia pinea*-induced cankers reduce the normal growth of trees (Nicholls 1977). Cankers are elongated, depressed areas at the whorl of the tree as a result of the cambium and cortical tissue being infected by the fungus (Gilmour 1964; Marks & Minko 1969; Swart *et al.* 1985). In severe cases, cankers can girdle the trees, causing mortality or crown wilt where only a portion of the crown is killed (Chou 1984, 1987).

### **3.2.3 Collar rot and a root disease**

*Diplodia pinea* causes collar rot in pine seedlings (Waterman 1943; Punithalingam & Waterston 1970). The root collar area discolors followed by foliage chlorosis and resin exudation (Swart *et al.* 1985). Wingfield & Knox-Davies (1980a) also reported a root disease on *P. elliottii* Engelm. and *P. taeda* L. as a result of *D. pinea* infection. The lateral roots of infected trees have dark blue, resinous lesions on the radial parts that can extend to a height of two meters up the tree trunks.

### 3.2.4 Blue stain

Blue stain normally refers to the discoloration of the sapwood due to dark pigmented sap-staining fungi growing in the ray parenchyma. It is a cosmetic, non-degrading defect that results only in the devaluation of timber. Blue stain of pine logs and timber is not only due to *D. pinea* but rather a combination of fungi that can also include *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *D. mutila* as well as, several *Ophiostoma* and *Ceratocystis* species (Laughton 1937; Da Costa 1955; Eldridge 1961; Butcher 1968; Swart & Wingfield 1991b; Seifert 1993; Mohali & Encinas 2001). Sometimes the undesirable staining of the sapwood is not only due to the mycelium of these blue stain fungi but the result of host cells reacting to metabolites produced by the fungi as was reported by Butcher (1968) for *Ceratocystis piceae* (Munch) Bakshi. and *Phialophora fastigiata* (Lagerb. and Melin) in red beech, or the production of pigments. Saprophytic *D. pinea* and *L. theobromae* infections can occur through bark abrasions caused during felling, bark butts after pruning or exposed ends of cut logs (Laughton 1937; Eldridge 1961; Gilmour 1964; Lückhoff 1964; Marks & Minko 1969; Peterson 1977; Swart *et al.* 1985; Swart & Wingfield 1991b; Zwolinski *et al.* 1995). *Ophiostoma* and *Ceratocystis* species on the other hand, are insect-vectored, mainly by bark beetles (Harrington 1993; Seifert 1993; Paine *et al.* 1997). Blue stain-associated fungi utilize only the extractives of the tree as carbon source while lignified walls and structural carbohydrates remain intact leaving the strength of the wood unaffected (Da Costa 1955; Eldridge 1961; Schirp *et al.* 2003).

### 3.3 Spore development, dispersal and infection

Pycnidia containing *D. pinea* conidia are formed on dead needles, bark, scales of two-year-old pine cones and the forest litter (Laughton 1937; Waterman 1943; Peterson 1981). Mature conidia are dispersed through water (Eldridge 1961; Brookhouser & Peterson 1971) or wind if

long distance dispersal is necessary (Swart *et al.* 1985). Conidia germinate and enter the host through wounds or stomata (Brookhouser & Peterson 1971), or through direct penetration of the epidermis of non-lignified shoots (Chou 1978). Hyphal aggregates in crevices at the needles bases and on the surfaces of the needles and bud scales, furthermore suggest that infection can potentially originate there (Waterman 1943; Rees & Webber 1988; Flowers *et al.* 2006).

Mycelium grows into the mesophyll from which it colonizes and spreads to the phloem and cortical tissue of the host (Laughton 1937). Latent *D. pinea* infections are localized in the outer stem cortex, while pathogenic infections occur throughout the shoot stem tissue (Flowers *et al.* 2006). The normal cambial function of the host is disrupted leading to bark and cambial necrosis, discoloration of the needles, girdling of the shoots and shedding of the needles (Laughton 1937; Marks & Minko 1969; Brookhouser & Peterson 1971; Brown *et al.* 1981).

Infection levels are high during the active elongation phase of the shoots and when environmental conditions are favorable for spore germination and penetration. This is especially true when the host is more susceptible either genetically or as a result of stress conditions (Millikan & Anderson 1957; Brookhouser & Peterson 1971; Minko & Marks 1973; Chou 1978, 1982). Optimal environmental conditions for infection include temperatures between 24 ° and 30 °C and a relative humidity higher than 90 % (Brookhouser & Peterson 1971; Chou 1982).

Infection is typically more severe in actively growing trees and it decreases with tree age (Laughton 1937; Marks & Minko 1969; Chou 1977, 1982). This is probably due to the change in the microenvironment and nutrition of the tree as it increases in size and complexity, making it less suitable for infection by *D. pinea* (Chou 1977). In older hail-damaged trees, infection was however, more severe than in younger trees (Smith *et al.* 2002a). A possible explanation for this is the presence more seed cones on older trees already containing latent *D. pinea*, which is able

to initiate a pathogenic infection with the onset of stress through hail (Smith *et al.* 2002a).

Altitude also plays a role in infection. *Diplodia pinea* infections are less in higher altitudes as the microenvironment necessary for infection is less suitable than in lower altitudes (Chou 1977; Zwolinski *et al.* 1990b).

### **3.4 Wounding, stress, virulence and host susceptibility**

For many years, it was assumed that *D. pinea*, especially the B morphotype, required wounds for infection (Wang *et al.* 1985; Palmer *et al.* 1987). These wounds would typically originate during pruning, hail damage or through insect feeding (Laughton 1937; Gilmour 1964; Lückhoff 1964; Marks & Minko 1969; Wright & Marks 1970; Peterson 1977; Swart *et al.* 1985; Zwolinski *et al.* 1995). This view has changed with various reports of both morphotypes of the fungus being able to infect unwounded stems and leaves through direct penetration of the stomatal pits (Waterman 1943; Brookhouser & Peterson 1971; Palmer 1991; Blodgett & Stanosz 1997a). *Diplodia pinea* has also been shown to persist in healthy, asymptomatic host tissue and mature, unopened seed cones in a latent state (Smith *et al.* 1996; Stanosz *et al.* 1997; Flowers *et al.* 2001, 2003).

Stanosz *et al.* 1997 demonstrated that both the A and B morphotypes of *D. pinea* isolated from asymptomatic shoots of *P. resinosa* and *P. banksiana* were able to develop characteristic *Diplodia* die-back symptoms when artificially inoculated.

*Diplodia pinea sensu lato*, like most species of the Botryosphaeriaceae, occur as latent infections in healthy tissue (Slippers & Wingfield 2007). One of the major obstacles in dealing with these latent infections is the lack of ability to detect them easily and to distinguish them from other epiphytic infections. Various quick assays have therefore, been developed to detect latent infections directly from asymptomatic host tissue. Flowers *et al.* (2003) developed a nested PCR using nuclear rDNA ITS primers to detect the presence of latent *D. pinea* and *B. obtusa*

infections in pine tissue but without being able to differentiate between these closely related species and the morphotypes of *D. pinea*. This was followed by a Real-time quantitative PCR assay based on the small ribosomal subunit able to rapidly detect and quantify *D. pinea* infections in inoculated *P. nigra* Arnold shoots (Luchi *et al.* 2005), as well as asymptomatic *P. nigra* shoots (Maresi *et al.* 2007). These assays were also unable to distinguish between the morphotypes of the fungus. A species-specific PCR assay, based on polymorphisms in the mitochondrial small subunit ribosome gene (mtSSU rDNA), has since been developed that is able to differentiate between the A and B morphotypes of *D. pinea* and *D. seriata* directly from dead red and jack pine tissue (Smith & Stanosz 2006).

Latent *D. pinea sensu lato* infections are hypothesized to be a survival mechanism of the fungus awaiting opportunity to overcome host defense responses and cause visible disease symptoms (Stanosz *et al.* 1997; Flowers *et al.* 2006). The onset of host stress as a result of adverse environmental or physical factors initiates pathogenic *D. pinea* infections (Laughton 1937; Waterman 1943; Buchanan 1967; Minko & Marks 1973; Brown *et al.* 1981; Swart *et al.* 1987a; Stanosz *et al.* 2001). Hail, drought, overstocking, poor site conditions and nutrient deficiencies are general predisposing factors (Laughton 1937; Lückhoff 1964; Wright & Marks 1970; Minko & Marks 1973; Chou 1977, 1982; Bega *et al.* 1978; Brown *et al.* 1981; Bachi & Peterson 1985; Stanosz *et al.* 2001). Maresi *et al.* (2007) demonstrated how water stress can potentially be a trigger that enables the fungus to switch from a latent phase to that of a more active pathogenic phase when they found a positive correlation between the presence of *D. pinea* and the normalized insolation index. The normalized insolation index is a measure of the amount of heat at a point that ultimately is an indication of water stress at a specific site. These predisposing factors decrease the rate of the host defense responses and consequently the growth of the

pathogen increases as a result of a larger carbohydrate pool available to it (Schoeneweiss 1981; Bachi & Peterson 1985).

Hail damage followed by *D. pinea*-induced die-back is a serious problem in South Africa resulting in huge economic losses (Zwolinski *et al.* 1990a, 1990b). The highest degree of mortality occurs four months after a hailstorm and can last for up to a year, after which regeneration of foliage normally occurs (Zwolinski *et al.* 1990b). Smith *et al.* (2002a) mapped the colonization of *D. pinea* in hail-damaged *P. patula* trees from the cone pith, through the stipe (connection between the cone and the branch), the branch and finally into the branch pith. In undamaged *P. patula* trees no discoloration due to *D. pinea* was found in the branch pith but *D. pinea* was present latently in the cone pith and in the stem.

Insects are commonly associated with *D. pinea* infection. They play a role in facilitating the colonization of healthy cambial tissue and thus, enhance the severity and impact of the infection rather than playing a role in the dissemination of the fungus (Haddow & Newman 1942; Wingfield & Palmer 1983; Zwolinski *et al.* 1995). Examples of insects that have been associated with *D. pinea* are the pine spittle bug (*Aphrophora parallela* Say) (Haddow & Newman 1942; Waterman 1943), the pitch nodule moth (*Petrova albicapitana* Busk) (Hunt 1969), the deodar weevil (*Pissodes nemorensis* Germar), the bark beetle (*Orthomicus erosus* Woll.) (Wingfield & Palmer 1983; Zwolinski *et al.*, 1995), the cone bug (*Gastrodes grossipes* De Geer) (Feci *et al.* 2002) and the pine shoot moth (*Dioryctria* sp.) (Feci *et al.* 2003). Zwolinski *et al.* (1995) made interesting observations regarding the association of *P. nemorensis* and *O. erosus* with post-hail associated *Diplodia* infections in South Africa. *Orthomicus erosus* is found only on post-hail *Diplodia*-infected trees while *P. nemorensis* can occur on healthy trees but exacerbates the spread of the fungus in post-hail *Diplodia*-infected trees.

Wounding and adverse environmental conditions, together with differences in host susceptibility and virulence of the pathogen play a role in *D. pinea* infections (Burdon *et al.* 1980; Palmer *et al.* 1987; Zwolinski *et al.* 1990b). Generally, *Pinus* species of the subgenus *Haploxyton* (white or soft pines, lacebark and foxtail pines) are less susceptible than those of the subgenus *Diploxyton* (yellow or hard pines) (Vujanovic *et al.* 2000). In the north hemisphere, *P. nigra* and *P. mugo* Turra seed cones were found to be more susceptible to *D. pinea* infection than *P. sylvestris*, and *P. resinosa* was the most tolerant species (Vujanovic *et al.* 2000). In South Africa, *P. radiata* D. Don was found to be the most susceptible species followed by *P. pinaster* Aiton (Swart *et al.* 1985). More resistant species are *P. taeda*, *P. elliottii* and *P. patula*, with the latter being more susceptible than the former two species (Swart *et al.* 1985). The northern *P. greggii* Engelm. ex Parl. provenance (*P. greggii* var. *greggii*) was also reported to be significantly more resistant to *D. pinea* than the southern provenance (*P. greggii* var. *australis*), even after hail damage (Smith *et al.* 2002b). These differences observed in host susceptibility are hypothesized to be the result of secondary metabolites like monoterpenes and phenolic compounds that have a fungistatic effect on *D. pinea* (Chou & Zabkiewicz 1976; Brown *et al.* 1981; Chou 1981; Blodgett & Stanosz 1997b).

The morphotypes of *D. pinea* differ in virulence. Isolates of the A morphotype are more virulent as those of the B. morphotype (Palmer *et al.* 1987; Palmer 1991; Blodgett & Stanosz 1997a). Interestingly, the C morphotype, which is known only from Northern Sumatra, has been shown to be the most virulent form of the fungus (De Wet *et al.* 2002). The differences in virulence observed for the A and B morphotypes was linked to the defense chemistry of the host (Blodgett & Stanosz 1997b). The phenolic extracts of *P. resinosa* reduced mycelial growth of the B

morphotype, resulting in a weak, localized infection, while the growth of the A morphotype was unaffected by these phenolics resulting in more aggressive infection that spread quickly.

#### 4. POPULATION GENETICS

The genetic structure of *D. pinea sensu lato* populations is relevant to the management and quarantine of Diplodia die-back and other *Diplodia*-associated diseases. A pathogen population with a highly diverse genetic structure can more easily adapt and overcome resistance. Because *D. pinea* is an endophyte (Smith *et al.* 1996; Stanosz *et al.* 1997; Burgess *et al.* 2001a; Flowers *et al.* 2001) and found on pine seed collected from cones in seed orchards (Peterson 1977; Fraedrich & Miller 1995; Vujanovic *et al.* 2000), it is fair to assume that wherever pine seed or seedlings have been introduced the fungus is likely to have been introduced with it.

In a study conducted by Smith *et al.* (2000), genotypic diversity of two *D. pinea* populations was assessed using vegetative compatibility groups (VCGs). They found the genotypic diversity of an introduced South African population to be unexpectedly higher than that of a native Indonesian population (Smith *et al.* 2000). In a subsequent study, simple sequence repeats (SSRs), were used to determine the genotypic diversity of four *Diplodia* populations from native and introduced *P. radiata* (Burgess *et al.* 2001a). The same trend was observed with higher genotypic diversities for the introduced South African, New Zealand and Australian populations, with those of South Africa being the highest, followed by New Zealand and Australia compared to a native Californian population (Burgess *et al.* 2001a).

With *D. pinea* being an asexually reproducing fungus, populations would be expected to be almost clonal with very low genotypic diversities. In the absence of sexual recombination and selective pressure, the assumption was made that the observed genotypic diversity reflects the number of introductions of the fungus into a region (Burgess *et al.* 2001a; Burgess & Wingfield



2002). Therefore, the high genotypic diversity observed for the introduced *D. pinea* populations is accounted for by multiple introductions of the fungus together with pine seed into the southern hemisphere (Smith *et al.* 2000; Burgess *et al.* 2001a; Burgess & Wingfield 2002). The much higher genotypic diversity calculated for the South African population was linked to the fact that afforestation in South African started about 100 years before Australia and New Zealand and that there has been little control on the importation of seed into the country (Burgess *et al.* 2001a; Burgess & Wingfield 2002). In contrast, Australia and New Zealand have strict quarantine regulations that significantly restrict the introduction of pine seed into those countries (Burgess *et al.* 2001a; Burgess & Wingfield 2002).

In the previous two studies, the genetic diversity of *D. pinea* populations were determined but the existence of *D. pinea sensu lato* as representing two different morphotypes and potential cryptic speciation were not considered. As previously discussed, the morphotypes of *D. pinea* differ with regards to their taxonomy, biology and virulence. Populations of the two morphotypes also have different genetic structures. The A morphotype or *D. pinea sensu stricto* is the main species associated with most *Pinus* spp. outside their native range (Burgess *et al.* 2004a). While the B morphotype is almost exclusively associated with *P. radiata* in its native range (Burgess *et al.* 2004b). The *D. pinea sensu stricto* populations have very low gene diversities, little population differentiation and share multilocus genotypes between populations on different continents (Burgess *et al.* 2004a). This suggests a long asexual history and constant selection pressure as selection is linked to the success of the endophyte. *D. pinea sensu stricto* populations are thus highly unlikely to overcome host resistance and breeding for resistance in the host will be a durable option. In contrast, populations of the B morphotype have high allelic diversity and no multilocus genotypes are shared between populations. The huge genetic

distance between populations with limited gene flow suggests a recent history of recombination and/or mutation as well as the presence of a cryptic sexual stage (Burgess *et al.* 2004b).

## 5. DISEASE MANAGEMENT

### 5.1 Conventional disease management

Management of *Diplodia*-associated diseases has most commonly been based on planting resistant pine species in combination with effective silvicultural practices (Lückhoff 1964; Wright & Marks 1970; Brookhouser & Peterson 1971; Peterson 1977; Gibson 1979; Swart *et al.* 1985; Swart & Wingfield 1991b). Although, these management strategies are implemented vigorously, substantial losses due to *D. pinea* infections are still experienced, especially in plantations of non-native *Pinus* spp. (Laughton 1937; Lückhoff 1964; Chou 1976; Zwolinski *et al.* 1990a). In South Africa, hybridization of *P. patula*, the most widely planted species, with *P. greggii* var. *greggii* for its drought tolerance, altitude adaption and *D. pinea* resistance even after hail damage, has been proposed as a robust solution to post-hail associated *Diplodia* die-back (Smith *et al.* 2002b). Breeding for *Diplodia* resistance in *Pinus* spp. under variable environmental conditions is, however a slow process and it is far from implementation in commercial forestry plantations.

In the meantime, management of *Diplodia*-induced diseases is being achieved by replacing susceptible *Pinus* spp. with more resistant species, especially in areas likely to be favorable for the initiation and spread of infection by this fungus (Lückhoff 1964; Wright & Marks 1970; Gibson 1979; Burdon *et al.* 1980; Palmer & Nicholls 1983). In South Africa, the very susceptible *P. radiata* is restricted to the winter rainfall areas where hailstorms are rare (Swart *et al.* 1987a, 1987b, 1988). The highly susceptible *P. patula* has been replaced by *P. elliottii* in hail sensitive areas of the summer rainfall region (Swart *et al.* 1987a, 1987b, 1988). There have

however, been reports of the tolerant *P. elliottii* experiencing post-hail associated Diplodia die-back and its replacement with *P. greggii* var. *greggii* might prove more resistance under those particular stress conditions (Smith *et al.* 2002b).

Appropriate silvicultural practices and sanitation are essential in managing *D. pinea*-associated diseases. Pruning must be done carefully to prevent wounds and should be scheduled for times when the density of fungal inoculum is low and environmental conditions are unfavorable for the dispersal and germination of the conidia (Gilmour 1964; Palmer & Nicholls 1983; Swart *et al.* 1985; Swart & Wingfield 1991a). The inoculum source can be reduced by removing slash after thinning and pruning ((Nicholls 1977; Bega *et al.* 1978; Gibson 1979). Premature thinning also reduces *D. pinea* infection as it lowers the atmospheric humidity and competition for water and nutrients is less (Bega *et al.* 1978; Gibson 1979). In nurseries, good sanitation practices are essential in reducing *D. pinea* infections (Nicholls 1977).

Fertilization has a profound influence on the incidence and severity of diseases. Generally, the application of fertilizers is believed to alleviate physiological stress on the host, ensuring overall well-being and vigorous growth. In the case of *D. pinea*, fertilization does not always lower the impact of the pathogen (Blodgett *et al.* 2005). The incidence of *D. pinea* was found to increase in areas with high rates of atmospheric ammonium deposition or when treated with fertilizers (De Kam *et al.* 1991; Blodgett *et al.* 2005). Blodgett *et al.* (2005) found that chemicals such as lignin and soluble phenolics, previously reported to play a role in host defense (Chou & Zabkiewicz 1976; Brown *et al.* 1981; Chou 1981; Blodgett & Stanosz 1997b), decrease when fertilizers were applied. The increased susceptibility of the host to *D. pinea* infections was thus hypothesized to be the result of a more suitable growth environment for the fungus or a tradeoff between growth and defense in the host.

Chemical control of *D. pinea* in commercial plantation forestry is not practical but has been useful in nurseries and in the case of small ornamentals like Christmas trees (Palmer & Nicholls 1983). Fungicides shown to be efficient in controlling *D. pinea* outbreaks usually have Benomyl (=Benlate) or thiophanate-methyl as active ingredient (Palmer & Nicholls 1983; Palmer *et al.* 1986; Stanosz & Smith 1996). These fungicides belong to the chemical family benzimidazoles. Control of blue stain is difficult as a combination of fungi is responsible for the unwanted discoloration of the sapwood and it is aggravated by storage conditions conducive to fungal growth. In the past, logs were forced-air dried to lower the moisture content necessary for fungal growth, followed by chemical dips like sodium azide and sodium petachlorophenate (Butcher 1968). These chemical dips are enzyme inhibitors that inhibit fungal metabolism. More recently, antisapstain chemicals that have been applied to exposed surfaces of felled logs through spraying or dipping are copper-8-quinolate and didecyldimethyl ammonium chloride (DDAC) (Thwaites *et al.* 2004). These chemicals form a protective layer on the exposed surfaces preventing fungal spores from germination and penetration for up to 10 weeks (Thwaites *et al.* 2004). No protection is however, provided against fungi that have already penetrated the wood or against chemical-tolerant sapstain fungi. A more successful antisapstain chemical that enables protecting against a broad spectrum of blue stain fungi for longer periods is a combination of two fungicides, registered under the name Sentry<sup>®</sup> (Wakeling *et al.* 2000). This is a solubilised concentration of methylenebisthiocyanate (MBT) and 2-*n*-octyl-4-isothiolin-3-one (OIA) formulated to form a micro-emulsion. An integrated approach, combining chemical control with biological control agents, has however been proposed as the best method for combating the undesirable effect of blue stain fungi (Behrendt *et al.* 1995b).

## 5.2 Biological control

Biological control is based on the ability of naturally occurring microorganisms that inhibit the growth or metabolic activity of pathogenic microorganisms (Cook 1993; Duffy *et al.* 2003; Howell 2003). Mechanisms of biocontrol can include antibiosis, competition, mycoparasitism, induction of defense responses in plants or hypovirulence (Day *et al.* 1977; Cook 1993; Duffy *et al.* 2003; Howell 2003). Biological control has environmental advantages over chemical control as it is safe and there is a reduced likelihood of the pathogen overcoming the control due to resistance (Duffy *et al.* 2003).

Biological control of blue stain fungi, mainly *D. pinea* and *Ophiostoma* spp., has been extensively studied and in combination with antisapstain fungicides proved to be highly effective (Behrendt *et al.* 1995b). Two methods of biocontrol can be employed. The first is based on inhibiting the growth of blue stain fungi by another fungus e.g. *Trichoderma* spp. or *Trichothecium roseum* (Vanneste *et al.* 2002). The second method is based on inhibition of blue stain fungi by secondary metabolites produced by plants or microorganisms. Oxygenated monoterpenes such as oxygenated alcohol or phenolic monoterpenes are secondary metabolites produced by the tree that are able to inhibit blue stain fungi preventing the unwanted discoloration of sapwood for up to nine months (Vanneste *et al.* 2002). Pine oil derivatives containing oxygenated monoterpenes is being developed for commercial use for the treatment of wood and wood products against blue stain fungi.

Several biocontrol agents specifically against blue stain-associated *Ophiostoma* species have been reported (Behrendt *et al.* 1995a) and two US patents have been registered (Patent no. 5096824; 5518921). These are based on Cartapip-97<sup>®</sup>, a commercially available formulation of a non-pigmented strain of *O. piliferum* (Behrendt *et al.* 1995a). The non-pigmented *O. piliferum*

strain competes with the blue stain-associated *Ophiostoma* spp. reducing the impact of the blue stain. Another biocontrol agent is *Phlebiopsis gigantea*, a white rot fungus that is able to parasitize on blue stain-associated *Ophiostoma* spp. reducing the undesirable blue stain (Behrendt & Blanchette 1997).

Biological control against *Diplodia*-induced disease symptoms other than blue stain is a relatively unexplored area. In this review, the focus is therefore, on biological control using fungal viruses as they have been studied in *D. pinea* in the past and they form a part of the research that makes up this dissertation.

### **5.2.1 Virus-like particles in fungi**

Virus-like particles (VLPs) associated with fungi were first isolated in 1950 from commercially produced mushrooms (*Agaricus bisporus*) associated with La France disease (Hollings 1962). These VLPs are normally associated with the cytoplasm of their hosts, have dsRNA genomes and are commonly known as mycoviruses (Buck 1986). VLPs associated with the mitochondria are referred to as mitoviruses (Polashock & Hillman 1994; Hong *et al.* 1999). The genetic composition of these viruses is very basic and therefore, cellular factors of the hosts sometimes play an important role in their transcription and replication (Lai 1998). Multiple infections with different viruses are common in all major classes of fungi (Hollings & Stone 1971; Barton & Hollings 1979; Buck 1986).

Mycoviruses are classified into virus families based on their nucleic acid composition. Those with dsRNA genomes, constituting the majority of mycoviruses thus far discovered, belong to the *Hypoviridae* (Nuss *et al.* 2005), *Totiviridae* (Wickner *et al.* 2005), *Partitiviridae* (Ghabrial *et al.* 2005), *Chrysoviridae* (Ghabrial & Castón 2005) and the genus *Mycoreovirus* (Mertens *et al.* 2005). Mycoviruses with ssRNA genomes belong to the *Barnaviridae* or *Narnaviridae* (Buck *et*

al. 2005). Mitoviruses that are associated with the mitochondria are classified in the genus *Mitovirus* belonging to the *Narnaviridae*. Mycoviruses with reverse transcribed RNA genomes belong to the *Metaviridae* (Boeke *et al.* 2005b) and *Pseudoviridae* (Boeke *et al.* 2005a) and those with dsDNA genomes to the genus *Rhizidiovirus* (Ghabrial & Buck 2000) (Fig. 2).

The origin of mycoviruses, especially the more common dsRNA mycoviruses, is believed to be polyphyletic (Koonin *et al.* 1989; Ghabrial 1998). This assumes there are multiple origins at different times, presumably from other cellular organisms (Hollings 1982; Koonin *et al.* 1989).

Mycoviruses and mitoviruses co-evolved with their fungal hosts and co-adapted over time (Lemke 1976; Hollings 1982; Koonin *et al.* 1989; Ghabrial 1998). The closest relative of fungal viruses is believed to be (+) ssRNA plant viruses of supergroups I and II (Koonin *et al.* 1989; Ghabrial 1998) (Fig. 3).

Most mycovirus infections are latent, having no affect on the phenotype or pathogenicity of the fungus they infect (Lemke & Nash 1974; Ghabrial 1980). Some VLPs do however, exhibit a range of phenomena in their hosts, such as killer traits in *S. cerevisiae* (Bevan *et al.* 1973) and *U. maydis* (Koltin & Kandel 1978), hypovirulence in *C. parasitica* (Day *et al.* 1977), *O. novo-ulmi* (Brasier 1983) and *H. victoriae* (Ghabrial 1986), modulation of virulence in *R. solani* (Tavantzis 1988) and gene silencing in a variety of hosts.

Multiple infections with different cytoplasmic and/or mitochondrial dsRNA elements are common in fungi (Hollings 1962; Barton & Hollings 1979; Buck 1986). These multiple virus infections can be from different or the same virus families. A few examples are, two viruses found in a single *Helminthosporium victoriae* isolate i.e. a totivirus (Huang & Ghabrial 1996) and a chrysovirus (Ghabrial *et al.* 2002). In *Gremmeniella abietina* var. *abietina* type A, three viruses were found i.e. a totivirus (*G. abietina* RNA virus L2 or GaRV-L2), a partitivirus (*G.*

*abietina* RNA virus MS2 or GaRV-MS2) and a mitovirus (*G. abietina* mitochondrial RNA virus S2 or GaMRV-S2) (Tuomivirta & Hantula 2005). Two viruses were found in a single *Rhizoctonia solani* isolate i.e. an unclassified virus related to plant bromoviruses (Jian *et al.* 1998) and a mitovirus (Lakshman *et al.* 1998). Four mitoviruses were found in a single *Ophiostoma novo-ulmi* isolate (Hong *et al.* 1998, 1999) and two partitiviruses in a single *Helicobasidium mompa* isolate (Osaki *et al.* 2004).

The interaction of multiple virus infections and their combined effects are less well studied. In *C. parasitica*, Sun *et al.* (2006) demonstrated the synergistic effect of dual infections with a hypovirus (CHV1-EP713) and a mycoreovirus (MyRV1-Cp9B21). More severe reductions in growth rate and sporulation were observed, relative to single infections with either virus (Sun *et al.* 2006). The dual infection however, only enhanced the replication and transmission of the mycoreovirus, while that of the hypovirus was unaffected.

DsRNA elements are ideal for genetic manipulation to potentially mediate biological control due to their small, elementary genomes and basic composition (Buck 1986; Nuss & Koltin 1990; Ghabrial 1994; Nuss *et al.* 2002). The only obstacle is the construction of transformation or transfection systems with which dsRNA-free isolates of the plant pathogenic fungus can be infected with the manipulated dsRNA elements. Transfection systems have however, been successfully developed for the *C. parasitica* hypovirus (CHV-1) (Dawe & Nuss 2001) and the *Diaporthe* RNA virus (DaRV) (Moleleki *et al.* 2003). These infectious cDNA-based reverse genetic systems enable detailed studies of virus-host interactions, fungal pathogenesis mechanisms, fungal signaling pathways, evolution of RNA silencing and engineering of mycoviruses for enhanced biocontrol properties (Nuss 2005).



### 5.2.2 Hypovirulence-mediated dsRNA elements as biocontrol agents

DsRNA elements that confer hypovirulence are increasingly being considered as biocontrol agents for plant pathogenic fungi. Hypovirulence refers to the spontaneous reduction in virulence of the pathogen and is linked to the presence of dsRNA elements (Anagnostakis 1988; McCabe & Van Alfen 2002). In order for this type of biocontrol to be effective, an understanding of the interaction among the tree, fungus and virus is essential. All factors reducing the rate of the disease epidemic and those enhancing the establishment of the hypovirus need to be considered (Heiniger & Rigling 1994; Milgroom & Cortesi 2004).

Effective transmission of the dsRNA elements is essential to allow natural spread of the virus through a population (Heiniger & Rigling 1994; Milgroom & Cortesi 2004). Transmission of mycoviruses occurs either horizontally (hyphal anastomosis) or vertically (cell division and spore production) and is controlled through vegetative incompatibility (*vic*) genes (Buck 1986; Liu & Milgroom 1996; Milgroom & Brasier 1997; Cortesi & Milgroom 1998; Milgroom 1999). If all the *vic*-genes of two fungal isolates are identical, they can anastomose, transmit dsRNA elements and produce heterokaryons (Buck 1986; Liu & Milgroom 1996). If all their *vic*-genes are different, they are incompatible, no heterokaryons are produced and programmed cell death occurs. Partial transmission of dsRNA elements has been reported where a few *vic*-genes are different (Liu & Milgroom 1996). *Vic* loci from several fungi have been characterized e.g. in *C. parasitica* in Europe, six unlinked *vic* loci with 2 alleles were described, which can result in 64 genotypes (Cortesi & Milgroom 1998).

The first successful implementation of dsRNA elements as hypovirulence-mediated biocontrol agents was achieved in the chestnut blight pathogen, *C. parasitica* (Anagnostakis 1982; Van Alfen 1982; Fulbright *et al.* 1983; Griffin 1986; MacDonald & Fulbright 1991; Nuss 1992;

Heiniger & Rigling 1994; Dawe & Nuss 2001; Milgroom & Cortesi 2004). Hypovirulence-inferring viruses of *C. parasitica* mainly belong to the family *Hypoviridae* (Shapira *et al.* 1991a; Hillman *et al.* 1994; Smart *et al.* 1999) and four species have been described namely *Cryphonectria parasitica hypovirus* (CHV-I, CHV-2; CHV-3; CHV-4) (Hillman & Suzuki 2004). However, only CHV-1, CHV-2 and CHV-3 confer hypovirulence (Milgroom & Cortesi 2004). Four other viruses with apparently no phenotypic effects on *C. parasitica* have also been found, of which one is a mitochondrion-associated dsRNA element (Peever *et al.* 1997). All the dsRNA elements associated with *C. parasitica*, as well as the presence of defective and satellite dsRNAs contribute to the complexity of the hypovirulence-associated phenotype (Shapira *et al.* 1991b).

Common phenotypic changes observed in CHV-infected *C. parasitica* isolates are a reduction in growth, reduction or absence in sexual or asexual reproduction, changes in pigment production and changes in virulence (Nuss & Koltin 1990; Nuss 1992; McCabe & Van Alfen 2002; Nuss *et al.* 2002). These phenotypic changes are the result of dsRNA elements disrupting the normal developmental processes of the fungus by producing secondary metabolites like antibiotics and toxins (Nuss 1996; McCabe & Van Alfen 2002). These foreign viral metabolites interact with the fungal G proteins (GTP-binding proteins) and consequently disrupt normal signal transduction pathways (Nuss 1996; McCabe & Van Alfen 2002).

Recently, the first evidence of RNA silencing as an antiviral defense mechanism was demonstrated in *C. parasitica* (Segers *et al.* 2007). RNA silencing refers to the RNA-mediated sequence-specific suppression of gene expression. In the study conducted by Segers *et al.* (2007), the effects of dicer gene disruptions upon mycovirus infections were examined. The dicer genes code for endonucleases that process structured or dsRNA into small interfering RNA

(siRNAs) of 21-24 nt. These siRNAs are incorporated into the RNA-induced silencing complex reversing the effect hypovirulence-associated dsRNA elements normally have on this fungus. Hypovirulence-mediated biocontrol using CHV-1 in controlling chestnut blight was only successful in Europe and in a few isolated cases in North America. This is primarily due to the larger number of vegetative incompatibility (*vc*) groups in the North American *C. parasitica* population compared to that of the European population (Anagnostakis *et al.* 1986; Heiniger & Rigling 1994; Nuss *et al.* 2002; Milgroom & Cortesi 2004). The diversity of the host thus gave an early indication of the success of the hypovirulence-associated dsRNA viruses as they are dependent on the host for migration (Peever *et al.* 1997). Any factors that further enhance the establishment of the hypovirus, like environmental conditions and host species play a role in ensuring successful biocontrol (Milgroom & Cortesi 2004).

### **5.2.3 DsRNA elements in *Diplodia pinea***

Several dsRNA elements ranging from 400 bp – 9 kb in size have been reported from *D. pinea sensu lato* (Wu *et al.* 1989; Preisig *et al.* 1998; Steenkamp *et al.* 1998; De Wet *et al.* 2001; Adams *et al.* 2002). Two of these elements, isolated from a single, South African *D. pinea* isolate, have been characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2) (Preisig *et al.* 1998). These two viruses belong to the family *Totiviridae* and the genus *Totivirus* (Fig.1). They are characterized by monopartite dsRNA genomes in the 5 kb size range with two open reading frames (ORFs), one coding for a capsid polypeptide and the other for a RNA-dependant RNA polymerase (RdRp).

Members of the *Totiviridae* are hypothesized to have the most ancient origin, most probably a non-infectious, single cell, virus progenitor that predates the differentiation of protozoans and fungi (Koonin *et al.* 1989; Bruenn 1993; Ghabrial 1998) (Fig. 3). The ability of this family of

viruses to infect a wide host range including yeasts, fungi and protozoa is furthermore, an indication of its ancient origin. Mycoviruses in the *Totiviridae* (SsRV1, SsRV2 and Hv190SV) have a higher degree of sequence homology to one another than to members of the same family infecting protozoa (*Giardiaviruses* and *Leishmaniaviruses*) or yeasts (*S. cerevisiae* L-A virus) (Preisig *et al.* 1998). These mycoviruses are also hypothesized to be the ancestors of those belonging to the *Partitiviridae* (Oh & Hillman 1995; Ghabrial 1998) (Fig. 3).

DsRNA elements in *D. pinea sensu lato* are transmitted via hyphal anastomosis. Transmission can also occur via conidia and Adams *et al.* (2002) reported a 70 - 100 % transmission rate. As mentioned previously, the genetic diversity of the host population is a restrictive factor in the transmission of dsRNA elements. Therefore, the implementation of dsRNA elements as biocontrol agents would be limited to populations of *D. pinea* with low genetic diversities.

DsRNA elements associated with *D. pinea sensu lato* have been reported to have no effect on the virulence of their hosts or result in any phenotypic changes (Wu *et al.* 1989; Preisig *et al.* 1998; Steenkamp *et al.* 1998; De Wet *et al.* 2001). Adams *et al.* (2002) did, however find one A morphotype isolate that was significantly more virulent when cured of its dsRNA, thus showing potential of having dsRNA-mediated hypovirulence. They used the AMMI (additive main effects and multiplicative interaction) model to obtain a more accurate estimation of relative virulence by partitioning the effects of genotype and environmental factors on the virulence. This model quantifies the sensitivity of the response of an isolate or tree species to the year, in the host-pathogen-year interaction. Interestingly, they found that dsRNA-containing parent strains could either be more or less virulent than dsRNA-free subcultures depending on year (environmental factors) and specific pine species. DsRNA-free subcultures also tended to be

more virulent in one year and less in the following year. DsRNA infections thus tend to moderate interactivity in *D. pinea* isolates of both the A and B morphotypes (Adams *et al.* 2002). Co-infections with both SsRV1 and SsRV2 are known to occur in *D. pinea* (Preisig *et al.* 1998) but the interaction between multiple infections and their cumulative effect has not been studied. Therefore, the unknown dsRNA elements associated with *D. pinea sensu lato* need to be characterized and their distribution in the different morphotypes of the fungus assessed, before they can be considered for exploitation as potential biocontrol agents against Diplodia die-back of pines.

## 6. CONCLUSIONS

The *Diplodia pinea sensu lato* species complex, like most species of the Botryosphaeriaceae, represents a suite of well-known pathogens causing disease symptoms such as die-back and cankers on numerous woody and non-woody hosts. These fungi have been notoriously difficult to identify accurately due to their having very similar morphological characteristics. This difficulty has been substantially alleviated since DNA-based phylogenetic studies have become available. The circumscription of members of the *D. pinea sensu lato* species complex, therefore, needs to be addressed.

Management of *Diplodia*-induced diseases is typically based on planting of resistant pine species and silvicultural practices that reduce stress on these trees. Huge economic losses, however, still occur especially in plantations of non-native pine species and in hail-prone areas such as the summer rainfall regions of South Africa. As our knowledge of the *D. pinea sensu lato* species complex increases and especially that pertaining to the distribution, virulence and genetic structure of the pathogen populations, it should be possible to refine management practices.

The exploitation of dsRNA elements that naturally occur in *D. pinea sensu lato* could potentially augment disease management strategies. This would need to follow an approach similar to that used for the chestnut blight pathogen, *C. parasitica*. Several dsRNA elements have been reported from *D. pinea sensu lato*, two of which been characterized. However, characterization of the novel dsRNA elements is needed. Furthermore, an assessment of the distribution of these dsRNA elements is needed in order to promote a deeper understanding of the genetic structure of virus populations in their fungal hosts. Although, no phenotypic effects have thus far been associated with dsRNA elements in *D. pinea sensu lato*, knowledge of their presence and of their genome organization could provide a foundation to engineer them towards inducing hypovirulence.

There is clearly much to learn regarding the biology, taxonomy and ecology of *D. pinea sensu lato* and the role these factors play in the evolution of the Botryosphaeriaceae. In the studies making up this dissertation, I will consider the taxonomy of *D. pinea sensu lato* and related fungi. Furthermore, I will characterize a novel dsRNA element more commonly associated with the B morphotype of *D. pinea* and determine the frequency and distribution of different viruses in isolates of *D. pinea sensu lato*. The longer term view is that these dsRNA elements might prove useful in promoting hypovirulence in the pathogen complex. In this way, they could contribute to an integrated biological control strategy for Diplodia die-back of pines in South Africa.

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Figure 1. Ten lineages of the Botryosphaeriaceae based on sequences of the large ribosomal subunit as described by Crous *et al.* 2006. Clade 11 and 12 represent *Camarosporium/Microdiplodia* and *Stenocarpella*, respectively. They are considered to group outside the Botryosphaeriaceae.

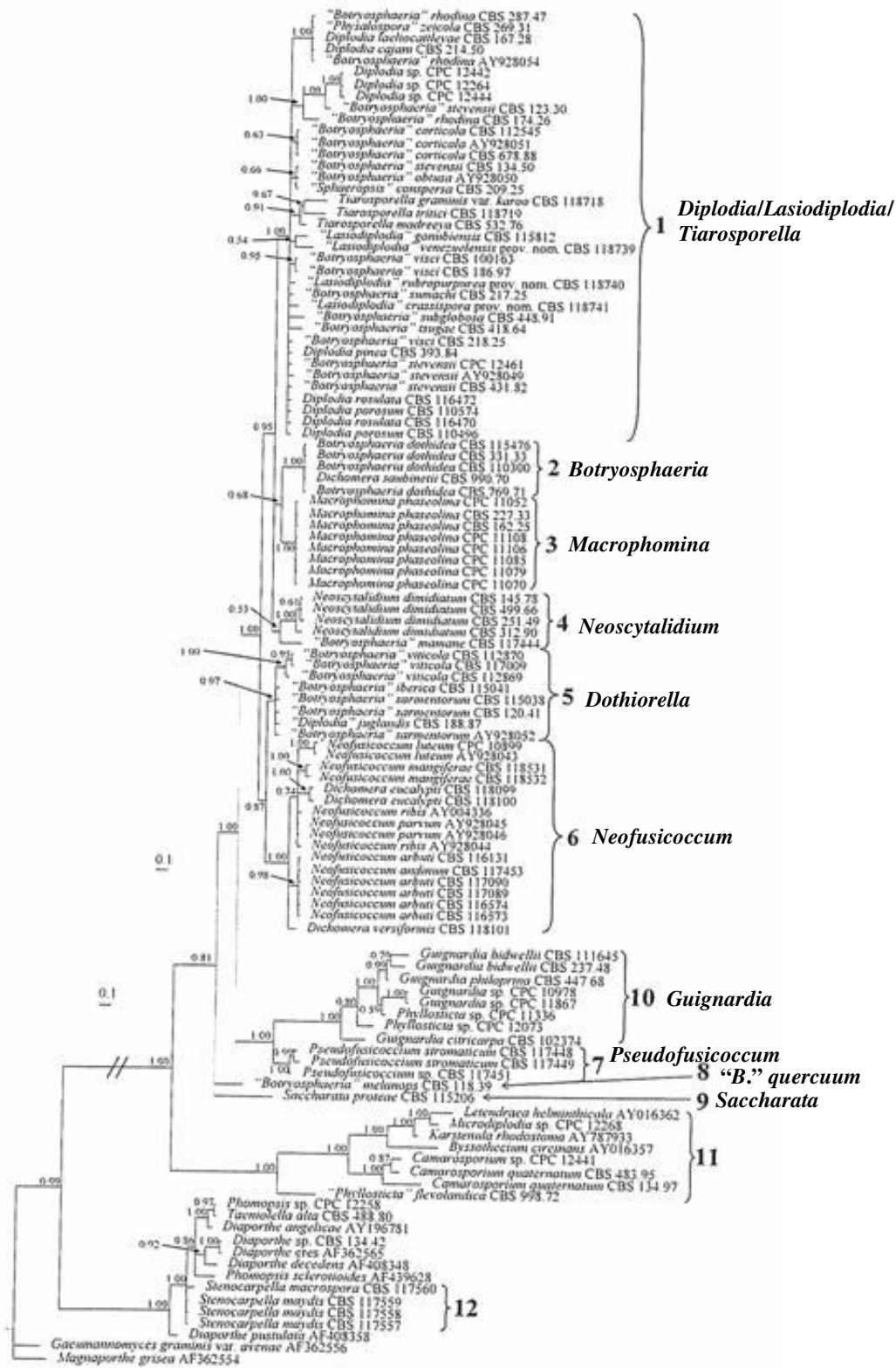




Figure 2. A schematic representation of the current classification system of mycoviruses according to The Universal Virus Database of the International Committee on the Taxonomy of Viruses (ICTVdB). Families, genera and types species of the mycoviruses are shown.

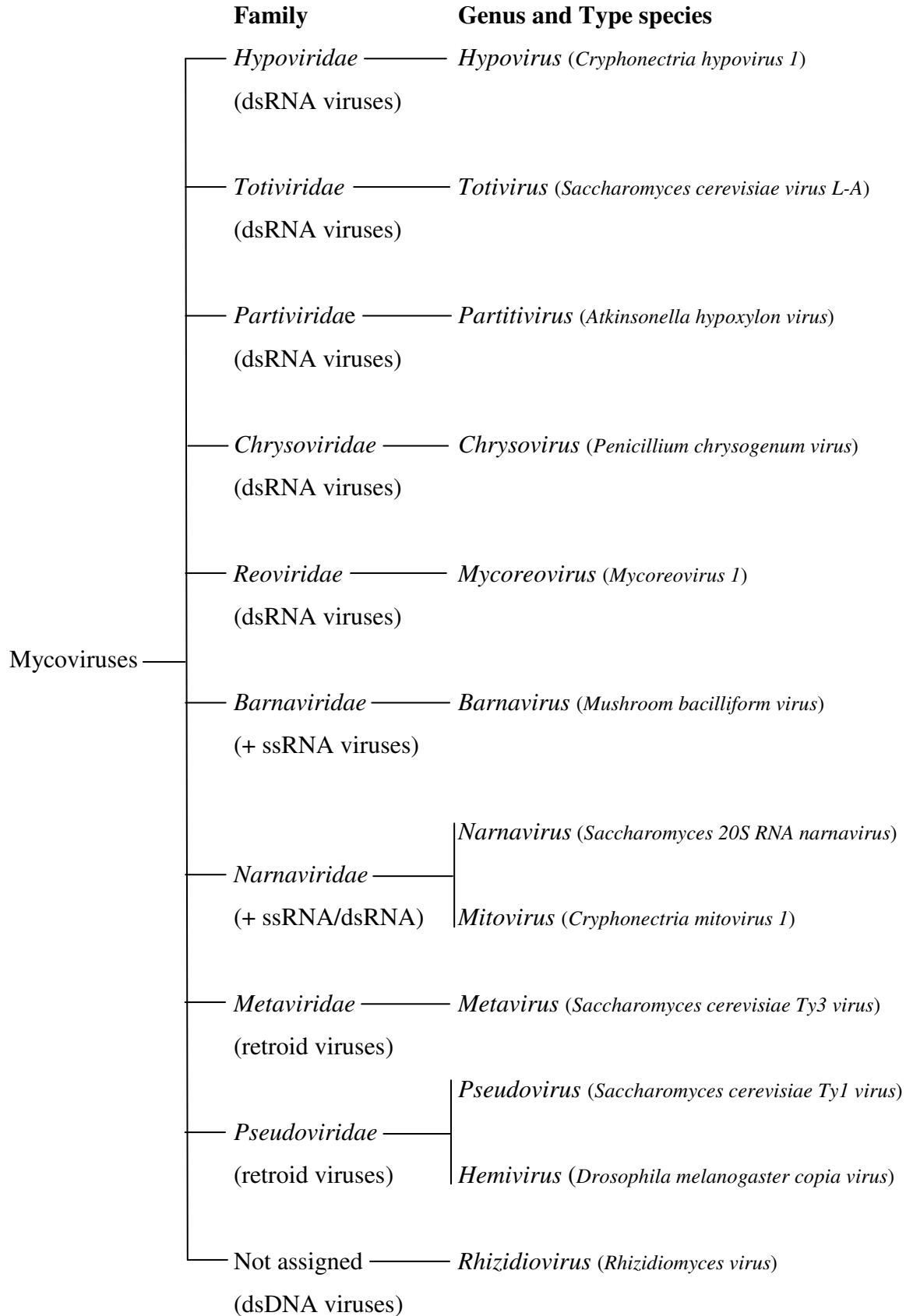
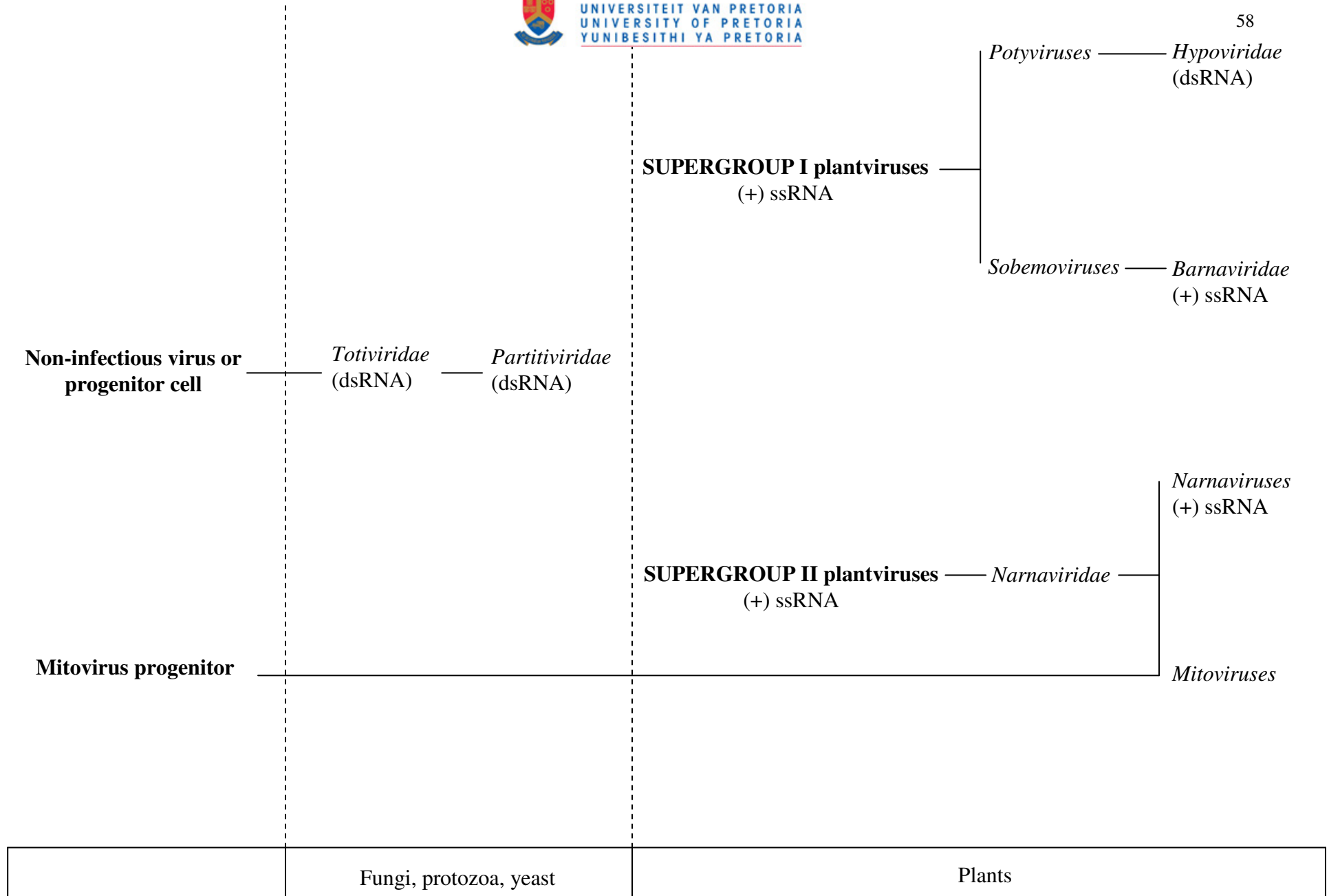


Figure 3. A schematic representation of the evolutionary pathways of mycoviruses and their closest relatives among the plant viruses.







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## CHAPTER 2

**MULTIPLE GENE GENEALOGIES AND MICROSATELLITE  
MARKERS REFLECT RELATIONSHIPS BETWEEN MORPHOTYPES  
OF *SPHAEROPSIS SAPINEA* AND DISTINGUISH A NEW SPECIES OF  
*DIPLODIA***

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## ABSTRACT

*Sphaeropsis sapinea* is an opportunistic pathogen causing serious damage to conifers, predisposed by adverse environmental conditions or mechanical damage. Three different morphological forms of the fungus have been described and are commonly referred to as the A, B and C morphotypes. Isolates of the different morphotypes have also been separated based on differences in pathogenicity and molecular characteristics. These differences, however, overlap and have not been considered sufficiently robust to justify the description of separate taxa. The aim of this study was to consider relationships between isolates representing different *S. sapinea* morphotypes, using multiple gene genealogies, inferred from partial sequences of six protein-coding genes and six microsatellite loci. Genealogies generated for the protein-coding genes and microsatellite loci were not congruent but both consistently grouped isolates representing the A and C morphotypes in separate but closely related clades. In both analyses, isolates of the B morphotype grouped together in a clade that was equally different to the A and C morphotypes, as it was to the clade encompassing isolates of *Botryosphaeria obtusa*. These results provide strong evidence to show that the B morphotype isolates are distantly related to *S. sapinea* and represent a discrete taxon, which we describe here as *Diplodia scrobiculata*.

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## INTRODUCTION

*Sphaeropsis sapinea* (Fr.) Dyko & Sutton (= *Diplodia pinea* (Desm.) Kickx.) is a latent, opportunistic pathogen of conifers occurring world-wide (Eldridge 1961; Swart & Wingfield 1991). It can have devastating effects on trees when it is associated with stress-inducing factors such as drought, hail, adverse temperatures or mechanical wounding (Purnell 1957; Chou 1987). *Sphaeropsis sapinea* causes extensive losses in commercial plantation forestry, especially where susceptible *Pinus* spp. are intensively propagated (Zwolinski *et al.* 1990). Three distinct morphotypes (A, B and C) have been described for *S. sapinea*. The A morphotype is characterised by fluffy mycelium and smooth conidial walls, while the B morphotype has mycelium appressed to the surface of the agar and pitted conidial walls (Wang *et al.* 1985; Wang *et al.* 1986; Palmer *et al.* 1987). C morphotype isolates have fluffy mycelium and smooth conidial walls similar to the A morphotype, but the conidia are significantly longer in the C morphotype (De Wet *et al.* 2000). Isolates of the C morphotype are also considerably more pathogenic than those of the A morphotype (De Wet *et al.* 2002). An I morphotype of *S. sapinea* has been described as being intermediate between the A and B morphotypes (Hausner *et al.* 1999), but subsequent studies based on SSR markers (Burgess *et al.* 2001a) showed that this fungus represents the anamorph state of *Botryosphaeria obtusa* (Schw.) Shoemaker.

The authenticity of the morphotypes of *S. sapinea* has been confirmed using DNA-based techniques, such as randomly amplified polymorphic DNA (RAPDs) (Smith & Stanosz 1995; De Wet *et al.* 2000), restriction fragment length polymorphisms (RFLPs) (Hausner *et al.* 1999) and DNA sequences of the rRNA operon (De Wet *et al.* 2000). More recently, ISSR (inter simple or short sequence repeats) fingerprinting and SSR (simple sequence repeats) markers have also been used to provide increased resolution to the differentiation between these morphotypes (Burgess *et al.* 2001a; Zhou *et al.* 2001). These techniques alone,

however, are not always informative when comparing closely related species or elements of the same species. This weakness can be resolved by using genealogies inferred from multiple protein-coding genes combined with highly polymorphic microsatellite loci (Geiser *et al.* 1998; Fisher *et al.* 2000; Koufopanou *et al.* 2001; Steenkamp *et al.* 2002). In this study, our aim was to construct multiple gene genealogies from partial sequences of six protein-coding genes (Bt2 of  $\beta$ -tubulin, chitin synthase [CHS], elongation factor 1 $\alpha$  [EF-1 $\alpha$ ], actin [ACT], calmodulin [CAL] and glutaraldehyde-6-phosphate [GPD]), and six microsatellite loci (SS5, SS7, SS8, SS9, SS10 and SS11) to elucidate the phylogenetic relationships between isolates of *S. sapinea* representing the different morphotypes.

## **MATERIALS AND METHODS**

### **Fungal isolates**

Eleven *S. sapinea* isolates (Table 1) from the United States, Australia, Mexico, California and Indonesia were used in this study. These isolates represented all three morphotypes described for *S. sapinea*. Four isolates of the closely-related species *B. obtusa*, were included for comparison and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (*B. rhodina* (Cooke) Arx) was used as an outgroup taxon (Table 1). The *S. sapinea* isolate from South Western Australia was obtained by direct isolation from the pith tissue of *P. radiata* cones, and those from Mexico from *P. greggii* cones. The Indonesian and Californian isolates were obtained from pycnidia on *P. patula* or *P. radiata* shoots, with die-back symptoms. Single conidial cultures were generated for all the isolates and cultured on 2 % Malt Extract Agar (MEA) (2 % m/v Biolab malt extract; 2 % m/v Biolab agar) in Petri dishes at 25 °C. All the single conidial cultures were transferred to 2 % MEA slants in McCartney bottles and stored at 4 °C. All isolates are maintained in the Culture Collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Representative isolates have also been deposited in the

Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands and the National Collection of Fungi (PREM), Pretoria, South Africa.

### **DNA extractions**

The single conidial isolates (Table 1) were grown in liquid ME medium in 1.5 ml Eppendorf tubes, for one week at 25 °C. After centrifugation, the mycelium pellet was freeze dried and DNA was extracted using the technique described by Raeder & Broda (1985). The DNA concentrations of the samples were determined against a standard molecular marker and diluted to 5 ng/μl for further studies.

### **Amplification of partial protein-coding genes and microsatellite loci**

The Bt2 regions of the β-tubulin gene (Glass & Donaldson 1995), parts of five other protein-coding genes (Carbone & Kohn, 1999) and six microsatellite loci (Burgess *et al.* 2001a) were amplified for 14 isolates (Table 1). The 25 μl reaction mixture consisted of 2.5 μl Expand PCR buffer (2 mM Tris-HCl, pH 7.5; 1.5 mM MgCl<sub>2</sub>; 10 mM KCl), 100 μM of each dNTP, 300 nM of each primer, 2 ng template and 0.25 U Expand High<sup>TM</sup> Fidelity *Taq* polymerase (Roche Biochemicals). The following temperature profile was followed: 2 min at 94 °C, 10 cycles of 30 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C, the last three temperature intervals were repeated for another 30 cycles with a 5 s increase per cycle for the elongation step at 72 °C.

### **Sequencing**

PCR products were visualised on a 1 % agarose gel containing ethidium bromide using UV illumination. The PCR products were purified using the Roche High Pure PCR product purification kit (Roche Diagnostics). Both DNA strands were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI Prism<sup>TM</sup> 377 DNA sequencer (Applied Biosystems, Warrington WA1 4SR, UK). All the reactions were done

using protocols recommended by the manufacturers. Sequence data for all the isolates (Table 1) were processed using Sequence Navigator version 1.0.1 (Perkin Elmer) and manually aligned.

### **Phylogenetic analyses**

Parsimony and distance analyses were performed on the individual data sets, as well as the combined data sets after partition homogeneity tests were performed on the individual protein-coding gene and microsatellite sequences using PAUP (Smithsonian Institution, 1993). A partition homogeneity test was also performed to test whether the protein-coding and microsatellite genealogies could be combined. In all cases, parsimony analyses were based on a strict heuristic search with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum length was zero. Bootstrap values were determined after 1000 replications and only groups with frequencies >50 % were retained. Distances were determined using “neighbour-joining” with an uncorrected “p” parameter.

## **RESULTS**

### **Amplification and sequencing of protein-coding genes and microsatellite loci**

Portions of six protein-coding genes and six microsatellite loci were successfully amplified for *S. sapinea* and *B. obtusa*, while only protein-coding gene regions could be amplified for *B. rhodina* isolates. Sequences generated from the amplification products ranged from 170 – 565 bp in length. Introns occurring in the partial gene sequences of Bt2 of  $\beta$ -tubulin, EF-1 $\alpha$ , ACT, CAL and GPD and the sequences flanking the microsatellites were included in the phylogenetic analyses.

### **Phylogenetic analyses**

Neighbour-joining distance phylograms were generated for each of the six protein-coding genes with bootstrap values (Fig. 1). The partition homogeneity test showed that no

significant conflict exists between the phylogenies of the individual protein-coding genes ( $P=0.01$ ). The individual sequences were consequently combined into one data set containing 2272 characters, of which 62 variable characters were parsimony informative, 238 were parsimony uninformative and the remainder were constant.

Neighbour-joining distance phylograms were also generated for each of the six microsatellite loci (Fig. 2). The partition homogeneity test on these data also showed that no significant conflict exists between the individual microsatellite phylogenies ( $P=0.01$ ). They were thus combined into one data set containing 1783 characters, of which 146 variable characters were parsimony informative, 263 were parsimony uninformative and the remainder were constant.

The partition homogeneity test showed that significant conflict exists between the combined microsatellite and the combined protein-coding gene phylogenies ( $P=0.26$ ) and that they could not be combined. Three distinct clades with bootstrap values higher than 50 % emerged from the combined neighbour-joining distance phylogram, generated from the protein-coding gene sequences, as well as the microsatellite sequences (Fig. 3). One clade included all the A and C morphotype isolates of *S. sapinea*. These isolates were closely related but clearly distinguishable from each other. A second clade contained all of the B morphotype isolates. A third clade contained *B. obtusa* isolates together with isolates (CMW8230 and CMW8231), previously described as the I morphotype of *S. sapinea* (Hausner *et al.* 1999) and now known to represent *B. obtusa* (Burgess *et al.* 2001a). The clade containing the B morphotype isolates was equally distant from the clade encompassing the A and C morphotype isolates, as it was from that including isolates of *B. obtusa*.

High levels of sequence similarity were observed for *S. sapinea* isolates representing the A and C morphotypes and no correlation to geographical distribution were observed for them. Isolates of the B morphotype encompassed a high degree of genetic diversity and groupings according to geographical origin were observed. Based on the protein-coding gene



genealogy, the B morphotype isolates from the United States (CMW189, CMW5870, CMW8228, CMW4898, CMW4900) grouped separately from the single European B morphotype isolate (CMW8753). The microsatellite genealogy could, furthermore, differentiate the B morphotype isolates from the United States into three sub-clades, one from the Central US (CMW189, CMW4334), one from California (CMW5870, CMW8228) and one from Mexico (CMW4898, CMW4900).

### **Taxonomy**

The results of the phylogenetic comparisons presented in this study provide robust evidence to justify treating isolates of the B morphotype of *S. sapinea* as a discrete taxon. We, therefore, provide the following description for the fungus:

*Diplodia scrobiculata* J. de Wet, B. Slippers & M.J. Wingfield anam. sp.nov.

(Figs 4-10)

Etym.: Latin, *scrobiculata* = minutely pitted, in reference to the texture of the conidial walls.

*Culturae* colonias supra submurinas vel murinas, infra atomurinas, marginibus sinuatis faciunt. Coloniae creverunt optime ad 25°, et superficiem medii in 8 diebus velabant.

Mycelium atratum septatum ad agarum appressum. *Conidiomata* in foliis pinorum pycnidialia, mycelio obtecta. Pycnidia atro-vinaceo-brunnea in foliis pinorum vel in agaro immersa, 150  $\Phi$ m diametro. *Cellulae conidiogae* holoblasticae, proliferatione percurrenti limitata, ut videtur annellationibus paucis, 10  $\Phi$ m diametro. *Conidia* clavata vel truncata, 1–3 septata, parietibus crassis, scrobiculatis, atrovinacea vel atrobrunnea, 39.4 H 14.1  $\Phi$ m.

*Cultures* (Fig 4) pale mouse grey(15''d) to mouse grey (15''m) viewed from the top of the Petri dish, dark mouse grey (15''k) to fuscous black (13''m) viewed from the bottom of the Petri dish, colonies with sinuate edges; optimal growth at 25°C, covering the medium surface in eight days. *Mycelium* dark, septate, appressed to the agar surface. *Conidiomata* (Fig 5) pycnidial, covered in mycelium, dark, immersed in pine needles or in the agar,

(100)150(250)  $\mu\text{m}$  diameter, single, papillate ostiole. *Conidiogenous cells* (Figs 6-7) discrete, dark, smooth, 10  $\mu\text{m}$  in diameter, holoblastic with limited percurrent proliferation seen as small numbers of annellations. *Conidia* (Figs 8-9) clavate to truncate, dark mouse grey (15''''k), (37.4)39.4(41.3)  $\mu\text{m}$  (12.8)14.1(15.5)  $\mu\text{m}$ , 1-3 septa, thick, pitted walls (Wang *et al.* 1986; Wang *et al.* 1985).

*Substratum*: Needles of *Pinus banksiana*, *P. resinosa*, *P. greggii*

*Distribution*: USA: Wisconsin, Minnesota, California; Mexico; Europe: France, Italy.

*Holotype*: **USA**: Wisconsin: Jackson County, *Pinus banksiana*. 1987, M.A. Palmer, (CMW189) in Herb. PREM57461.

*Paratypes*: **USA**: Minnesota, Wadena County, *Pinus resinosa*. 1987, G.R. Stanosz, (CMW4334); California, *Pinus radiata*. 2000, T. Gordon, (CMW5870, CMW8228);

**Mexico**: *Pinus greggii*. 1998, M.J. Wingfield, (CMW4898, CMW4900/CBS117836); all in Herb. PREM57462, PREM57463, PREM57464, PREM57465, PREM57466.

## DISCUSSION

Using multiple gene genealogies constructed from six protein-coding gene regions and six microsatellite-rich loci, we have been able to provide robust evidence showing that the B morphotype isolates of *S. sapinea* represent a distinct species. We have thus provided the name *D. scrobiculata* to this fungus. Our results also reinforce those of Zhou *et al.* (2001) using dominant ISSR markers and Burgess *et al.* (2001a) using co-dominant SSR markers, suggesting the A and B morphotypes of *S. sapinea* represent distinct taxa.

The construction of multiple gene genealogies has enabled us to infer reliable and consistent phylogenetic relationships between the morphotypes of *S. sapinea*. We found that isolates of the A and C morphotypes are much more closely related to each other, than they are to *D. scrobiculata*. *Diplodia scrobiculata* isolates were equally distant from those of the A and C

morphotypes of *S. sapinea*, as they were from isolates of *B. obtusa*. Phylogenetic relationships inferred from these gene genealogies corroborate results obtained using SSR markers, based on sizes (Burgess *et al.* 2001a). Therefore, in this case SSR markers alone would have been adequate to infer species level relationships, even though initial empirical studies have suggested otherwise (Fisher *et al.* 2000).

*Botryosphaeria* spp. are very difficult to distinguish based on their teleomorph morphology, but they can more easily be divided into two groups using anamorph characteristics. These represent a group with dark-spored conidia, best treated in the genus *Diplodia* and a group with predominantly hyaline conidia residing in *Fusicoccum* (Denman *et al.* 2000).

*Sphaeropsis sapinea* closely resembles the *Diplodia*-anamorphs of *Botryosphaeria* spp. (Denman *et al.* 2000; Jacobs & Rehner 1998) and was segregated from *Diplodia* based primarily on characteristics of conidial development (Sutton 1980). Phylogenetic data derived from this study provide substantial additional evidence to justify reverting to the name *Diplodia pinea* and in future, we recommend doing so.

No sexual state is known for any form of *S. sapinea*, although, together with *D. scrobiculata*, molecular evidence (Burgess *et al.* 2001a) shows that it clearly represents an anamorph of *Botryosphaeria*. Burgess, Wingfield & Wingfield (2001b) have also shown the A morphotype isolates representing *S. sapinea sensu stricto* are overwhelmingly clonal.

*Diplodia scrobiculata* isolates occasionally produce spermatia-like spores (Palmer *et al.* 1987), suggesting the presence of a sexual state in this fungus. Recent studies using SSR markers have shown a considerably higher degree of genetic diversity amongst isolates of *D. scrobiculata*, than those of the A and C morphotypes of *S. sapinea* (Burgess unpublished). *Diplodia scrobiculata* could represent a recently derived lineage of *Botryosphaeria*, which has only recently lost its ability to reproduce sexually. Alternatively, sexual reproduction in this fungus may possibly be suppressed by unfavourable conditions such as those in culture

and sexual structures may yet be found in nature. In contrast, we believe the A and C morphotypes of *S. sapinea* represent ancient lineages that have stabilised over time and have acquired a virtually clonal existence.

*Sphaeropsis sapinea*, as reflected by the A and C morphotypes of this fungus, appears to be native to and widely distributed across the natural range of *Pinus* species. The two morphotypes that represent this species differ in their distribution, host specificity and virulence. The A morphotype is common and has a wide distribution in Southern hemisphere countries including South Africa, Australia and New Zealand, where it was probably introduced together with pine seed imports (Burgess & Wingfield 2001; Swart *et al.* 1991). The C morphotype of *S. sapinea* has, thus far, been found only on *Pinus* spp. in Indonesia and isolates are significantly more virulent than those of the A morphotype (De Wet *et al.* 2002). *Diplodia scrobiculata* has a much more restricted distribution. The fungus was initially known only on *Pinus banksiana* and *P. resinosa* in the north central United States (Wang *et al.* 1985; Palmer *et al.* 1987), but has recently been reported from other conifers in Europe (Stanosz *et al.* 1999). There is no conclusive evidence to show that it has been introduced into pine growing areas of the southern hemisphere.

The wide array of phylogenetic comparisons presented in this study, provide robust evidence to support the description of *D. scrobiculata*. This is also supported by the results of other molecular genetic comparisons (Burgess *et al.* 2001a; Zhou *et al.* 2001), as well as useful morphological and ecological data previously published (Wang *et al.* 1985; Palmer *et al.* 1987; De Wet *et al.* 2002). Isolates of *D. scrobiculata* are characterized by dark, septate mycelium appressed to the surface of the agar. This is consistently different to *S. sapinea* isolates that have fluffy, aerial mycelium. Conidia of *D. scrobiculata* are dark brown with thick, pitted walls and 1-3 septa (Wang *et al.* 1985; Wang *et al.* 1986; Palmer *et al.* 1987).

Conidiogenous cells are holoblastic with annelidic proliferations and based on this characteristic, *D. scrobiculata* and *S. sapinea* are apparently indistinguishable.

*Sphaeropsis sapinea* was one of the earliest fungi to be recognised as a common inhabitant of *Pinus* spp. (Fisher 1912). It is also one of the best-known pathogens of *Pinus* spp. grown as exotics in the tropics and southern hemisphere (Burgess & Wingfield 2001). Thus, the discovery of taxonomically and ecologically meaningful differences in isolates of *S. sapinea* in the north central United States in the late 1980's, was relatively recent. During the past 15 years, substantial evidence has accumulated to show that these differences reflect both inter- and intraspecies variation. While the description of *D. scrobiculata* represents an important step in this process, the fungus is probably not of particular relevance in terms of pathology. *Diplodia scrobiculata* is known to be a very weak pathogen (Palmer *et al.* 1987) and it is probably best recognised as a relatively harmless endophyte. This is in contrast to the A and C morphotypes of *S. sapinea* that are important pathogens whose movement should be carefully managed.

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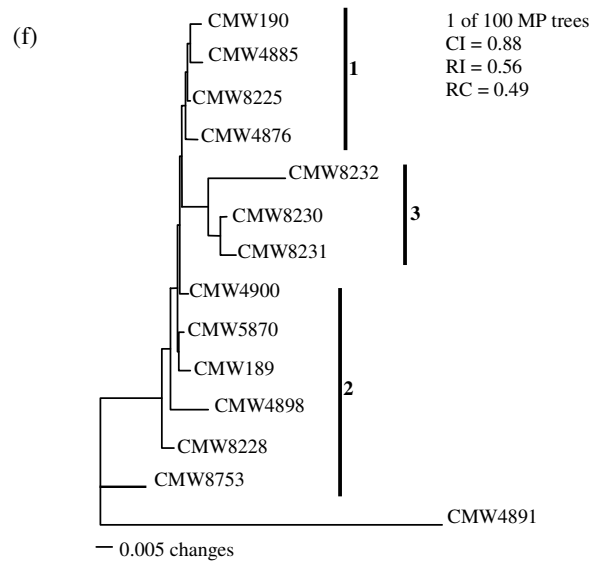
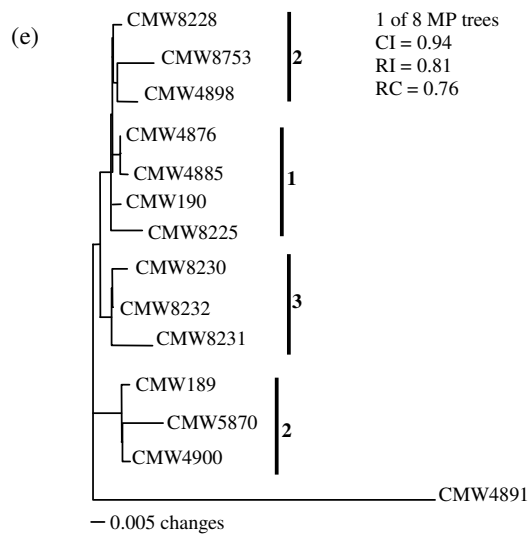
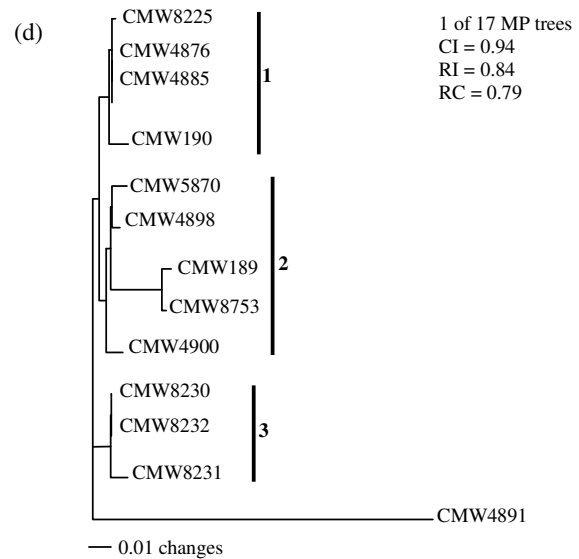
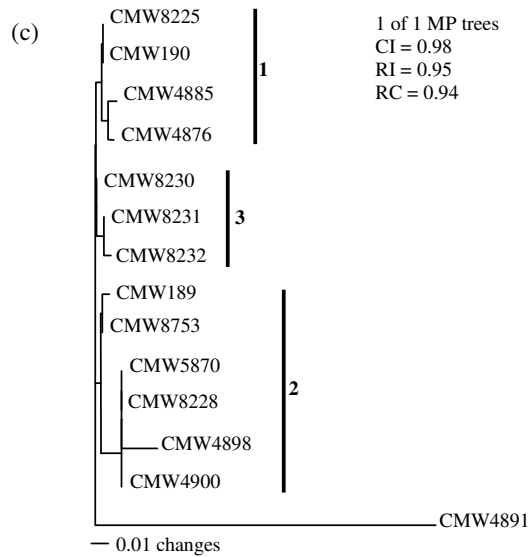
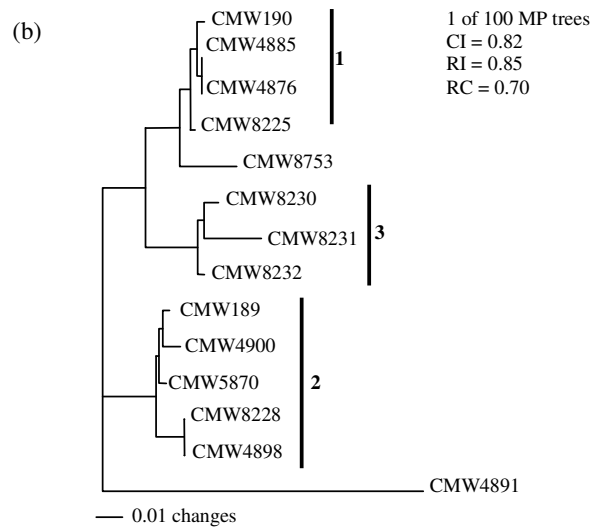
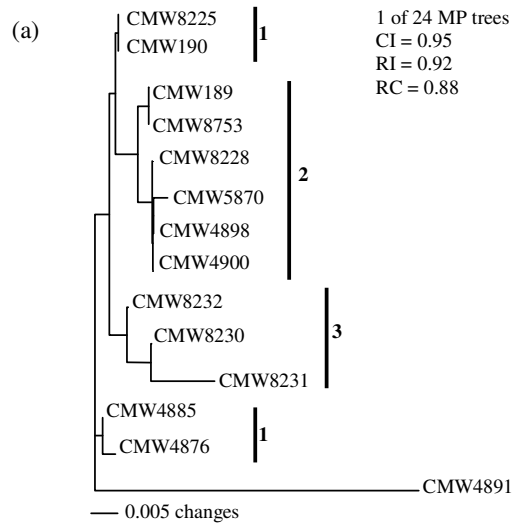
**Table 1.** Isolates used in this study.

| Isolates <sup>a/</sup> | Type/Species <sup>b/</sup>      | Origin        | Host                   | Collector      | Other collections <sup>c/</sup> | References                 |
|------------------------|---------------------------------|---------------|------------------------|----------------|---------------------------------|----------------------------|
| CMW8225                | A                               | Australia     | <i>Pinus radiata</i>   | T. Burgess     |                                 |                            |
| CMW190                 | A                               | United States | <i>P. resinosa</i>     | M.A. Palmer    |                                 |                            |
| CMW4885                | C                               | Indonesia     | <i>P. patula</i>       | M.J. Wingfield |                                 |                            |
| CMW4876                | C                               | "             | "                      | "              |                                 |                            |
| CMW5870                | B                               | California    | <i>P. radiata</i>      | T. Gordon      |                                 |                            |
| CMW8228                | B                               | "             | "                      | "              |                                 |                            |
| CMW4898                | B                               | Mexico        | <i>P. greggii</i>      | M.J. Wingfield |                                 |                            |
| CMW4900                | B                               | "             | "                      | "              |                                 |                            |
| CMW189                 | B                               | United States | <i>P. banksiana</i>    | M.A. Palmer    | 124                             | Palmer <i>et al.</i> 1987  |
| *CMW4334               | B                               | "             | <i>P. resinosa</i>     | G.R. Stanosz   | 474                             | Blodgett & Stanosz 1999    |
| ^CMW8753               | B                               | Italy         | <i>Pinus. sp.</i>      | L. Sparapano   | 97-73                           | Stanosz <i>et al.</i> 1999 |
| CMW8230                | <i>B. obtusa</i>                | Canada        | <i>Picea glauca</i>    | J. Reid        | 920729                          | Hausner <i>et al.</i> 1999 |
| CMW8231                | "                               | "             | <i>P. banksiana</i>    | "              | 810704                          | "                          |
| CMW8232                | "                               | South Africa  | <i>Malus domestica</i> | W.A. Smit      |                                 |                            |
| *CMW8233               | "                               | "             | "                      | "              |                                 |                            |
| ^CMW4891               | <i>Lasiodiplodia theobromae</i> | Indonesia     | <i>P. patula</i>       | M.J. Wingfield |                                 |                            |

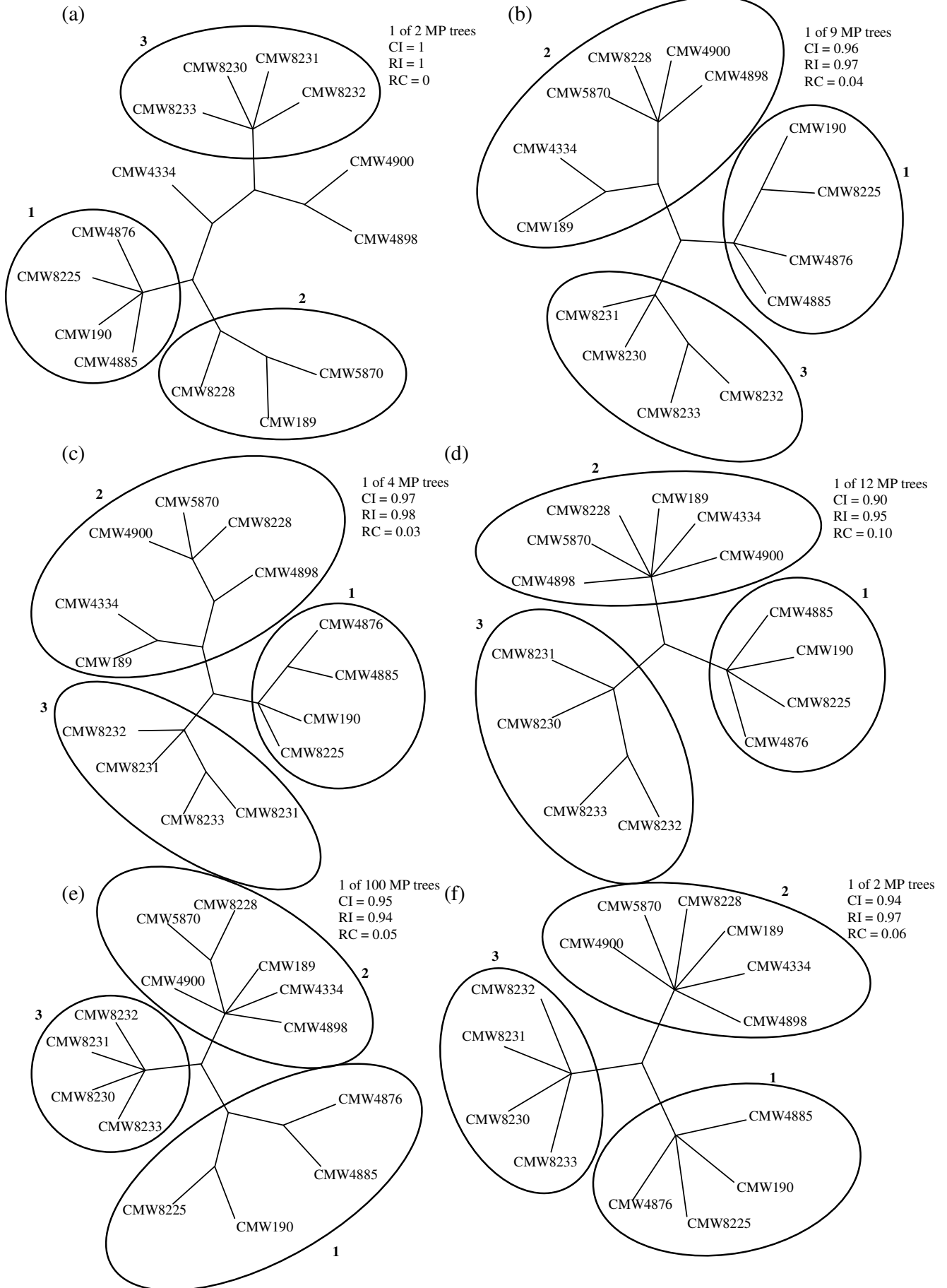
<sup>a/</sup>Isolates marked (\*) were included only in the microsatellite genealogy and those marked (^) were included only in the protein-coding gene genealogy. <sup>b/</sup>Morphotype designation for *D. pinea* is based on morphotype descriptions provided by Palmer *et al.* 1987; De Wet *et al.* 2000.

<sup>c/</sup>Isolation numbers used in previous studies for which references are provided in the last column.

**Figure 1.** Phenograms constructed for the partial sequences of six protein coding genes (a) Bt2 of the  $\beta$ -tubulin gene; (b) ACT; (c) EF-1 $\alpha$ ; (d) CAL; (e) CHS; (f) GPD using neighbour-joining distance analysis with an uncorrected “p” parameter and parsimony based on a strict heuristic search to determine bootstrap values. Bootstrap values were determined after 1000 replications and only groups with frequencies >50% were retained. Clade 1 = *Sphaeropsis sapinea* (A and C morphotypes), Clade 2 = *Diplodia scrobiculata*, Clade 3 = *Botryosphaeria obtusa*. MP = most parsimonious, CI = consistency index, RI = retention index, RC = reconstructed consistency index.



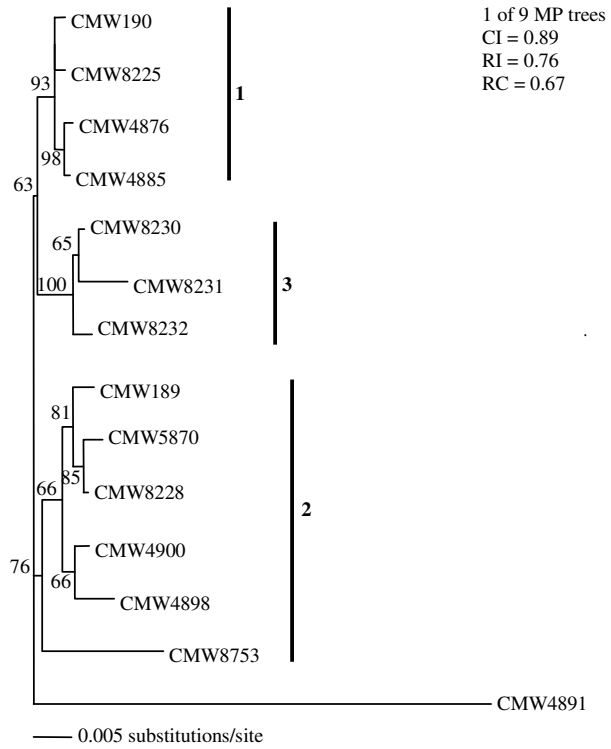
**Figure 2.** Phenograms constructed for sequence data of six SSR loci (a) SS5, (b) SS7, (c) SS8, (d) SS9, (e) SS10, (f) SS11 using neighbour-joining distance analysis with an uncorrected “p” parameter and parsimony based on a strict heuristic search to determine bootstrap values. Bootstrap values were determined after 1000 replications and only groups with frequencies >50% were retained. Clade 1 = *Sphaeropsis sapinea* (A and C morphotypes), Clade 2 = *Diplodia scrobiculata*, Clade 3 = *Botryosphaeria obtusa*. MP = most parsimonious, CI = consistency index, RI = retention index, RC = reconstructed consistency index.



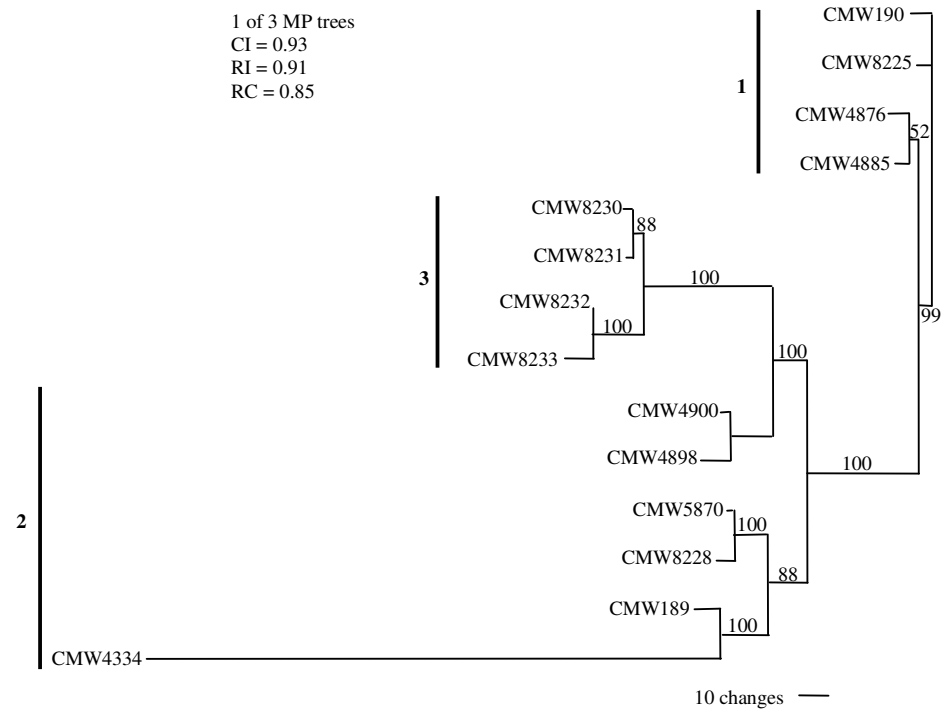
**Figure 3.** (a) Phenogram constructed for the combined sequences of the six protein-coding genes (b) phenogram constructed for the combined sequence data of six SSR loci using neighbour-joining distance analysis with an uncorrected “p” parameter and parsimony based on a strict heuristic search to determine bootstrap values. Bootstrap values were determined after 1000 replications and only groups with frequencies >50% were retained. Clade 1 = *Sphaeropsis sapinea* (A and C morphotypes), Clade 2 = *Diplodia scrobiculata*, Clade 3 = *Botryosphaeria obtusa*. MP = most parsimonious, CI = consistency index, RI = retention index, RC = reconstructed consistency index.



(a)

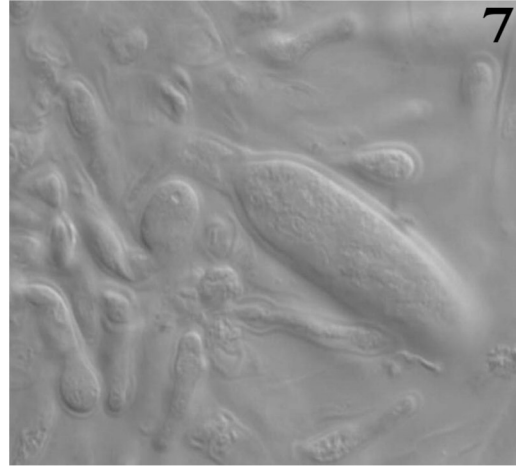
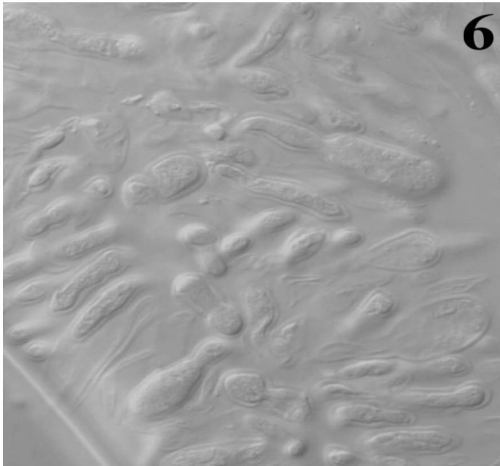
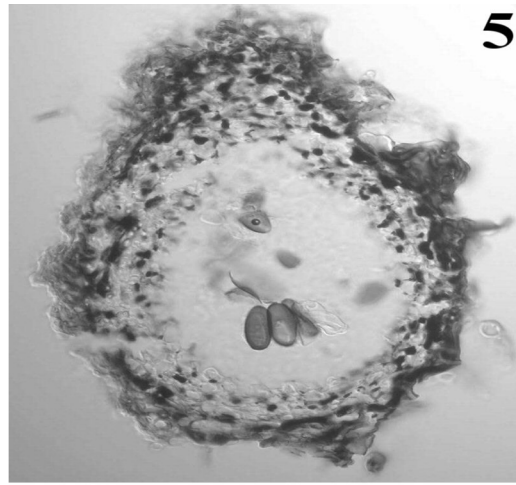
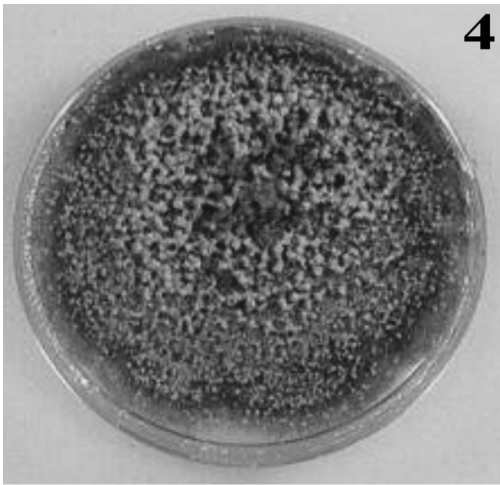


(b)



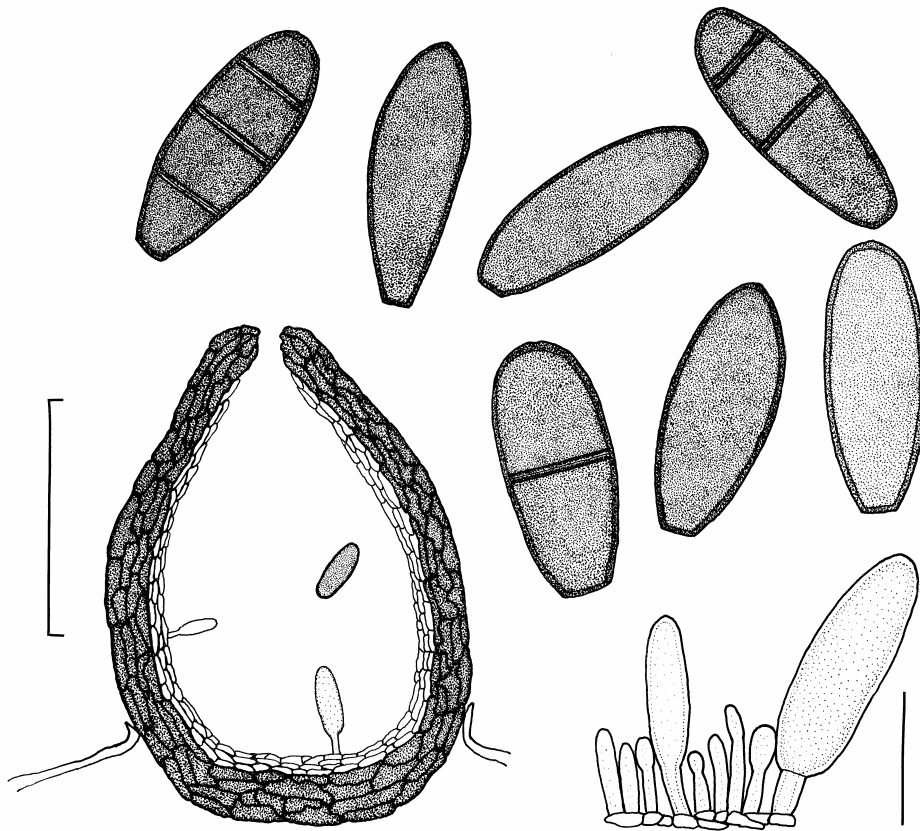


**Figures 4-9.** *Diplodia scrobiculata*. **Fig. 4.** Colony characteristics on malt extract agar. **Fig. 5.** Section through pycnidium with conidia. **Fig. 6-8.** Conidiophores with conidiogenous cells. **Fig. 9.** Conidia with up to three septa, scale bar = 10  $\mu\text{m}$ .





**Figure 10.** Illustration of (a) pycnidium, scale bar = 100  $\mu\text{m}$ , (b) conidiogenous cells and (c) conidia, scale bar = 20  $\mu\text{m}$ .





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## CHAPTER 3

# MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF *DOTHIORELLA CASUARINI* SP. NOV. AND OTHER BOTRYOSPHAERIACEAE WITH *DIPLODIA*-LIKE CONIDIA

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Submitted to *Mycologia*: De Wet J, Slippers B, Preisig O, Wingfield BD, Tsopelas P & Wingfield MJ.

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## ABSTRACT

Following recent changes to the taxonomy of the Botryosphaeriaceae, species with Diplodia-like (=dark, pigmented) conidia are considered to belong to at least three genera including *Diplodia*, *Lasiodiplodia* and *Dothiorella*. In a recent molecular phylogenetic study, it became apparent that two groups of isolates with Diplodia-like conidia required taxonomic revision. One group of isolates originated from *Cupressus sempervirens* in Greece and Cyprus and had previously been identified as *D. pinea* f.sp. *cupressi* based on morphological characteristics. The other isolates originated from a *Casuarina* sp. in Australia and were superficially similar to those in the first group based on their morphologically similar Diplodia-like conidia. The aim of this study was to resolve the taxonomy of these two groups of isolates by combining the information from the multiple gene genealogies with morphological characters. The results showed that the isolates from *C. sempervirens* in Greece and Cyprus represent *D. cupressi*. The isolates from *Casuarina* in Australia belong to the more distantly related genus *Dothiorella* and represent a distinct species that is described here as *Do. casuarini* sp. nov.

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## INTRODUCTION

Species of the Botryosphaeriaceae represent both pathogens and saprophytes of woody and non-woody plants (Denman *et al.* 2000; Crous *et al.* 2006). Some well-known species include the conifer pathogen, *Diplodia pinea* (Desm.) J. Kickx f. (Eldridge 1961; Swart & Wingfield 1991), the fruit tree pathogen, *D. seriata* De Not. (Phillips *et al.* 2007; Slippers *et al.* 2007), the blue stain-associated, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Mohali *et al.* 2005) and *Botryosphaeria dothidea* (Moug. Fr.) Ces. & De Not (Slippers *et al.* 2004a). In recent years, analyses of DNA sequence data have had a significant influence on the taxonomy of the Botryosphaeriaceae resulting in the description of ten generic lineages and various cryptic species (e.g. De Wet *et al.* 2003; Crous *et al.* 2006). Of particular relevance to this study is the fact that various investigations have shown that the genera *Diplodia*, *Lasiodiplodia* and *Dothiorella*, which all have anamorphs characterized by dark, pigmented conidia (Diplodia-like) and have been regarded as synonyms (Denman *et al.* 2000), are phylogenetically distinct (Phillips *et al.* 2005; Crous *et al.* 2006; De Wet *et al.* 2008).

*Diplodia* and *Lasiodiplodia* are well characterized genera of the Botryosphaeriaceae, but *Dothiorella* has only recently been re-erected as anamorph genus in this family (Phillips *et al.* 2005). Species of *Dothiorella* are morphologically most similar to those of *Diplodia*. However, the conidia of *Dothiorella* turn brown and 1-septate while still in the pycnidium and sometimes even when they are still attached to the conidiogenous cells. In contrast, those of *Diplodia* typically become dark and septate only after discharge from the pycnidium. Furthermore, in *Dothiorella* percurrent proliferation of the conidiogenous cells is extremely rare, while this form of conidium development is common in *Diplodia*. Interestingly, based on phylogenetic

inference, *Dothiorella* spp. are more closely related to *Neofusicoccum* spp. with hyaline conidia than they are to other genera with Diplodia-like conidia (Phillips *et al.* 2005).

*Dothiorella* is currently represented by four species namely *Do. pyrenophora* Sacc., *Do. sarmentorum* A.J.L. Phillips, Alves & Luque, *Do. iberica* A.J.L. Phillips, Luque & Alves and *Do. viticola* A.J.L. Phillips & Luque. *Dothiorella pyrenophora* is the type species of *Dothiorella* having conidia that are brown and one-septate while inside the pycnidial cavity and often still attached to the conidiogenous cells (Crous & Palm 1999; Phillips *et al.* 2005). *Dothiorella sarmentorum* has been reported from *Malus*, *Ulmus*, *Pyrus*, *Prunus* and *Menispermum*, and probably has a world-wide distribution (Phillips *et al.* 2005). *Dothiorella iberica* is known from *Quercus* and *Malus*, only in Italy and Spain (Phillips *et al.* 2005) and *Do. viticola* occurs on *Vitis vinifera* in South Africa and Spain (Luque *et al.* 2005).

In a recent molecular phylogenetic study (De Wet *et al.* 2008), it became apparent that two groups of isolates require taxonomic revision. Both had superficially similar Diplodia-like conidia. The one set of isolates from *Cupressus sempervirens* in Greece and Cyprus of which those from Greece have previously been identified as *D. pinea* f.sp *cupressi* based only on morphology (Xenopoulos & Tsopelas 2000). The other group of isolates originated from *Casuarina* in Canberra, Australia and appeared to represent an undescribed *Dothiorella* species. The aim of this study was to combine molecular phylogenetic data with morphological characters to characterize these isolates.

## **MATERIALS AND METHODS**

### **Fungal isolates and morphological characterization**

A collection of 11 isolates with Diplodia-like conidia were characterized (Table 1). Sequence data for various Botryosphaeriaceae not generated in this study were obtained from GenBank



(Table 1). All the isolates were accessed from the Culture Collection (CMW) of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates from this study have also been deposited in the Culture Collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Isolates were transferred to 2 % water agar (WA) (Biolab Diagnostics, Midrand, South Africa), to which a few sterile pine needles had been placed on the agar surface to induce sporulation, and incubated at 25 °C in constant light to induce sporulation. Single conidial isolates were generated by breaking pycnidia that were formed on the pine needles, spreading the conidia out and allowing them to germinate. A single, germinating conidium was then transferred and grown on 2 % malt extract agar (MEA) (Biolab Diagnostics, Midrand, South Africa) at 25 °C. All cultures were stored at 4 °C for further study.

For morphological characterization, fruiting structures were sectioned by hand and mounted in clear lactic acid. Morphological observations were made and images were recorded using a Zeiss Axioskop light microscope and Axiocam digital camera (Carl Zeiss, Germany). Growth rate and colony morphology of the isolates were determined on 2 % MEA at 25 °C. Color descriptions of cultures, mycelium and conidia were made according to Rayner (1970).

### **DNA extractions**

DNA was extracted (Raeder & Broda 1985) from the freeze-dried mycelium of the 11 single conidial isolates (Table 1). The isolates were grown in 500 µl of 2 % malt extract (ME) (Biolab Diagnostics, Midrand, South Africa) broth in 1.5 ml Eppendorf tubes, incubated at 25 °C, one week prior to the DNA extraction. The broth was then removed by centrifugation, 20 min at 13 000 rpm, washed with distilled water and freeze-dried.

## **DNA amplification and sequencing**

Part of the elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) (Carbone & Kohn 1999) gene was amplified for 11 Diplodia-like isolates (Table 1) using primers and conditions as described previously (De Wet *et al.* 2000 & 2003). The ITS regions of the rDNA operon (White *et al.* 1990) for four of these isolates (Table 1) were also amplified, while those of the rest were obtained from a previous study (De Wet *et al.* 2008). PCR products were visualized on a 1 % agarose gel containing ethidium bromide using UV illumination. The PCR products were purified using the Roche High Pure PCR product purification kit (Roche Diagnostics, Germany). Both DNA strands were sequenced using the ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing kit and an ABI PRISM<sup>®</sup> 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). All the reactions were done using protocols recommended by the manufacturers. All the sequence data were processed using Sequence Navigator version 1.0.1 (Perkin Elmer) and aligned using MAFFT version 5 (Katoh *et al.* 2005).

## **Phylogenetic analyses**

ITS and EF-1 $\alpha$  sequence data were combined after a partition homogeneity test was performed to determine whether there is congruency between the different phylogenies using PAUP\* (Swofford 2002), and the combined dataset was submitted to TreeBase (SN3866). The homogeneity test was based on strict heuristic searches with a tree-bisection reconnection (TBR) branch swapping algorithm and 1000 replicates. Parsimony, distance (NJ) and Bayesian analyses were applied to the combined data set. Introns occurring in the partial EF-1 $\alpha$  gene sequences were included in the phylogenetic analyses. All characters were treated as unordered and having equal weight. The phylogenetic signal (G1) of the data sets was determined using

PAUP\* and compared with critical values (Hillis & Huelsenbeck 1992) at the 0.01 and 0.05 confidence levels.

Parsimony was based on strict heuristic searches with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum length is zero.

Neighbour-joining distance analysis was done in PAUP\* using the most appropriate model of DNA substitution as determined with MODELTEST 3.5 (Posada & Crandall 1998). Bayesian analysis using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) implementing the Markov Chain Monte Carlo (MCMC) technique and the parameters predetermined with MODELTEST 3.5 was performed. Four simultaneous Markov chains were run from random starting trees for 500 000 generations and trees were sampled every 100 generations. The first 700 of 5001 trees generated were discarded as burnin. The Bayesian analysis was repeated to test the independence of the results from topological priors. Bootstrap support was determined after 1000 replications and only groups with frequencies >50% were retained. All phylogenetic trees were viewed in TreeView and monophyletically rooted to *Mycosphaerella* spp. as outgroups (*M. kona* Crous, Joanne E. Taylor & M.E. Palm: ITS=AY260085, EF-1 $\alpha$ =AY752185; and *M. citri* Whiteside: ITS=AY752145, EF-1 $\alpha$ =AY752179).

## RESULTS

### Phylogenetic analyses

260 bp of the EF-1 $\alpha$  gene were amplified and sequenced for 11 *Diplodia*-like isolates (Table 1). For four of these isolates, 540 bp of the rDNA operon including the ITS1, ITS2 and 5.8S sub-unit were also amplified and sequenced (Table 1). GenBank sequences of 26 isolates, representing *Diplodia*, *Lasiodiplodia*, *Dothiorella*, *Botryosphaeria* and *Neofusicoccum* were added for comparative purposes (Table 1). A partition homogeneity test showed that no significant conflict

exists between the phylogenies of the rDNA and EF-1 $\alpha$  ( $P=0.1$ ). The G1-value ( $G1 = -0.33$ ) was lower than the predicted critical values at both the 95% ( $P = -0.08$ ) and 99% ( $P = -0.09$ ) confidence levels, implying a strong phylogenetic signal. The combined data set contained 808 characters of which 327 characters were constant, 71 were variable, parsimony uninformative characters and 410 were variable, parsimony informative characters. The data set had a tree length of 1113, a consistency index (CI) of 0.73, a retention index (RI) of 0.92 and a homoplasy index (HI) of 0.27. These indices measure the level of homoplasy which is an indication of the reliability of the parsimonious cladograms. MODELTEST 3.5 tested 56 models and predicted the Tamura-Nei model with unequal frequencies (TrN) and a gamma distribution shape parameter (G) as the most appropriate model of DNA substitution.

Two major clades were observed after analyses of the combined dataset (Fig. 1) and these results were confirmed when analyses were done on the two datasets independently. One major clade represented *Diplodia* and *Lasiodiplodia* and the other *Botryosphaeria*, *Dothiorella* and *Neofusicoccum*. The *Diplodia/Lasiodiplodia* clade was comprised of seven sub-clades namely *D. cupressi*, *D. mutila*, *D. scrobiculata*, *D. pinea*, *D. seriata*, *L. theobromae* and *D. porosum* Van Niekirk & Crous. The *Dothiorella/Neofusicoccum/Botryosphaeria* clade also consisted of seven sub-clades including *Do. sarmentorum*, *Do. iberica*, an undescribed *Dothiorella* species, *N. eucalyptorum*, *N. luteum*, *N. ribis* and *B. dothidea*.

The isolates from *C. sempervirens* from Greece and Cyprus grouped with *D. cupressi* from Israel. While isolates from *Casuarina* in Australia grouped in a distinct clade representing an undescribed *Dothiorella* species, with strong bootstrap and Bayesian posterior probability support (100 % and 1.0, respectively). Support for the undescribed *Dothiorella* species as a

distinct member of the genus *Dothiorella* was also provided when the two datasets were analyzed separately.

### **Taxonomy**

Results of the phylogenetic and morphological analyses provide robust evidence to support treatment of the isolates from a *Casuarina* sp. as a discrete taxon for which the following description is provided:

*Dothiorella casuarini* J. De Wet, Slippers & M.J. Wingfield anam. sp. nov. (Figs. 2—7)

(Mycobank 510856)

Etym.: named for *Casuarina* the host from which the fungus was isolated.

Margines coloniarum irregulariter rosulatae. Mycelium cum seriebis tumorum hyphorum chlamydoformas semblantium. Conidiomata pycnidialia, nigra, globosa. Cellulae conidiogenae cellulis parietum pycnidiorum proxime portatae, holoblasticae, hyalinae, subcylindricae, in plano eodem in concretionibus periclinalibus proliferantes, raro percurrente proliferantes bis vel ter indistincte annulatae. Conidia 22—38 x 8—13.5  $\mu\text{m}$  (mediocriter 27.1 x 10.8  $\mu\text{m}$ ), primo non septata hyalina subcylindrica, dum etiam in pycnidio brunnescentia vel atrobrunnescentia, uniseptata raro 2—3 septata, ellipsoidea vel ovoidea, raro anguste ellipsoidea, apice late rotundata, basi truncata.

*Cultures* smooth to fluffy, pale greenish grey to greenish grey from above, becoming lighter or white around the edges, light bluish of sky grey from below, colony margins irregular, rosette-like. *Mycelium* thick walled, branched, septate, melanized light to dark brown, with strings of dark brown chlamydo-spore-like hyphal swellings. *Conidiomata* pycnidia, black, globose, ostiole central, solitary, scattered and immersed in water agar, few on pine needles supplied as substrate. *Conidiophores* absent. *Conidiogenous cells* emerging directly from cells

lining the pycnidial cavity, holoblastic, hyaline, smooth-walled, sub-cylindrical, determinate or indeterminate and proliferating at the same level resulting in periclinal thickening, very rarely proliferating percurrently to produce two or three indistinct annellations. *Conidia* (22—)23—31(—38) x (8—) 9—12 (—13.5)  $\mu\text{m}$  (ave. of 60 conidia = 27.1 x 10.8  $\mu\text{m}$ ), initially aseptate and hyaline, becoming brown to dark brown or sepia and 1-septate within the pycnidium, rarely 2-3 septate, ellipsoid to ovoid, rarely narrow ellipsoid, as obtuse apex and truncate base.

*Known host.* *Casuarina* sp.

*Known geographical range.* Canberra, Australia.

*Holotype: Australia:* Canberra: Cotter River. On *Casuarina* sp., 2000, M.J. Wingfield (CMW4855/CBS120688); in Herb. PREM59650.

*Paratypes: Australia:* Canberra: Cotter River. On *Casuarina* sp., 2000, M.J. Wingfield (CMW4856/CBS120689, CMW4857/CBS120690, CMW4854, CMW4858); all in Herb. PREM59651, PREM59652, PREM59649, PREM59653.

## DISCUSSION

The gene genealogy generated from ITS rDNA and partial EF-1 $\alpha$  sequence data, combined with morphological observations provide robust evidence to justify the description of a set of Diplodia-like isolates from *Casuarina* in Australia as the new species, *Dothiorella casuarini*. This is the fifth species to be described in *Dothiorella*. All except the type species, *Do. pyrenophora* for which no cultures are available, are phylogenetically distinct. In contrast, it would be very difficult to distinguish them based only on morphological characteristics as these often overlap and the more easily distinguishable teleomorphs are rare. This is a problem that is encountered increasingly commonly for fungi (Crous 2005), with the Botryosphaeriaceae providing an excellent example (Crous *et al.* 2006).

*Dothiorella* are distinguished from other anamorph genera of the Botryosphaeriaceae based on conidial morphology and DNA sequence comparisons (Luque *et al.* 2005; Phillips *et al.* 2005). In this regard, *Do. casuarini* has conidia that are ellipsoid to ovoid, initially aseptate and hyaline turning brown to dark brown and 1-septate while still in the pycnidium. Conidia of this species are longer than those of *Do. sarmentorum*, *Do. iberica* and *Do. viticola*. It is also characterized by chlamydospore-like hyphal swellings, which are frequently observed and that have not been reported in other *Dothiorella* spp. Furthermore, *Do. casuarini* has very obvious smooth to fluffy grey-green cultures with typical irregular, rosette-like borders.

No teleomorph structures have been observed for *Do. casuarini*. This is not unusual as sexual states are typically less common in the Botryosphaeriaceae than anamorph states. The known teleomorphs of other *Dothiorella* sp. were previously described as “*Botryosphaeria*” *sarmentorum* A.J.L. Phillips, Alves & Luque, “*Botryosphaeria*” *iberica* A.J.L. Phillips, Luque & Alves and “*Botryosphaeria*” *viticola* A.J.L. Phillips & Luque (Phillips *et al.* 2005). The teleomorph of *Dothiorella* has since been placed in the genus *Dothidotthia*, but the above mentioned teleomorphs have not been formally renamed (Crous *et al.* 2006). If a teleomorph were to be found for *Do. casuarini* this would be expected to have the characteristics of *Dothidotthia*.

Phylogenetic analyses of the ITS rDNA and partial EF-1 $\alpha$  sequence data, grouped a set of isolates from Greece and Cyprus with the ex-type cultures of *D. cupressi* from Israel. This fungus was recently described by Alves *et al.* (2006) and was previously known as *D. pinea* f. sp. *cupressi*, the causal agent of a canker disease on *Cupressus sempervirens* in Israel (Solel *et al.* 1987), South Africa (Linde *et al.* 1997), Greece (Xenopoulos & Tsopelas 2000) and Tunisia (Intini & Panconesi 2005). This is the first report of the pathogen from *C. sempervirens* in

Cyprus. *Diplodia cupressi* is phylogenetically most closely related to *B. tsugae* and *D. mutila* (Alves *et al.* 2006) and clearly has no logical association with *D. pinea*. *Diplodia cupressi* is also the name given to the pathogen found on *Juniperus* spp. previously identified as *D. mutila* (Alves *et al.* 2006, De Wet *et al.* 2008).

Phylogenetic analyses in this study showed that *D. cupressi* is more closely related to species from hardwoods, such as *D. mutila* from *Fraxinus*, than to *D. pinea*. Interestingly, *D. pinea* is also more closely related to the hardwood-infecting species, *D. seriata*, than to other softwood-infecting species. Clearly, distantly related hosts have been colonized by ancestors of these fungi. These host jumps (Slippers *et al.* 2005), rather than co-evolution with the hosts, most likely contributed to the speciation of the taxa. These results also support results of a recent study (De Wet *et al.* 2008) in which we showed that species of *Diplodia* and *Lasiodiplodia* were common on both gymnosperms and angiosperms (*D. seriata*, *D. porosum*, *L. theobromae*). This was in contrast to species of *Dothiorella*, *Neofusicoccum* and *Botryosphaeria* that were virtually all from angiosperms, which is the likely ancestral host group of the Botryosphaeriaceae (De Wet *et al.* 2008).

*Diplodia*, *Lasiodiplodia* and *Dothiorella* are all morphologically similar members of the Botryosphaeriaceae. These genera all have conidia that are similar in size and shape (ellipsoidal to ovoid), initially hyaline, but becoming pigmented with age, and sometimes septate. Isolates belonging to these three genera included in this study could, however, easily be assigned to these genera using a multiple gene sequence comparisons. This underscores the importance of combining morphological and DNA sequence data when identifying and describing new species with *Diplodia*-like characteristics (Denman *et al.* 2000; De Wet *et al.* 2003; Alves *et al.* 2004; Pavlic *et al.* 2004; Alves *et al.* 2006).



*Diplodia* and *Lasiodiplodia* are clearly sister genera and it is not surprising that they share similar conidial morphology. *Dothiorella* is, however, more closely related to morphologically distinct genera such as *Neofusicoccum* and *Botryosphaeria*. The latter taxa have conidia that are mostly hyaline and fusoid in shape and only rarely become pigmented, thus very different from those of *Dothiorella*. Pigmented older conidia that are ovoid to ellipsoid thus represent a polyphyletic character, which has been lost or gained independently amongst the lineages of the Botryosphaeriaceae.

Results of this study, confirm the value of generating multiple gene genealogies to resolve the status of species of the Botryosphaeriaceae with *Diplodia*-like anamorphs. It has further shown that neither morphology, nor host association, necessarily reflect the evolutionary history of the genera of the Botryosphaeriaceae. Much remains to be understood regarding the role of host association in shaping the diversity and distribution of species in this group of fungi. Studies considering conidial morphology, and factors that influence this character based on a more complete taxon set are likely to reflect important aspects of the evolutionary histories for members of the Botryosphaeriaceae.

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**Table 1.** *Diplodia* and *Dothiorella* isolates included in this study as well as other members of the Botryosphaeriaceae used for comparative purposes.

| Isolates <sup>a/</sup> | Species                         | Origin        | Host                          | Reference/Collector <sup>b/</sup>                           | GenBank Accession numbers <sup>c/</sup> |               |
|------------------------|---------------------------------|---------------|-------------------------------|---|---|---------------|
|                        |                                 |               |                               |   | ITS                                     | EF-1 $\alpha$ |
| CMW19954               | <i>Diplodia cupressi</i>        | Greece        | <i>Cupressus sempervirens</i> | P. Tsopelas (SH-1/CBS120691)                                | DQ846775                                | DQ875334      |
| CMW19955               | "                               | "             | "                             | P. Tsopelas (SH-2/CBS120692)                                | DQ846776                                | DQ875335      |
| CMW19956               | "                               | Cyprus        | "                             | P. Tsopelas (SH-4/CBS120693)                                | DQ846777                                | DQ875336      |
| CMW19957               | "                               | "             | "                             | P. Tsopelas (SH-7/CBS121027)                                | DQ846779                                | DQ875338      |
| CMW4854                | <i>Dothiorella casuarini</i>    | Australia     | <i>Casuarina</i> sp.          | MJ. Wingfield   | EF107752                                | EF107758      |
| CMW4855<br>(CBS120688) | "                               | "             | "                             | "   | DQ846773                                | DQ875331      |
| CMW4856<br>(CBS120689) | "                               | "             | "                             | "   | DQ846772                                | DQ875332      |
| CMW4857<br>(CBS120690) | "                               | "             | "                             | "   | DQ846774                                | DQ875333      |
| CMW4858                | "                               | "             | "                             | "   | EF107753                                | EF107759      |
| CMW1182                | <i>D. cupressi</i>              | Israel        | <i>C. sempervirens</i>        | W. Swart (Swart <i>et al.</i> 1993)                         | EU220433                                | EU220487      |
| CMW1183                | "                               | "             | "                             | "   | EU220434                                | EU220488      |
| CBS168.87              | "                               | "             | "                             | Alves <i>et al.</i> 2006                                    | DQ458893                                | DQ458878      |
| CMW190                 | <i>D. pinea</i> (A)             | United States | <i>Pinus resinosa</i>         | Palmer <i>et al.</i> 1987, De Wet <i>et al.</i> 2000 & 2003 | AY253290                                | AY624251      |
| CMW4876                | <i>D. pinea</i> (C)             | Indonesia     | <i>P. patula</i>              | De Wet <i>et al.</i> 2000 & 2003                            | AY253294                                | AY624252      |
| CMW5870                | <i>D. scrobiculata</i>          | California    | <i>P. radiata</i>             | De Wet <i>et al.</i> 2003                                   | AY623704                                | AY624254      |
| CMW4900                | "                               | Mexico        | <i>P. greggii</i>             | "   | AY623705                                | AY624255      |
| CMW189                 | "                               | USA           | <i>P. banksiana</i>           | Palmer <i>et al.</i> 1987, De Wet <i>et al.</i> 2000 & 2003 | AY253292                                | AY624253      |
| CMW8230                | <i>D. seriata</i>               | Canada        | <i>Picea glauca</i>           | De Wet <i>et al.</i> 2003                                   | AY972104                                | DQ280418      |
| CMW8232                | "                               | South Africa  | <i>Malus domestica</i>        | De Wet <i>et al.</i> 2003                                   | AY972105                                | DQ280419      |
| CMW9074                | <i>Lasiodiplodia theobromae</i> | Mexico        | <i>Pinus</i> sp.              | Slippers <i>et al.</i> 2004a                                | AY236952                                | AY236901      |
| CMW10130               | "                               | Uganda        | <i>Vitex doniana</i>          | "   | AY236951                                | AY236900      |
| CMW7060                | <i>D. mutila</i>                | Netherlands   | <i>Fraxinus excelsior</i>     | "   | AY236955                                | AY236904      |
| CMW7776                | "                               | Italy         | "                             | "   | AY972106                                | DQ280420      |

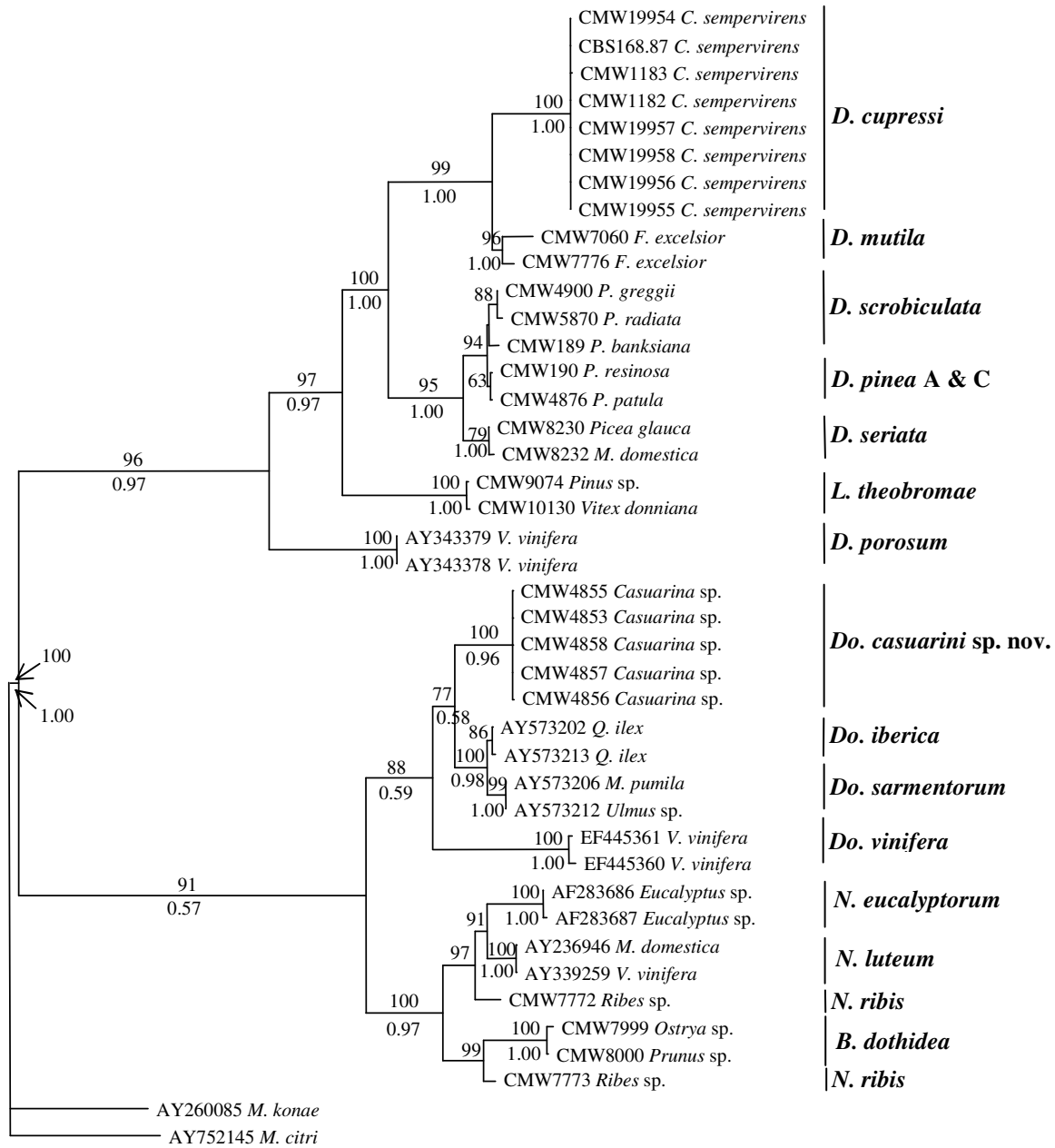


| Isolates <sup>a/</sup> | Species                              | Origin           | Host                  | Reference/Collector <sup>b/</sup> | GenBank Accession numbers <sup>c/</sup> |               |
|------------------------|--------------------------------------|------------------|-----------------------|-----------------------------------|---|---------------|
|                        |                                      |                  |                       |                                   | ITS                                     | EF-1 $\alpha$ |
| CMW7999                | <i>B. dothidea</i>                   | Switzerland      | <i>Ostrya</i> sp.     | Slippers <i>et al.</i> 2004a      | AY236948                                | AY236897      |
| CMW8000                | "                                    | Switzerland      | <i>Prunus</i> sp.     | "                                 | AY236949                                | AY236898      |
| CBS110574              | <i>D. porosum</i>                    | South Africa     | <i>Vitis vinifera</i> | Van Niekerk <i>et al.</i> 2004    | AY343378                                | AY343340      |
| CBS110496              | "                                    | "                | "                     | "                                 | AY343379                                | AY343339      |
| IMI63581b              | <i>Do.</i><br><i>sarmentorum</i>     | England          | <i>Ulmus</i> sp.      | Phillips <i>et al.</i> 2005       | AY573212                                | AY573223      |
| CBS115038              | "                                    | Netherlands      | <i>M. pumila</i>      | "                                 | AY573206                                | AY573235      |
| CBS115041              | <i>Do. iberica</i>                   | Spain            | <i>Quercus ilex</i>   | "                                 | AY573202                                | AY573222      |
| CBS115035              | "                                    | "                | "                     | "                                 | AY573213                                | AY573228      |
| CBS121117              | <i>Do. viticola</i>                  | South Africa     | <i>V. vinifera</i>    | Damm <i>et al.</i> 2007           | EF445361                                | EF445394      |
| CBS121118              | "                                    | "                | "                     | "                                 | EF445360                                | EF445393      |
| CMW7772                | <i>Neofusicoccum</i><br><i>ribis</i> | New York,<br>USA | <i>Ribes</i> sp.      | Slippers <i>et al.</i> 2004a      | AY236935                                | AY236877      |
| CMW7773                | "                                    | "                | "                     | "                                 | AY236936                                | AY236878      |
| BOT24                  | <i>N. eucalyptorum</i>               | South Africa     | <i>Eucalyptus</i> sp. | Smith <i>et al.</i> 2001          | AF283686                                | AY339264      |
| BOT16                  | "                                    | "                | "                     | "                                 | AF283687                                | AY236892      |
| CMW9076                | <i>N. luteum</i>                     | New Zealand      | <i>M. domestica</i>   | Slippers <i>et al.</i> 2004b      | AY236946                                | AY339265      |
| CMW10310               | "                                    | Portugal         | <i>V. vinifera</i>    | "                                 | AY339259                                | AY339267      |

<sup>a/</sup> CMW refers to the Culture Collection (CMW) of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. <sup>b/</sup> Reference refers to previous publications where the same isolates were used and collector refers to the collector and isolation numbers of isolates not previously published. <sup>c/</sup> Sequences for isolates in bold were generated in the present study while the remainder were obtained from GenBank.

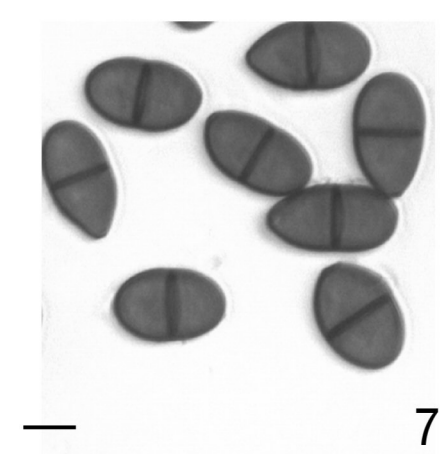
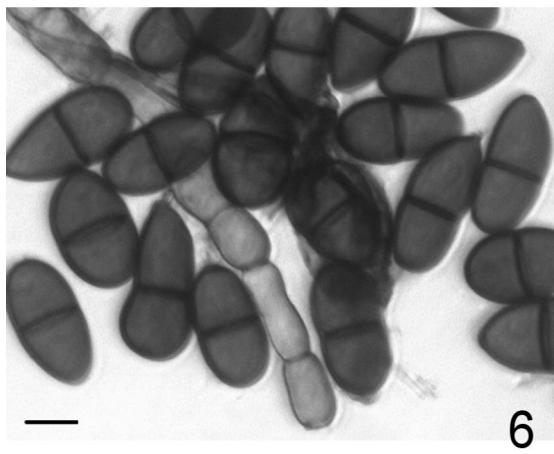
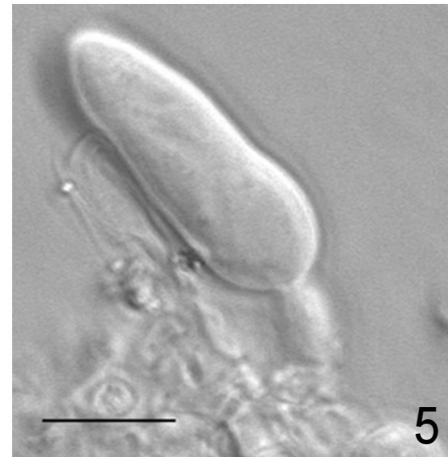
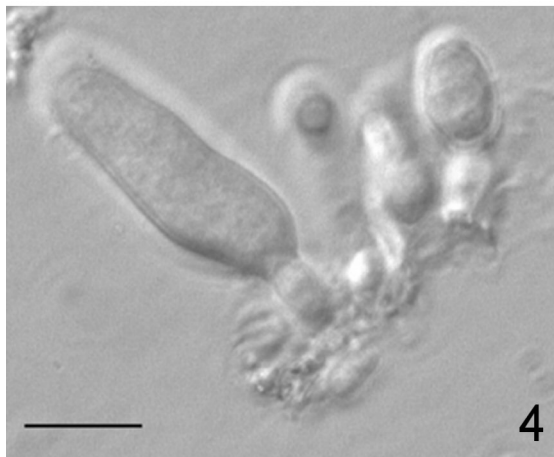
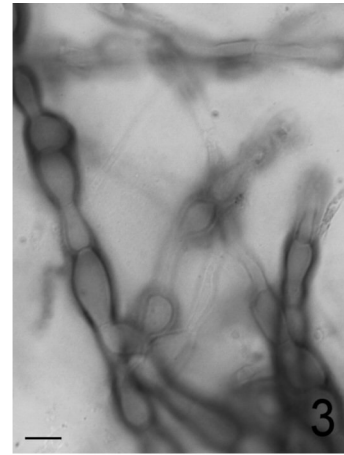
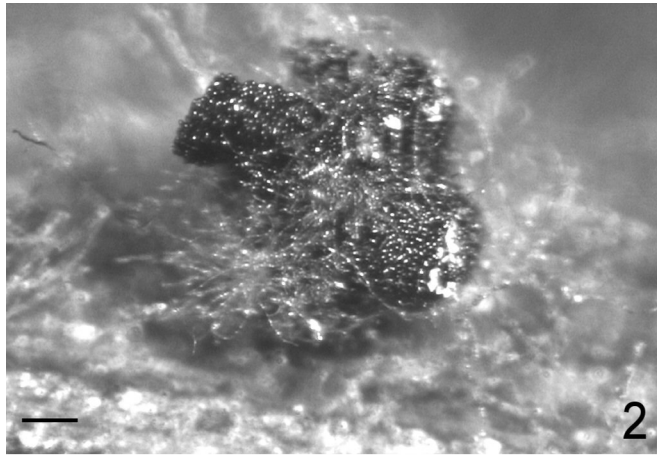


**Figure 1.** Phylogram constructed from the sequences of the rDNA operon (ITS regions and 5.8S ribosomal sub-unit) and partial elongation factor 1 alpha (EF-1 $\alpha$ ) based on strict heuristic searches with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum length is zero with branch support values (maximum parsimony bootstrap proportions/Bayesian posterior probabilities). Bootstrap values were determined after 1000 replications in PAUP\*. Only groups with frequencies >50% were retained. The Bayesian posterior support values were determined using MrBayes 3.1.2 with the Tamura-Nei model and a gamma distribution shape parameter (TrN+ G). The bar represents 10 changes.





**Figures 2-7.** *Dothiorella casuarini* sp. nov. **Fig. 2.** Pycnidium formed on a sterile pine needle in culture on water agar. **Fig. 3.** Pigmented chlamydospore-like hyphal cells in chains. **Fig. 4-5.** Conidiogenous cells and immature developing conidia. **Fig. 6-7.** Mature, septate, dark conidia. Bars = 10  $\mu\text{m}$





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## CHAPTER 4

# PHYLOGENY OF THE BOTRYOSPHAERIACEAE REVEALS PATTERNS OF HOST ASSOCIATION

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## ABSTRACT

Three anamorph genera of the Botryosphaeriaceae namely *Diplodia*, *Lasiodiplodia* and *Dothiorella* have typically dark, ovoid conidia with thick walls, and are consequently difficult to distinguish from each other. These genera are well-known pathogens of especially pine species. We generated a multiple gene genealogy to resolve the phylogenetic relationships of Botryosphaeriaceae with dark conidial anamorphs, and mapped host associations based on this phylogeny. The multiple gene genealogy separated *Diplodia*, *Lasiodiplodia* and *Dothiorella* and it revealed trends in the patterns of host association. The dataset was expanded to include more lineages of the Botryosphaeriaceae, and included all isolates from different host species for which ITS sequence data are available. Results indicate that *Diplodia* species occur mainly on gymnosperms, with a few species on both gymnosperms and angiosperms. *Lasiodiplodia* species occur equally on both gymnosperms and angiosperms, *Dothiorella* species are restricted to angiosperms and *Neofusicoccum* species occur mainly on angiosperms with rare reports on Southern Hemisphere gymnosperms. *Botryosphaeria* species with *Fusicoccum* anamorphs occur mostly on angiosperms with rare reports on gymnosperms. Ancestral state reconstruction suggests that a putative ancestor of the Botryosphaeriaceae most likely evolved on the angiosperms. Another interesting observation was that both host generalist and specialist species were observed in all the lineages of the Botryosphaeriaceae, with little evidence of host associated co-evolution.

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## INTRODUCTION

Most of the species of the Botryosphaeriaceae cause disease symptoms such as die-back and cankers on numerous woody and non-woody hosts, especially in combination with stress-inducing environmental conditions (Eldridge 1961; Buchanan 1967; Punithalingam & Waterston 1970). Species of the Botryosphaeriaceae include well-recognized pathogens of forestry trees including the important pine pathogen, *Diplodia pinea* (Desm.) J. Kickx f. (Eldridge 1961; Swart & Wingfield 1991), and *Botryosphaeria dothidea* (Moug. Fr.) Ces. & De Not. and *Neofusicoccum eucalyptorum* Crous, H. Smith & M.J. Wingf. that cause serious canker diseases on *Eucalyptus* L'Hér (Smith *et al.* 1994; Smith *et al.* 2001). These fungi also include pathogens of fruit trees such as *Diplodia seriata* De Not. (= *Botryosphaeria obtusa*) and *D. mutila* (Fr.) Mont. (Phillips *et al.* 2007; Slippers *et al.* 2007), grape vines including *N. australe* Crous, Slippers & A.J.L. Phillips and *N. luteum* Crous, Slippers & A.J.L. Phillips (Van Niekerk *et al.* 2004) and the Proteaceae including *Saccharata proteae* (Wakef.) Denman & Crous (Denman *et al.* 2003).

The taxonomy of species in the Botryosphaeriaceae is commonly based on the morphology of the anamorph states, which are most frequently encountered in nature. However, overlapping morphological characteristics has emphasized the utility of applying DNA sequence comparisons to resolve species. In a more recent and broadly-based phylogenetic study, ten lineages were identified for the Botryosphaeriaceae and these were shown to represent several newly described genera (Crous *et al.* 2006). The genera currently treated in the Botryosphaeriaceae are thus *Diplodia* Fr./*Lasiodiplodia* Ellis & Everh./*Tiarosporella* Höhn, *Botryosphaeria* Ces. & De Not. (*Fusicoccum* anamorphs), *Macrophomina* Petr., *Neoscytalidium* Crous & Slippers, *Dothidotthia* Höhn (*Dothiorella* anamorphs), *Neofusicoccum* Crous, Slippers & A.J.L. Phillips

(*Botryosphaeria*-like teleomorphs, *Dichomera*-like synanamorphs), *Pseudofusicoccum* Mohali, Slippers & M.J. Wingf., *Saccharata* Denman & Crous (*Diplodia*- and *Fusicoccum*-like synanamorphs), “*Botryosphaeria*” *quercuum* (Schwein.) Sacc. (*Diplodia*-like anamorph) and *Guignardia* Viala & Ravaz (*Phyllosticta* anamorphs). The genus *Botryosphaeria* now applies only to *B. dothidea*, *B. mamane* D.E. Gardner and *B. corticis* (Demaree & Wilcox) Arx & E. Müll. Where the taxonomy remain uncertain the name “*Botryosphaeria*” is used in the broad sense and as is the case for “*Botryosphaeria*” *quercuum*. While the study of Crous *et al.* (2006) brought new clarity to the taxonomy of the Botryosphaeriaceae, it also highlighted many remaining taxonomic problems. Particularly the identity and phylogenetic relationships of genera with *Diplodia*-like anamorphs of the Botryosphaeriaceae that either belongs to *Diplodia*, *Dothiorella* or *Lasiodiplodia*, remains unclear.

The taxonomy of genera of the Botryosphaeriaceae with *Diplodia*-like anamorphs (*Diplodia*, *Lasiodiplodia* and *Dothiorella*) is commonly confused. Their conidia are similar in size and shape (mostly ovoid with a length:width ratio of 2-3:1), thick-walled, and often only becoming pigmented and dematiaceous as they age. These characters make the *Diplodia*-like anamorph genera distinctly different from other anamorph genera of the Botryosphaeriaceae having hyaline, *Fusicoccum*-like conidia, and they might thus be expected to be related. It is therefore, not surprising that they have also previously been treated as synonyms of each other (Punithalingam & Waterston 1970; Denman *et al.* 2000). Phillips *et al.* (2005), however, provided strong evidence to re-erect *Dothiorella* to accommodate isolates with dark and single septate conidia early in development unlike conidia of *Diplodia*-like anamorphs turning dark and multi-septated over time. The finding that they are phylogenetically more closely related to *Neofusicoccum* than to *Diplodia* provided strong support for this view (Phillips *et al.* 2005;



Crous *et al.* 2006). The taxonomic status of *Diplodia* and *Lasiodiplodia* remains uncertain (Crous *et al.* 2006).

One well studied example, which illustrates the complexities of identifying species of the Botryosphaeriaceae with *Diplodia*-like anamorphs, is found in the *D. pinea* species complex. All species with dematiaceous conidia associated with disease symptoms on *Pinus* L. spp. were initially treated as *D. pinea* (= *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton) (Waterman 1943; Punithalingam & Waterston 1970). *Diplodia pinea* has been differentiated based on different morphological types, that have been referred to as the A, B, C and I morphotypes (Wang *et al.* 1985; Palmer *et al.* 1987; Smith & Stanosz 1995; Hausner *et al.* 1999; De Wet *et al.* 2000, 2002). Multiple gene genealogies for these fungi have, however, shown that the A, B and C morphotypes represent two distinct species. *Diplodia pinea* is the best known species and an important pine pathogen that occurs in two morphological forms referred to as the A and C morphotypes (De Wet *et al.* 2000, 2002). The B morphotype of *D. pinea* has been described as *D. scrobiculata* J. de Wet, Slippers & M.J. Wingf. (De Wet *et al.* 2003). Isolates designated as the I morphotype of *D. pinea* represent *D. seriata* (Burgess *et al.* 2001).

In the past, host association was often used to distinguish or describe species of the Botryosphaeriaceae. It has, however, become clear that host association is not always a good indication of species delineation in this family. Certain Botryosphaeriaceae are clearly generalist species, able to infect a wide range of unrelated hosts (e.g. *B. dothidea*, *L. theobromae* (Pat.) Griffon & Maubl. and *D. seriata*). Others are more specialized and appear to infect only a specific host genus or group of related host genera (e.g. *N. eucalyptorum* and *N. eucalypticola* Slippers, Crous & M.J. Wingf.). The difficulties associated with identifying many members of the Botryosphaeriaceae using morphological characteristics has, however, made it difficult to

study host association patterns in the group. Such host association patterns are important when seeking to understand the driving forces of evolution in the group, patterns of co-evolution with specific hosts, as well as, for pathology and epidemiology studies. Large numbers of sequences are becoming available for species in the Botryosphaeriaceae, and a consideration of host association patterns has become possible.

The primary aim of this study was to generate a multiple gene genealogy for species of the Botryosphaeriaceae with *Diplodia*-like anamorphs. In order to further explore the host association patterns that became apparent amongst *Diplodia*-like anamorphs of the Botryosphaeriaceae, we expanded the initial sampling set by including all isolates of six of the ten lineages of the Botryosphaeriaceae as described by Crous *et al.* (2006) with ITS sequence representation in GenBank, and for which host data are available.

## **MATERIAL AND METHODS**

### **Fungal isolates**

A collection of 23 *Diplodia*-like isolates from various regions and hosts was included in this study (Table 1). Sequence data for various Botryosphaeriaceae not generated in this study were obtained from Genbank (Table 2). European isolates used in the study were provided by Dr. Pierre Chandelier (INRA-French National Institute for Agricultural Research, Nancy, France). All the other isolates were accessed from the Culture Collection (CMW) of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Isolates were transferred to 2 % water agar (WA) (Biolab Diagnostics, Midrand, South Africa), with a few sterile pine needles placed on the agar surface, and incubated at 25 °C in constant light to induce sporulation. Single conidial isolates were generated, and these were grown on 2 % malt

extract agar (MEA) (Biolab Diagnostics, Midrand, South Africa) at 25 °C. All cultures were stored at 4 °C for further study.

### **DNA extractions, amplification and sequencing**

DNA was extracted from the freeze-dried mycelium of the 23 single conidial isolates (Table 1). The isolates were grown in 500 µl of 2 % ME broth in 1.5 ml Eppendorf tubes, incubated at 25 °C, one week prior to the DNA extraction. The broth was then removed through centrifugation, 20 min at 13 000 rpm, washed with distilled water and freeze-dried. DNA was extracted using the technique described by Raeder & Broda (1985).

The internally transcribed spacer (ITS) regions 1 and 2 and the 5.8S ribosomal subunit (White *et al.* 1990), Bt2 regions of the  $\beta$ -tubulin gene (Glass & Donaldson 1995) and part of the protein-coding gene, actin (ACT) (Carbone & Kohn 1999) were amplified (Table 1). The gene regions were amplified using primers and conditions as described previously (De Wet *et al.* 2000, 2003).

PCR products were visualised on a 1 % agarose gel containing ethidium bromide using UV illumination. The PCR products were purified using the Roche High Pure PCR product purification kit (Roche Diagnostics, Germany). Both DNA strands were sequenced using the ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing kit and an ABI PRISM<sup>®</sup> 3100 DNA sequencer (Applied Biosystems, Foster City, CA 94404 USA). All the reactions were done using protocols recommended by the manufacturers. All the sequence data were processed using Sequence Navigator version 1.0.1 (Perkin Elmer) and aligned using MAFFT version 5 (Kato *et al.* 2005).

### **Phylogenetic analyses**

BLAST searches in GenBank were performed using ITS sequence data. Two data sets were generated. One of these combined ITS, Bt2 of  $\beta$ -tubulin and ACT sequence data to distinguish

between closely related *Diplodia*-like isolates from different coniferous hosts and geographical regions. The other data set was based only on ITS sequence data for selected species of the Botryosphaeriaceae, from all hosts available on GenBank. Six of the ten lineages as described by Crous *et al.* (2006) were included. *Macrophomina*, *Guignardia*, “*Botryosphaeria*” *quercuum* and *Saccharata* were excluded as either their taxonomy is uncertain, or they group outside the phylogeny considered here. *Tiarosporella*, which grouped with *Diplodia* in Crous *et al.* (2006), was not included in this study as corresponding ITS sequence data was not available on GenBank.

At the time of analysis, 771 ITS sequences were available in GenBank for the Botryosphaeriaceae. A total of 134 of these sequences were used in this study, representing one ITS sequence for each species from a unique host. The aim of this analysis was to generate a global view of as many species of the Botryosphaeriaceae from unique hosts as possible and thus to consider their host associations. When more than one sequence was available representing the same species from the same host, one was chosen randomly. Because these data in GenBank was not expected to represent the full host ranges of all the species we compared the of host ranges represented by the ITS sequence data with published host ranges (e.g. SBML Fungus-Host Distribution Database <http://nt.ars-grin.gov/fungaldatabases/fungushost/FungusHost.cfm> and other published literature). The value of literature records of these species on various hosts is, however, weakened by the uncertainty surrounding reports of species of the Botryosphaeriaceae based solely on morphology. Following this process we were convinced that the overall patterns of host association for the genera were as accurate as possible.

Parsimony, distance (NJ), maximum likelihood (ML) and Bayesian analyses were applied to all data sets. Introns occurring in the partial gene sequences of Bt2 of  $\beta$ -tubulin and ACT were

included in the phylogenetic analyses. All characters were treated as unordered and having equal weight. Partition homogeneity tests were performed on the combined data sets to determine whether there was congruency between the different phylogenies using PAUP\* (Swofford 2002). The phylogenetic signal (G1) of the data sets was determined using PAUP\* and compared with critical values (Hillis & Huelsenbeck 1992) at the 0.01 and 0.5 confidence levels.

Parsimony was based on strict heuristic searches with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum length is zero.

Neighbour-joining distance analysis was done in PAUP\* using the most appropriate model of DNA substitution as determined with MODELTEST 3.5 (Posada & Crandall 1998). Maximum likelihood was also performed in PAUP\* using the parameters as determined with MODELTEST 3.5 (Posada & Crandall, 1998). Bayesian analysis using MrBayes 3.0b4, implementing the Markov Chain Monte Carlo (MCMC) technique (Huelsenbeck & Ronquist 2001) and the parameters predetermined with MODELTEST 3.5 was used. Trees were sampled every 100 generations. The first 500 of 500,000 trees were discarded (burnin=200). The Bayesian analysis was repeated to test the independence of the results from topological priors. Bootstrap support for all four analyses was determined after 1000 replications and only groups with frequencies >50% were retained. The character state reconstruction was done in MacClade ver. 4 (Maddison & Maddison 2000). All phylogenetic trees were viewed in TreeView and monophyletically rooted to *Mycosphaerella* spp. as outgroups (*M. konae* Crous, Joanne E. Taylor & M.E. Palm: ITS=AY260085, BT2=AY725606, ACT=AY752213, EF-1 $\alpha$ =AY752185 and *M. citri* Whiteside: ITS=AY752145; EF-1 $\alpha$ =AY752179). *Mycosphaerella konae* was used in both data sets as an outgroup because it has sequences for all the relevant gene areas available on GenBank.

## RESULTS

### Phylogenetic analyses of the Botryosphaeriaceae with *Diplodia*-like anamorphs (Fig. 1)

A collection of *Diplodia*-like isolates from coniferous hosts were included in this data set to determine their identity, as well as to derive information regarding specificity. The ITS region of the rDNA operon and parts of two protein-coding genes were successfully amplified for all the isolates included in this study (Table 1). Sequences generated from the amplification products ranged from 266 – 554 bp in length. A partition homogeneity test showed no significant conflict between the phylogenies of the rDNA, BT2 of  $\beta$ -tubulin or ACT ( $P > 0.01$ ). The G1-value ( $G1 = -0.73$ ) was lower than the predicted critical values at both the 95 % ( $P = -0.08$ ) and 99 % ( $P = -0.09$ ) confidence levels, implying strong phylogenetic signal for the data set. The combined data set contained 1306 characters of which 587 characters were constant, 296 were variable, parsimony uninformative characters and 423 were variable, parsimony informative characters. The data set had a consistency index (CI) of 0.65, a retention index (RI) of 0.81 and a homoplasy index (HI) of 0.35. MODELTEST 3.5 tested 56 models and predicted a transitional (TIM) model with a proportion of invariable sites (I) and gamma distribution shape parameter (G) as the most appropriate model of DNA substitution.

Two major clades emerged from the constructed phylogram (Fig. 1). One of these represented *Diplodia* and *Lasiodiplodia* and the other included *Botryosphaeria*, *Dothiorella* and *Neofusicoccum*. The *Diplodia/Lasiodiplodia* clade consisted of seven sub-clades including the A and C morphotypes of *D. pinea*, *D. scrobiculata*, *D. seriata*, *D. cupressi*, *D. mutila* and *L. theobromae*. The *Botryosphaerial/Neofusicoccum/Dothiorella* clade consisted of *B. dothidea*, *N. ribis* Slippers, Crous & M.J. Wingf., and an undescribed species of *Dothiorella* from *Casuarina*.

All isolates in the sub-clade containing the A morphotype of *D. pinea* were from various conifer hosts including *P. resinosa* Sol. ex Aiton, *Pseudotsuga menziesii* (Mirb.) Franco, *Cedrus deodora* (Roxb.) G. Don and a *Larix* Miller sp. These host species reside in the Pinales and Pinaceae, and they are represented by three sub-families, i.e. Pinoideae (*Pinus*), Laricoideae (*Larix* and *Pseudotsuga*) and Abietoideae (*Cedrus*).

The sub-clade representing *D. pinea* C morphotype, included three isolates (CMW14654, CMW14655 and CMW14656) recognized for the first time originating from *P. merkusii* in Sulawesi (Indonesia). They grouped with the previously described C morphotype isolate (CMW4876) from *P. patula* in Northern Sumatra (Indonesia).

The *D. scrobiculata* sub-clade contained isolates from *P. greggii* Engelm. ex Parl., *P. radiata* D. Don, *P. banksiana* Lamb., *Picea mariana* (Mill.) Britton, Sterns & Poggenburg and *C. deodora*. These hosts are all conifers residing in the Pinales and Pinaceae and they are represented by three sub-families, i.e. Pinoideae (*Pinus*), Piceoideae (*Picea*) and Abietoideae (*Cedrus*).

The *D. seriata* sub-clade contained isolates from a diverse range of hosts that includes angiosperms (*Malus domestica* Borkh.) as well as gymnosperms residing in the Pinales and Pinaceae and they are represented by three sub-families i.e. Piceoideae (*Picea*), Abietoideae (*Abies*, *Cedrus*) and Laricoideae (*Pseudotsuga*)

The *Lasiodiplodia* sub-clade is represented only by *L. theobromae* isolates from *Pinus* spp. and *Vitex doniana* Sweet.

### **Phylogenetic analyses for six lineages of the Botryosphaeriaceae (Fig. 2)**

A total of 134 ITS sequences representing six of the ten lineages of the Botryosphaeriaceae from every distinct host species available on GenBank were included. The G1-value (G1 = -0.43) was less than the predicted critical values at both the 95 % (P = -0.08) and 99 % (P = -0.09)

confidence levels implying strong phylogenetic signal for the dataset. The data set contained 564 characters of which 236 characters were constant, 51 were variable, parsimony uninformative characters and 277 were variable, parsimony informative characters. The data set had a consistency index (CI) of 0.52, a retention index (RI) of 0.90 and a homoplasy index (HI) of 0.48. MODELTEST 3.5 tested 56 models and predicted a transitional (TIM) model with a proportion of invariable sites (I) and a gamma distribution shape parameter (G) as the most appropriate model of DNA substitution.

In the resulting phylogram, seven lineages can be distinguished (Fig. 2). *Diplodia* and *Lasiodiplodia* isolates grouped in two separate lineages and were not unresolved as one lineage as was found based on large subunit sequence data (Crous *et al.* 2006). The *Diplodia* clade includes *D. seriata*, *D. pinea*, *D. scrobiculata* and *D. mutila*. *Diplodia seriata* occurs on a wide range of angiosperms and gymnosperms. *Diplodia pinea* and *D. scrobiculata* occur only on gymnosperms, and *D. mutila* only on angiosperms. Some species such as *D. corticola* Phillips, Alves & Luque from *Quercus* L., *D. porosum* from *Vitis* L., *D. rosulata* Gure, Slippers & Stenlid from *Prunus* L. and *D. cupressi* from *Cupressus* appear to be restricted to a single host genus. In previous studies, isolates from cankers on *Juniperus* L. were identified as *D. mutila* and they were considered to be closely related to *D. cupressi* (Swart *et al.* 1993; Stanosz *et al.* 1998; Zhou & Stanosz 2001).

Results of this study, however, indicate that *D. mutila* from *Juniperus* represents *D. cupressi*.

In the *Lasiodiplodia* clade, isolates of *L. theobromae* all grouped together and they originated from a wide variety of hosts including both angiosperms and gymnosperms. *Lasiodiplodia venezuelensis* Burgess, Barber & Mohali from *Acacia* Miller, *L. rubropurpurea* Burgess, Barber & Pegg from *Eucalyptus*, *L. crassispora* Burgess & Barber from *Eucalyptus* and *Santalum* L., and *L. gonubiensis* Pavlic, Slippers & M.J. Wingf. from *Syzygium* Gaertn. also resided in this clade.



The *Neofusicoccum* clade included two species complexes. These were *N. ribis/N. parvum* and *N. luteum/N. australe* that occur on hosts including a wide variety of angiosperms and gymnosperms including *Araucaria* Juss., *Wollemia* Jones, Hill & Allen, *Widdringtonia* Endl., *Pinus* and *Podocarpus* Labill. Each of the other nine *Neofusicoccum* species in this clade was associated with only one host. These were *N. vitifusiforme* Crous, Slippers & A.J.L. Phillips from *Vitis*, *N. viticlavatum* Crous, Slippers & A.J.L. Phillips from *Vitis*, *N. eucalyptorum* from *Eucalyptus*, *N. eucalypticola* from *Eucalyptus*, *N. arbuti* Crous, Slippers & A.J.L. Phillips from *Arbutus* L., *N. andinum* Mohali, Slippers & M.J. Wingf. form *Eucalyptus*, *N. macroclavatum* T. Burgess, Barber & L.M. Hardy from *Eucalyptus*, *N. mangiferae* Crous, Slippers & A.J.L. Phillips from *Mangifera* L. and *N. protearum* Crous, Slippers & A.J.L. Phillips from *Protea* spp.

The *Dothiorella* clade included *Do. iberica* and *Do. sarmentorum*. These fungi are associated with various host genera but they are all angiosperms. The other two species in this clade were associated with only one host. They are *Do. viticola* from *Vitis* and a potentially undescribed species of *Dothiorella* from *Casuarina*.

The *Botryosphaeria* clade included two species. One of these is *B. dothidea* that occurs on a wide variety of angiosperms and occasionally on gymnosperms. The other species that resides in this clade is *Botryosphaeria corticis* (Demaree & Wilcox) Arx & E. Müll. from *Vaccinium* L.

The *Neoscytalidium* clade included two species, *N. dimidiatum* Crous & Slippers from *Mangifera* and “*Botryosphaeria*” *mamane* D.E. Gardner from *Sophora* L. They are known only from these hosts. The *Pseudofusicoccum* clade included *Ps. stromaticum* Mohali, Slippers & M.J. Wingf. only known from *Eucalyptus*.

## DISCUSSION

In this study we provide strong supportive evidence for the distinction between *Diplodia*, *Lasiodiplodia* and *Dothiorella* as separate genera, based on sequence data from two protein-coding loci, as well as the ITS region of the rDNA operon. The study also confirms the phylogenetic relationship of these genera to genera with *Fusicoccum* anamorphs such as *Botryosphaeria* and *Neofusicoccum* (Jacobs & Rehner 1998; Denman *et al.* 2000; Zhou & Stanosz 2001). Furthermore, based on results of all available sequence data, *Diplodia* and *Lasiodiplodia* species are shown to commonly occur on both gymnosperms and angiosperms. All the other Botryosphaeriaceae lineages (excluding *Macrophomina*, *Guignardia*, *Saccharata* and “*Botryosphaeria*” *quercuum*) are predominantly found on angiosperms, with rare exceptions on gymnosperms. Interestingly, these are only from Southern Hemisphere conifers in the Araucariaceae and single reports from non-native pines in the Southern Hemisphere. These results suggest that the ancestors of the Botryosphaeriaceae evolved on angiosperms, and only later colonized and speciated on gymnosperms.

The multiple gene genealogy generated in this study, supports the separation of all three genera with *Diplodia*-like anamorphs. Despite the morphological similarities between *Diplodia*, *Lasiodiplodia* and *Dothiorella*, *Dothiorella* shares a more recent common ancestor with morphologically distinct genera such as *Neofusicoccum* and *Botryosphaeria*. This could be due to convergent evolution or simply because this character (*Diplodia*-like conidia) predates the separation of the main genera in Botryosphaeriaceae. The latter hypothesis might be most feasible because there are groups with both conidial forms for example *Saccharata* and *Dichomera* anamorphs of *Neofusicoccum* and *Botryosphaeria* that are superficially more similar to anamorphs with *Diplodia*-like conidia than those with *Fusicoccum*-like conidia.

Several species in the *Diplodia* clade could be distinguished in this study. These include both morphological forms (A and C morphotypes) of *D. pinea*, the well-known opportunistic, stress-associated die-back pathogen of pines (Swart & Wingfield 1991; De Wet *et al.* 2000), *D. scrobiculata* that was previously known as the B morphotype of *D. pinea* (De Wet *et al.* 2003), *D. cupressi* previously treated as *D. pinea* f.sp. *cupressi* (Alves *et al.* 2006), *D. mutila* and *D. seriata* (Phillips *et al.* 2007). Many of these species have been confused in the past due to their morphological similarity (Wang *et al.* 1985; Swart *et al.* 1993; Smith & Stanosz 1995; Stanosz *et al.* 1998; Burgess *et al.* 2001; Zhou & Stanosz 2001). Cryptic species can, however, be distinguished when using multiple gene genealogies as has been shown previously (De Wet *et al.* 2000, 2003; Alves *et al.* 2006) and in the present study.

The multiple gene genealogy generated in this study confirms the wide host range of the A morphotype of *D. pinea* that includes various *Pinus* spp., *C. deodora*, *Pseudotsuga menziesii* and a *Larix* sp. This supports previous studies that have demonstrated a wide distribution and host range of the A morphotype of *D. pinea* (Stanosz *et al.* 1999; Zhou & Stanosz 2001). The C morphotype of *D. pinea* is very closely related to the A morphotype based on DNA sequence data, but is morphologically distinct, more pathogenic and has a very restricted distribution (De Wet *et al.* 2000). This form of *D. pinea* was initially described from *P. patula* in Northern Sumatra, Indonesia (De Wet *et al.* 2000) and in this study it is also recognized from *P. merkusii* in Sulawesi, Indonesia. Unlike *P. patula*, this is a native pine in Asia and it is most likely the source of isolates found on the former species, which is grown as a non-native in plantations. Together these data strongly suggest that the C morphotype of *D. pinea* should be recognized and described as a distinct species.

*Diplodia scrobiculata* was initially found to be different from *D. pinea* (Palmer *et al.* 1987) and mainly associated with *P. resinosa* and *P. banksiana* in the North Central United States (Smith & Stanosz 1995). It was later also reported from other *Pinus* spp., as well as *Cedrus* spp. in Europe and Israel (Stanosz *et al.* 1999; De Wet *et al.* 2000). Results of the present study have expanded the host range of *D. scrobiculata* to include *Picea mariana*. The host ranges of *D. pinea* and *D. scrobiculata* include only gymnosperms in the Pinaceae but both species appear not to be host-specific below this phylogenetic level.

Hosts of *D. seriata* include both gymnosperms and angiosperms. It is a generalist species reported from a wide variety of host genera (Punithalingam & Waller 1973). *Diplodia mutila* is also a generalist species able to infect a wide range of angiosperms (Jacobs & Rehner 1998; Zhou & Stanosz 2001) and the single report of this fungus from a *Juniperus* sp. (Tisserat *et al.* 1988) was shown in this study to be *D. cupressi*. The host range of *D. cupressi* includes only gymnosperms in the Cupressaceae (Alves *et al.* 2006).

In most previous studies, the *Lasiodiplodia* clade of the Botryosphaeriaceae has been represented by sequence data from only one species, *L. theobromae*. In GenBank this species is represented by isolates from *Pinus*, *Vitis*, *Musa*, *Santalum*, *Carica papaya*, *Acacia*, *Camptotheca*, *Syzygium*, *Fraxinus*, *Vitex* and *Eucalyptus*. This fungus is thus a generalist species able to infect both angiosperms and gymnosperms. It is well-known that *L. theobromae* is generally found in tropical and subtropical regions on an extremely wide host range (Punithalingam 1976). Other *Lasiodiplodia* species are also predominant in tropical and subtropical regions, and most are also not host specific. These include *L. gonubiensis* (Pavlic *et al.* 2004), *L. venezuelensis*, *L. rubropurpurea* and *L. crassispora* (Burgess *et al.* 2006). They do, however, seem to be associated only with angiosperms. *Lasiodiplodia* remains undersampled in most studies,

including in this one, and needs dedicated collections and taxonomic attention if its true status is to be confirmed.

*Dothiorella* is represented by four species. These are *Do. sarmentorum* from *Malus*, *Ulmus*, *Pyrus* and *Prunus*, *Do. iberica* from species of *Quercus* and *Malus*, *Do. viticola* from *Vitis* spp and a potentially undescribed species from *Casuarina* spp. The latter species should be compared to other species described from this host and area to determine its species status, and be formally described if none exist. All the *Dothiorella* species, for which sequence data are available, are only known from angiosperms (Phillips *et al.* 2005).

Interesting trends were observed in host association for the lineages of the Botryosphaeriaceae investigated. Some *Diplodia* species (*D. pinea*, *D. scrobiculata* and *D. cupressi*) occur exclusively on gymnosperms, and other *Diplodia* species (*D. mutila* and *D. seriata*) on both gymnosperms and angiosperms. *Lasiodiplodia* species occur on both gymnosperms and angiosperms, and the phylogenetically more distant *Dothiorella* species only on angiosperms. *Neoscytalidium* and *Pseudofusicoccum* are known only from angiosperms. *Botryosphaeria* spp. are also known exclusively from angiosperms although there is a single report from *P. nigra* in Lexington, Kentucky (Flowers *et al.* 2003). This, however, represents only one isolate, and extensive world-wide studies on conifers in native and introduced environments have shown that this is not a general trend (De Wet *et al.* 2000; Burgess *et al.* 2004). Species of *Neofusicoccum* also occur mostly on angiosperms. There are, however, some interesting exceptions, all on Southern Hemisphere conifers. These include an undescribed *Neofusicoccum* sp. from *Wollemia* and *Araucaria*, *N. australe* from *Wollemia* and *Widdringtonia* in Australia and South Africa (Slippers *et al.* 2005b), and single reports of *N. parvum* on *P. patula* (Gezahgne *et al.* 2003) and *Podocarpus falcatus* (Gure *et al.* 2005) in Ethiopia.

Analyses of host association for the six lineages of the Botryosphaeriaceae have shown that most species have been reported only from angiosperms, or in a few cases both angiosperms and gymnosperms. Very few species are known exclusively from gymnosperms. Angiosperms thus appear to be the most common, and possibly ancestral, host group of the Botryosphaeriaceae (excluding *Macrophomina*, *Guignardia*, *Saccharata* and “*Botryosphaeria*” *quercuum*). Infection of gymnosperms most likely occurred more recently in specific groups via host shifts, as there appears to be little evidence for host associated co-evolution amongst species of the Botryosphaeriaceae. This is perhaps not surprising, given that many species are not host specific. The close relationship between some species occurring predominantly on either gymnosperms or angiosperms (or different families within the gymnosperms) indicates that host shifts between distantly related groups of plants are not uncommon, and could have been an important driver of speciation in the group. Understanding these patterns of host shift is important, as they can often lead to disease or epidemic outbreaks (Slippers *et al.* 2005a). Host association patterns in the Botryosphaeriaceae are largely unexplored. This is partly due to taxonomic problems that have been associated with the group and particularly a reliance on morphology to identify species. The many recent reports of incorrectly identified or cryptic species aptly illustrates this view. The profusion of ITS sequence data that has become available for members of the Botryosphaeriaceae in recent years has made it possible here to explore general patterns of host association in the group. In some cases, the environment appears to be a dominating determinant (e.g. *L. theobromae*; Punithalingam 1976; Mohali *et al.* 2005), while in others specificity might be restricted to a single host genus (e.g. *Eucalyptus* spp. for *N. eucalyptorum* and *N. eucalypticola*; Slippers *et al.* 2004b) or host families (e.g. Pinaceae for *D. pinea* and *D. scrobiculata*; Stanosz *et al.* 1999; De Wet *et al.* 2003). An improved understanding



of these patterns and factors that drive them will be important determinants in understanding the evolution of this group of fungi, their epidemiology, the emergence of new diseases, and characterizing and managing their threat to forestry and agriculture.

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**Table 1.** *Diplodia* and *Dothiorella* isolates included in this study.

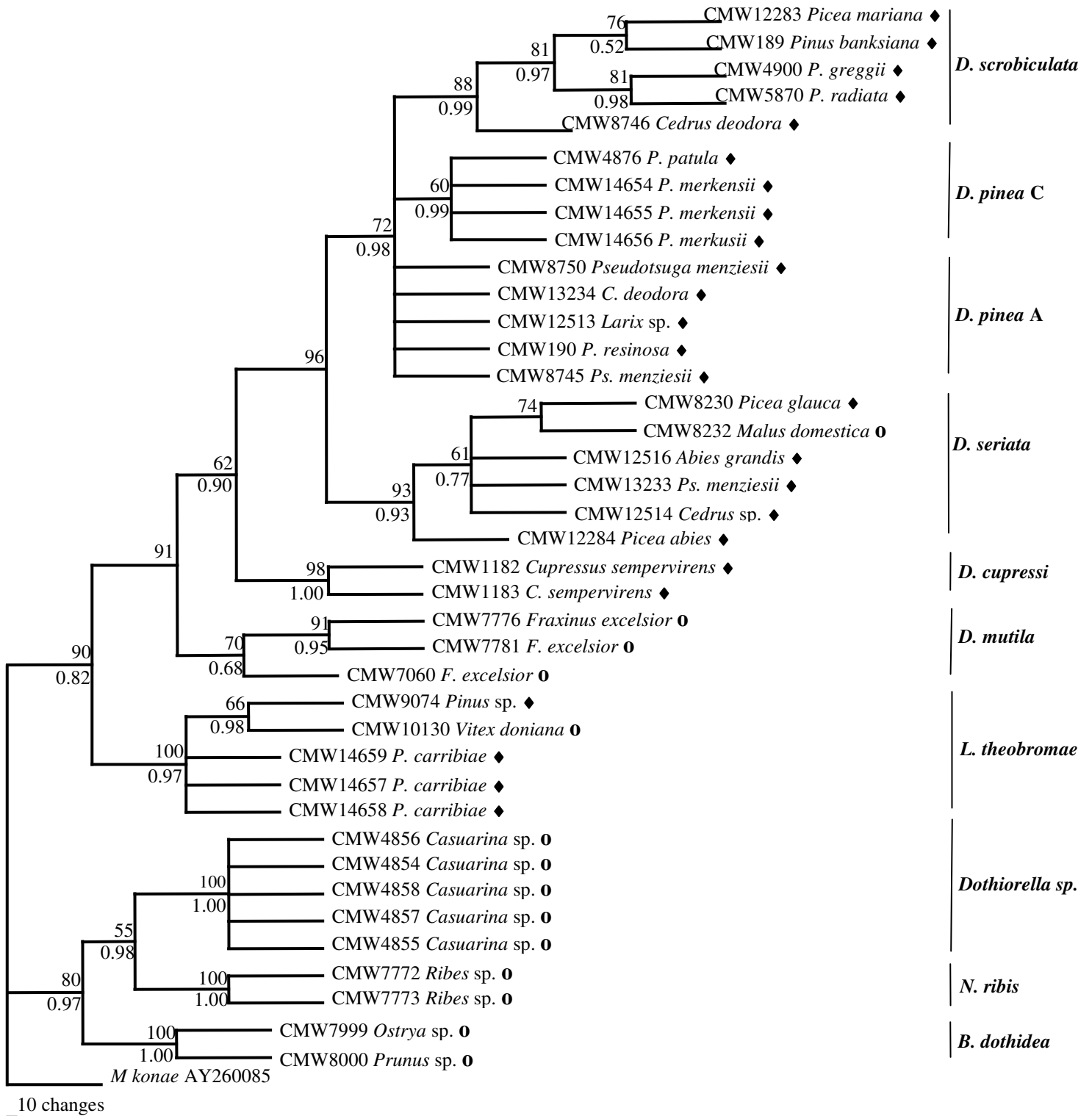
| Isolates | Identification         | Origin              | Host                          | Other collections | Collector                            | GenBank Accession numbers |            |          |
|----------|------------------------|---------------------|-------------------------------|-------------------|--------------------------------------|---------------------------|------------|----------|
|          |                        |                     |                               |                   |                                      | ITS                       | $\beta$ T2 | ACT      |
| CMW1182  | <i>D. cupressi</i>     | Israel              | <i>Cupressus sempervirens</i> |                   | W. Swart (Swart <i>et al.</i> 1993)  | EU220433                  | EU220469   | EU220451 |
| CMW1183  | "                      | "                   | "                             |                   | "                                    | EU220434                  | EU220470   | EU220452 |
| CMW8745  | <i>D. pinea</i> (A)    | Michigan            | <i>Pseudotsuga menziesii</i>  | 150               | M. Palmer                            | EU220435                  | EU220471   | EU220453 |
| CMW8750  | "                      | Great Britain       | "                             | 94-165            | J. Gibbs                             | EU220436                  | EU220472   | EU220454 |
| CMW13233 | <i>D. seriata</i>      | France              | "                             | BOT1109           | P. Chandelier                        | EU220437                  | EU220473   | EU220455 |
| CMW8746  | <i>D. scrobiculata</i> | "                   | <i>Cedrus deodora</i>         | 94-17             | "                                    | EU220438                  | EU220474   | EU220456 |
| CMW12514 | <i>D. seriata</i>      | "                   | <i>Cedrus</i> sp.             | BOT1101           | "                                    | EU220439                  | EU220475   | EU220457 |
| CMW13234 | <i>D. pinea</i> (A)    | "                   | <i>C. deodora</i>             | BOT1112           | "                                    | EU220440                  | EU220476   | EU220458 |
| CMW12513 | "                      | France              | <i>Larix</i> sp.              | BOT1100           | "                                    | EU220441                  | EU220477   | EU220459 |
| CMW12516 | <i>D. seriata</i>      | "                   | <i>Abies grandis</i>          | BOT1097           | "                                    | EU220442                  | EU220478   | EU220460 |
| CMW12284 | "                      | Minnesota           | <i>Picea abies</i>            | MNS3/BOT2834      | M.J. Wingfield                       | EU220443                  | EU220479   | EU220461 |
| CMW12283 | <i>D. scrobiculata</i> | Ontario             | <i>P. mariana</i>             | U9596/BOT2833     | J. Reid (Hausner <i>et al.</i> 1999) | EU220444                  | EU220480   | EU220462 |
| CMW4854  | <i>Dothiorella</i> sp. | Canberra, Australia | <i>Casuarina</i> sp.          |                   | M.J. Wingfield                       | EF107752                  | EF107756   | EF107754 |
| CMW4855  | "                      | "                   | "                             | CBS120688         | "                                    | DQ846773                  | DQ875340   | DQ846781 |
| CMW4856  | "                      | "                   | "                             | CBS120689         | "                                    | DQ846772                  | DQ875339   | DQ846780 |
| CMW4857  | "                      | "                   | "                             | CBS120690         | "                                    | DQ846774                  | DQ875341   | DQ846782 |
| CMW4858  | "                      | "                   | "                             |                   | "                                    | EF107753                  | EF107757   | EF107755 |
| CMW14654 | <i>D. pinea</i> (C)    | Sulawesi, Indonesia | <i>P. merkusii</i>            | [1]1              | "                                    | EU220445                  | EU220481   | EU220463 |
| CMW14655 | "                      | "                   | "                             | [1]6              | "                                    | EU220446                  | EU220482   | EU220464 |
| CMW14656 | "                      | "                   | "                             | [2]1              | "                                    | EU220447                  | EU220483   | EU220465 |
| CMW14657 | <i>L. theobromae</i>   | Cuba                | <i>P. caribaea</i>            | Cuba5             | "                                    | EU220448                  | EU220484   | EU220466 |
| CMW14658 | "                      | "                   | "                             | Cuba12            | "                                    | EU220449                  | EU220485   | EU220467 |
| CMW14659 | "                      | "                   | "                             | Cuba17            | "                                    | EU220450                  | EU220486   | EU220468 |

**Table 2.** Isolates of *Diplodia pinea*, *D. scrobiculata* and various *Botryosphaeria* spp. included in this study for comparative purposes.

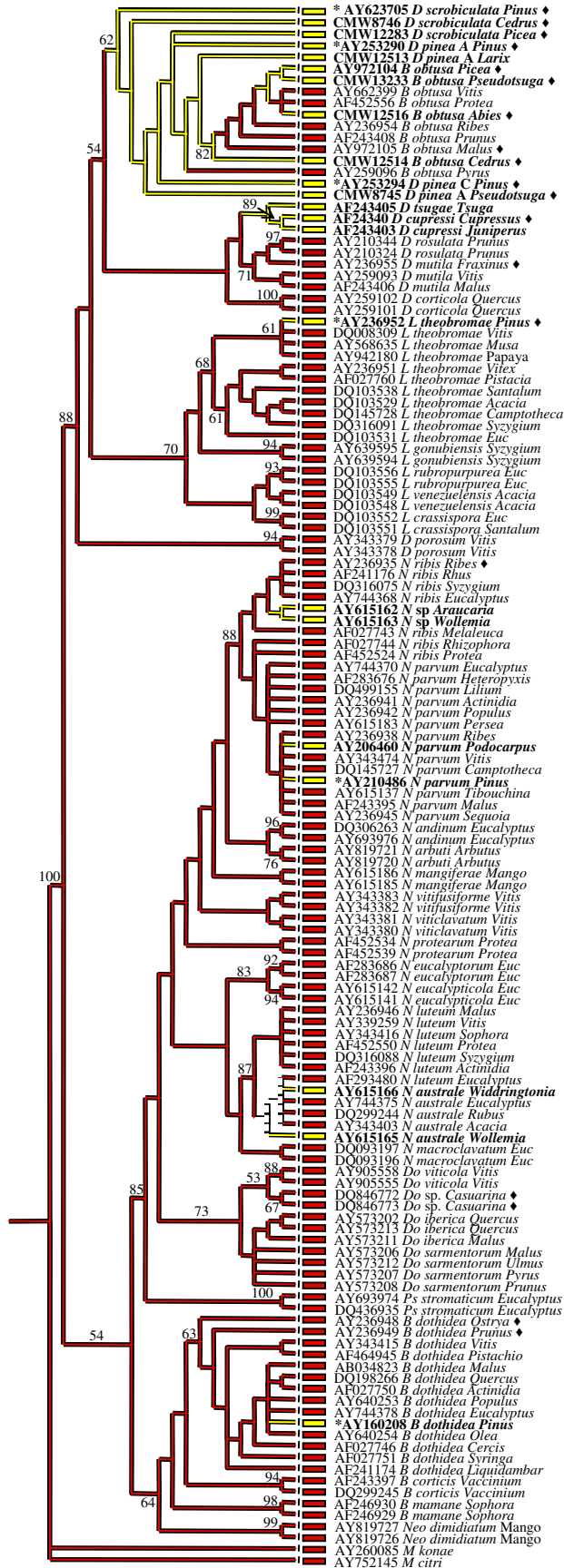
| Isolates | Identification                  | Origin                      | Host                      | Reference  | GenBank Accession numbers |          |          |
|----------|---------------------------------|-----------------------------|---------------------------|--|---------------------------|----------|----------|
|          |                                 |                             |                           |  | ITS                       | βT2      | ACT      |
| CMW190   | <i>Diplodia pinea</i> (A)       | United States               | <i>P. resinosa</i>        | Palmer <i>et al.</i> 1987; De Wet <i>et al.</i> 2000, 2003 | AY253290                  | AY624256 | AY624261 |
| CMW4876  | <i>D. pinea</i> (C)             | Northern Sumatra, Indonesia | <i>P. patula</i>          | De Wet <i>et al.</i> 2000, 2003                            | AY253294                  | AY624257 | AY624262 |
| CMW5870  | <i>D. scrobiculata</i>          | California                  | <i>P. radiata</i>         | De Wet <i>et al.</i> 2003                                  | AY623704                  | AY625259 | AY624264 |
| CMW4900  | "                               | Mexico                      | <i>P. greggii</i>         | "  | AY623705                  | AY624260 | AY624265 |
| CMW189   | "                               | USA                         | <i>P. banksiana</i>       | Palmer <i>et al.</i> 1987; De Wet <i>et al.</i> 2000, 2003 | AY253292                  | AY624258 | AY624263 |
| CMW8230  | <i>D. seriata</i>               | Canada                      | <i>Picea glauca</i>       | De Wet <i>et al.</i> 2003                                  | AY972104                  | AY972119 | AY972110 |
| CMW8232  | "                               | South Africa                | <i>Malus domestica</i>    | De Wet <i>et al.</i> 2003                                  | AY972105                  | AY972120 | AY972111 |
| CMW9074  | <i>Lasiodiplodia theobromae</i> | Mexico                      | <i>Pinus sp.</i>          | Slippers <i>et al.</i> 2004a                               | AY236952                  | AY236930 | AY972108 |
| CMW10130 | "                               | Uganda                      | <i>Vitex doniana</i>      | "  | AY236951                  | AY236929 | AY972109 |
| CMW7060  | <i>D. mutila</i>                | Netherlands                 | <i>Fraxinus excelsior</i> | "  | AY236955                  | AY236933 | AY972112 |
| CMW7776  | "                               | Pusiona, Italy              | "                         | "  | AY972106                  | AY972121 | AY972113 |
| CMW7781  | "                               | Porza, Switzerland          | "                         | "  | AY972107                  | AY972122 | AY972114 |
| CMW7772  | <i>Neofusicoccum ribis</i>      | New York, USA               | <i>Ribes sp.</i>          | "  | AY236935                  | AY236906 | AY972115 |
| CMW7773  | "                               | "                           | "                         | "  | AY236936                  | AY236907 | AY972116 |
| CMW7999  | <i>B. dothidea</i>              | Crocifisso, Switzerland     | <i>Ostrya sp.</i>         | "  | AY236948                  | AY236926 | AY972117 |
| CMW8000  | "                               | "                           | <i>Prunus sp.</i>         | "  | AY236949                  | AY236927 | AY972118 |



**Figure 1.** Phylogram constructed for the combined sequence data of the ITS regions and 5.8S rDNA operon and two partial protein-coding genes (Bt2 of  $\beta$ -tubulin and ACT) based on neighbour-joining distance analysis with branch support values (maximum parsimony bootstrap proportions/Bayesian posterior probabilities). Bootstrap values were determined after 1000 replications using parsimony based on a strict heuristic search with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum length is zero. Only groups with frequencies >50% were retained. Isolates marked with  $\blacklozenge$  are from Gymnosperms and isolates marked with  $\bullet$  are from Angiosperms.



**Figure 2.** Phylogram constructed for the ITS and 5.8S rDNA based on neighbour-joining distance analysis with branch support values (maximum parsimony bootstrap proportions). Bootstrap values were determined after 1000 replications using parsimony based on a strict heuristic search with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum length is zero. Only groups with frequencies >50% were retained. Gymnosperm/angiosperm character states were traced in MacClade. Isolates marked with ■ are from Gymnosperms and isolates marked with □ are from Angiosperms. Isolates marked with an asterisk \* are from *Pinus* spp. *Pinus* is arguably the most extensively sampled host for the Botryosphaeriaceae. The dominating species are *D. pinea*, *D. scrobiculata* and *L. theobromae*. Reports of *B. dothidea* and *N. parvum* on this host are two rare exceptions, only observed once in each case. Isolates marked with ♦ were included in Figure 1.



*Diplodia*

*Lasiodiplodia*

*Neofusicoccum*

*Dothiorella*

*Pseudofusicoccum*

*Botryosphaeria*

*Neoscytalidium*



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## CHAPTER 5

# PATTERNS OF MULTIPLE VIRUS INFECTIONS IN THE CONIFER PATHOGENIC FUNGI, *DIPLODIA PINEA* AND *DIPLODIA SCROBICULATA*

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## ABSTRACT

*Diplodia pinea* and *D. scrobiculata* are opportunistic pathogens associated with various disease symptoms on conifers that most importantly include die-back and stem cankers. Two viruses with dsRNA genomes, *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2) are found in *D. pinea* and an undescribed dsRNA element is known to occur in *D. scrobiculata*. In this study, we partially characterized the putative RNA dependent RNA polymerase (RdRp) of the undescribed dsRNA element and designed virus-specific primers from the RdRp regions of all three virus genomes. This made it possible to screen for the presence of the three viruses in a collection of *D. pinea* and *D. scrobiculata* isolates using Real-Time PCR. Triple infections with all three viruses occurred in *D. pinea* and *D. scrobiculata*. Co-infections with SsRV1 and SsRV2 were common but found only in *D. pinea*. Co-infection with SsRV1 and the undescribed dsRNA element was rare and observed only in *D. pinea*. Single infections with either SsRV1 or SsRV2 were equally common, while the undescribed dsRNA element never occurred alone. SsRV1 occurred alone in both *D. pinea* and *D. scrobiculata* while SsRV2 occurred alone only in *D. pinea*. There were only two instances where the undescribed dsRNA element was observed in *D. pinea* and it was otherwise found only in *D. scrobiculata*. This study highlights the complex interactions between the viruses found in the closely related plant pathogenic fungi, *D. pinea* and *D. scrobiculata*. It illustrates the importance of not only characterizing viruses infecting fungi but also of determining the interactions between mycoviruses and their fungal hosts.

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## INTRODUCTION

*Diplodia pinea* (Desm.) Kickx (= *Sphaeropsis sapinea* (Fr.) Dyko & Sutton and *D. scrobiculata* J. de Wet, Slippers & M.J. Wingf., previously known as the B morphotype of *D. pinea* (Palmer *et al.* 1987; De Wet *et al.* 2003), are opportunistic pathogens on conifers (Eldridge 1961; Punithalingam & Waterston 1970; Swart *et al.* 1985). Both fungi are endophytes that can exist in healthy asymptomatic trees and the onset of disease is typically associated with stress (Smith *et al.* 1996; Stanosz *et al.* 1997; Flowers *et al.* 2001, 2003). In association with unfavourable environmental conditions or harsh physical factors, they commonly cause disease symptoms including die-back, whorl cankers and seedling collar rot (Eldridge 1961; Gibson 1979; Swart & Wingfield 1991). *Diplodia pinea* is an important pathogen of *Pinus* spp. in natural forests and plantations of non-native species, in many parts of the world (Punithalingam & Waterston 1970; Swart *et al.* 1985; Stanosz *et al.* 1999; Burgess *et al.* 2001). In contrast, *D. scrobiculata* has a more limited distribution known only from the North Central USA and Western Europe and it tends to be weakly pathogenic (Palmer *et al.* 1987; Blodgett & Stanosz 1997; Stanosz *et al.* 1999; Burgess *et al.* 2004).

Diseases caused by *D. pinea* and *D. scrobiculata* are managed through the exploitation of resistant host species and the implementation of optimal management strategies and silvicultural practices (Swart *et al.* 1985; Swart & Wingfield 1991). Significant economic losses due to *D. pinea* infections are however incurred, especially in plantations of non-native pine species in the southern hemisphere (Gibson 1979; Zwolinski *et al.* 1990a, 1990b). An alternative to conventional control might be found in the application of hypovirulence-mediated mycoviruses as biocontrol agents (Anagnostakis 1982; Heiniger & Rigling 1994). Various studies have,

therefore, considered whether dsRNA elements occur in *D. pinea* (Wu *et al.* 1989; Preisig *et al.* 1998; Steenkamp *et al.* 1998; De Wet *et al.* 2001; Adams *et al.* 2002).

Several dsRNA elements ranging from 600 bp – 7 kb in size have been reported from *Diplodia* isolates (Wu *et al.* 1989; Preisig *et al.* 1998; Steenkamp *et al.* 1998; De Wet *et al.* 2001; Adams *et al.* 2002). Two of these elements have been characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2) (Preisig *et al.* 1998). A third dsRNA element associated with *D. scrobiculata* is known, but it has not been characterized (De Wet *et al.* 2001). SsRV1 and SsRV2 belong to the family *Totiviridae* and are treated as unclassified *Totiviridae*. They are characterized by unipartite dsRNA genomes with two open reading frames, one coding for a capsid polypeptide and the other one for a RNA-dependant RNA polymerase (RdRp). SsRV1 and SsRV2 genomes are in the 5 kb size range, while the undescribed dsRNA element in *D. scrobiculata* is slightly larger. The dsRNA elements associated with *D. pinea* and *D. scrobiculata* do not appear to result in phenotypic changes to the pathogen or to reduce its virulence (Steenkamp *et al.* 1998; De Wet *et al.* 2001). Despite the fact that these viruses have no apparent phenotypic effect, the presence of specific viruses in their host populations serve as a useful marker in studying movement of fungal pathogens.

Multiple infections with different cytoplasmic dsRNA elements, as well as, mitochondrial dsRNA elements are common in fungi (Buck 1986). For example, in a single *Helminthosporium victoriae* isolate, two viruses were found, one belonging to the *Totiviridae* (Huang & Ghabrial, 1996) and the other to the *Chrysoviridae* (Ghabrial *et al.* 2002). Likewise, three different viruses a totivirus (*G. abietina* RNA virus L2 or GaRV-L2), a partitivirus (*G. abietina* RNA virus MS2 or GaRV-MS2) and a mitovirus (*G. abietina* mitochondrial RNA virus S2 or GaMRV-S2) have been found in a single *Gremmeniella abietina* var. *abietina* type A isolate (Tuomivirta &



Hantula, 2005). Thus, the discovery of two different totiviruses, SsRV1 and SsRV2, in a single *D. pinea* isolate (Preisig *et al.* 1998) was not unusual.

In a previous study, using SsRV1- and SsRV2-specific primers, it was shown that some isolates are infected by either SsRV1 or SsRV2, or a combination of the two viruses (De Wet *et al.* 2001). The ability to detect these genomes in infected strains is inconsistent as the titre of the viruses can be low and variable depending on the culture conditions of the fungus. Traditionally, detection of specific viral genomes has depended on the use of blotting techniques, which have a limited sensitivity. Real-Time PCR is increasingly being used for virus detection, especially in the medical field (Mackay *et al.* 2002). This technology is not only extremely sensitive but it also allows for relatively accurate and rapid quantification of the concentration of the viral genomes.

*Diplodia pinea* and *D. scrobiculata* are closely related fungi that have only recently been recognized as distinct. Thus, an intriguing question relates to the relative distribution of the three viruses in isolates of the two fungi. Consequently, this study was undertaken to screen a collection of *D. pinea* and *D. scrobiculata* isolates from various parts of the world for the presence of SsRV1, SsRV2 and the undescribed virus element known only from *D. scrobiculata*. To achieve this goal, we partially characterized a putative RdRp of the undescribed dsRNA element associated with *D. scrobiculata*.

## **MATERIALS AND METHODS**

### **DsRNA extraction, cDNA synthesis and cloning of a putative RdRp gene**

A single conidial isolate (CMW5870) of *D. scrobiculata* from California was grown in 2 ml Eppendorf tubes containing 2 % malt extract (ME) broth (Biolab Diagnostics, Midrand, South Africa), incubated at room temperature for at least two weeks. Mycelium was harvested by

centrifugation and washed with 0.1 % diethylpyrocarbonate (DEPC) treated double distilled water. The mycelium was homogenized using a Retsch MM301 homogenizer (30 freq/s; 30 s). Trizol (Invitrogen Corporation, Carlsbad, CA, USA) was used to extract dsRNA from the mycelium (1ml Trizol per 0.5 g mycelium). After centrifugation, the supernatant was passed through a QIAquick gel extraction column (QIAGEN, GmbH, Germany) following the manufacturer's specifications. The eluted sample was separated on a 1 % agarose (w/v) gel (Biolab Diagnostics, Midrand, South Africa) stained with ethidium bromide, using a 1 x Tris-Acetic Acid-EDTA (TAE) (pH 8) electrophoresis buffer. The dsRNA band was cut from the gel and purified using the QIAquick gel extraction kit (QIAGEN, GmbH, Germany).

Synthesis of cDNA from dsRNA was performed using a Roche cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). The dsRNA and random hexamer primers were subjected to denaturing conditions (10 min at 99 °C), which was followed by the first and second strand cDNA syntheses done as specified by the manufacturer. The synthesized cDNA was purified using a QIAquick gel extraction kit (QIAGEN, GmbH, Germany) and cloned using the Lucigen PCR-SMART non-proofreader cloning kit (Lucigen® Corporation, Middleton, WI, USA). A colony PCR was performed using CL3 and SR2 pcrSMART™ vector-specific primers. Colony PCR products with inserts were purified using the Roche PCR product purification kit (Roche Diagnostics, Basel, Switzerland) and sequenced.

Genome-specific primers were designed from the random amplified cDNA fragments to amplify larger pieces of the putative RdRp using the Roche Titan One Tube RT-PCR system (Roche Diagnostics, Basel, Switzerland). Single band RT-PCR products were purified using the Roche PCR product purification kit and sequenced. Non-specific RT-PCR products were gel purified using the PCR product purification kit (Roche Diagnostics, Basel, Switzerland) and cloned using

the pGEM<sup>®</sup>-T Easy Vector System II (Promega Corporation, Madison, WI, USA). A colony PCR was performed using T7 and SP6 pGEM<sup>®</sup>-T Easy vector-specific primers. Colony PCR products with inserts were purified using the Roche PCR product purification kit (Roche Diagnostics, Basel, Switzerland) and sequenced.

Sequencing was achieved using the ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing kit and an ABI PRISM<sup>®</sup> 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). All reactions were done using protocols recommended by the manufacturers. All the sequence data were processed using Chromas 2.3 (<http://www.technelysium.com.au>) and contigs were assembled using Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Contigs were aligned in BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsberg, CA, USA).

### **Primer development**

Virus-specific primers for SsRV1 and SsRV2 were designed from the open reading frame (ORF) coding for the RdRp of these viruses (Preisig *et al.* 1998) The full-length virus genomes are available on GenBank (SsRV1 = AF038665; SsRV2 = AF039080)

(<http://www.ncbi.nlm.nih.gov/>). The SsRV1-specific primers are SsRV1-F1 (5'-

GACGGCCCCGTCTACAACACAGAC-3') and SsRV1-R1 (5'-

GGGCGGCGCGTTCCACCTCCGAC-3') (1951-2102). The SsRV2-specific primers are

SsRV2-F1 (5'-GCCGTTGCGCCCAACCGCTCGAGG-3') and SsRV2-R2 (5'-

GGTCTGCGCCTCACTGGGCCGAGG-3') (2033-2183). The sequence of the putative RdRp

ORF of the undescribed dsRNA element associated with *D. scrobiculata* as determined in this

study was deposited in GenBank (EF568774). Primers specific to this undescribed dsRNA

element were designed and these primers are DsRV1-F2 (5'-

GGTATCGCTGGTTACCCGATCCGC-3') and DsRV1-R2 (5'-CAGATGGGGCTCAAAGGCACCTCC-3') (1781-1934).

### **Fungal isolates used for genotyping**

A total of 32 *Diplodia pinea* and *D. scrobiculata* isolates from South Africa, North Central United States, Mexico, Madagascar, Colombia and California were used in this study (Table 1). The identity of these isolates had been determined in previous studies (Stanosz *et al.* 1999; De Wet *et al.* 2000; Adams *et al.* 2002; De Wet *et al.* 2003). Single conidial cultures of these isolates were obtained from the Culture Collection (CMW) of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

### **Total RNA isolations**

Single conidial cultures were transferred to 2 % malt extract agar (MEA) (Biolab Diagnostics, Midrand, South Africa) and incubated at 25 °C under cool white light. After four days, mycelium was scraped from the borders of the cultures and transferred to 500 µl 2 % malt extract (ME) broth (Biolab Diagnostics, Midrand, South Africa) in 2 ml Eppendorf tubes. These were incubated at 25 °C for at least two weeks or until sufficient biomass had been produced for total nucleic acid isolations. Mycelium was harvested by centrifugation and washed with DEPC-treated double distilled water. The mycelium was homogenized using a Retsch MM301 homogenizer (30 freq/s; 30 s).

Trizol (Invitrogen Corporation, Carlsbad, CA, USA) was used to extract total RNA from the homogenized mycelium (1ml Trizol per 500 mg mycelium). The suspension was incubated at room temperature for 5 min, 200 µl chloroform was added, vigorously shaken and incubated a second time at room temperature for 5 min. The supernatant containing total RNA was

recovered through centrifugation at 12 000 rpm (14 463 x g) at 4 °C for 10 min. Supernatant was passed through a QIAquick gel extraction column (QIAGEN, GmbH, Germany) according to the manufacturer's specifications. Samples were stored at – 20 °C.

### **cDNA synthesis and Real-Time PCR genotyping**

Synthesis of cDNA from the extracted total RNA was performed using the Roche Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland). The total RNA and virus-specific primers was firstly denatured for 5 min at 99 °C followed by the first strand cDNA synthesis done according to the manufacturer's instructions. For each isolate, cDNA was synthesized using all three anti-sense primers, separately.

Real-Time amplification was achieved using the LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Basel, Switzerland). A one in ten dilution was made of the cDNA and 5 µl was added to 10 µl SYBR Green Master Mix and each specific primer pair to a final concentration of 0.5 µM (final volume = 20 µl). Amplification was carried out in a 384-well plate in the LightCycler® 480 Real Time PCR system (Roche Diagnostics, Basel, Switzerland) using an initial denaturation of 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 62 °C for 5 s and 72 °C for 8 s. Fluorescence was recorded at the annealing step for each cycle. The amplification cycles were followed by a melting cycle, in which DNA was denatured at 95 °C for 30 s, cooled to 50 °C using a rate of 1 °C /s and held for 30 s. Temperature was then raised to 95 °C with a transition rate of 10 acquisitions/ °C. Fluorescence was continuously monitored during the melting cycle. This was followed by a cooling cycle to 40 °C for 20 s.

Melting curves were converted into negative derivative curves of fluorescence with respect to temperature ( $-dF/dT$ ) by the LightCycler® Data Analysis software. These showed whether a sequence-specific product with a unique melting temperature ( $T_m$ ) had been obtained. Non-

specific amplification products such as primer dimers could be distinguished from sequence-specific products based on their lower melting points. The final amplification products were electrophoresed on a 1.5 % agarose gel to confirm that the melting curve analysis reflects the amplicons of *ca.* 150 bp.

### **Amplicon sequence confirmation**

The identity of a sub-set of amplicons was confirmed by sequencing (Table 1). A one in ten dilution of the amplified product was made and 5 µl was used in a 25 µl reaction mixture consisted of 10 x Fast Start PCR buffer (50 mM Tris-HCl, pH 8.3; 2 mM MgCl<sub>2</sub>; 10 mM KCl; 5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), 200 µM of each dNTP, 0.2 µM of each primer and 2 U FastStart Taq DNA polymerase (Roche Diagnostics, Basel, Switzerland). The following temperature profile was followed: 95 °C for 5 min, 45 cycles of 95 °C for 10 s, 62 °C for 5 s and 72 °C for 8 s, followed by a final elongation at 72 °C for 5 min. PCR products were electrophoresed on a 1.5 % agarose gel. Products without primer dimers were purified using the Roche High Pure PCR Product Purification kit (Roche Diagnostics, Basel, Switzerland), and those with primer dimers were gel purified using the same kit. Purified products were then sequenced using the ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing kit and an ABI PRISM<sup>®</sup> 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). All the reactions were done using protocols recommended by the manufacturers. The sequence data were processed in Chromas 2.3 (<http://www.technelysium.com.au>) and aligned in Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). DNA sequences of fragments obtained were subjected to BLAST using the NCBI translated database (Blastx) to confirm their identities. Sequences obtained for SsRV1 and SsRV2 were aligned to the original genome sequences. Sequences obtained for the

undescribed dsRNA element associated with *D. scrobiculata* were aligned to the putative RdRp to confirm similarity, as well as, to determine whether any differences were present.

## RESULTS

### Partial characterization of a putative RdRp gene

DsRNA was extracted from a Californian isolate of *D. scrobiculata* (CMW5870). DNA fragments of various sizes were obtained after cDNA synthesis with random hexamer primers. These sequences were subjected to BLAST searches using the NCBI translated database (Blastx). Sequences with homology to the RdRp of *Trichomonas vaginalis* virus II (TVV2) were retained. These fragments were aligned according to the RdRp of TVV2 and specific primers were designed to amplify a total of 2174 bp. The nucleotide sequence that was obtained translated to 724 amino acids containing all eight conserved motifs as described by Bruenn (1993). The obtained RdRp gene for the undescribed virus associated with *D. scrobiculata* has 24 % homology to the RdRp of TVV2, 24 % homology to the RdRp of SsRV1 and 27 % homology to the RdRp of GaRV-L1. The sequence of the putative RdRp associated with *D. scrobiculata* was deposited in GenBank (EF568774), while the rest of the genome is being determined.

### cDNA synthesis and Real-Time PCR genotyping

Total RNA was extracted from 32 *D. pinea* and *D. scrobiculata* isolates collected from a wide range of geographic locations (Table 1). cDNA was then synthesized and amplified for all the isolates. Melting curve analyses revealed three unique melting points for SsRV1, SsRV2 and the undescribed dsRNA element associated with *D. scrobiculata*. The  $T_m$  for SsRV1 ranged from 88.5 – 90.3 °C, that for SsRV2 from 86.2 - 89.1 °C and the  $T_m$  for the dsRNA element associated with *D. scrobiculata* ranged from 87.0 – 87.7 °C. Multiplexing was not possible as the  $T_m$ -

values of the three viruses overlapped. Agarose gel electrophoresis revealed the desired 150 bp amplicon and where primer dimers were observed, these correlated with the lower  $T_m$  values (81 – 84) that were detected with the melting curve analyses.

### **Amplicon sequence confirmation**

Sequences of a sub-set of amplicons gave a 100 % confirmation with the respective GenBank sequences of SsRV1, SsRV2 and the putative RdRp of the undescribed dsRNA element. A representative sample of amplicons was sequenced for SsRV1 and SsRV2 and all six amplicons of the undescribed dsRNA element were sequenced. Single base pair differences were in some case observed between the sequenced amplicons and the original genome sequence probably as a result of amplification errors. Sequence data did, however, provide confidence in the  $T_m$ -values of each specific virus as detected by Real-Time PCR.

### **Virus distribution in isolates**

All three dsRNA elements (SsRV1, SsRV2 and the undescribed dsRNA element in *D. scrobiculata*) occurred in five of the 32 isolates (16 %) included in this study (Table 1). Of these five isolates, only one was of *D. pinea* and the other four were *D. scrobiculata*. SsRV1 and SsRV2 occurred together in 13 of the 32 isolates (41 %) (Table 1). All of these isolates were of *D. pinea*. Single infections with only SsRV1 occurred in six of the 32 isolates (19 %) and six isolates contained only SsRV2. Isolates infected with only SsRV1 were of *D. pinea* and *D. scrobiculata*. Isolates infected only with SsRV2 were all of *D. pinea*. One *D. pinea* isolate contained both SsRV1 and the undescribed dsRNA element. One *D. scrobiculata* isolate was not infected with any of the dsRNA elements.



## DISCUSSION

This study is the first to consider the presence of dsRNA elements in a relatively large panel of *D. pinea* and *D. scrobiculata* isolates from different parts of the world including South Africa, North Central United States, Mexico, Madagascar, Colombia and California. Results were obtained through highly reliable Real-Time PCR and revealed intriguing patterns of mixtures of the three different dsRNA elements, which have never previously been considered in conjunction. Triple infections were less frequent than double infections with SsRV1 and SsRV2. Single infections with either SsRV1 or SsRV2 were equally common while the undescribed dsRNA element never occurred alone.

The dsRNA element in *D. scrobiculata* has not been fully characterized but it nevertheless was possible to sequence the putative RdRp of this dsRNA element. This putative RdRp has relative low homology to the RdRps of SsRV1 and SsRV2, as well as, those of other members of the *Totiviridae* namely *G. abietina* RNA virus L1 (GaRV-L1) and *Trichomonas vaginalis* virus 2 (TVV2). The generic position of the undescribed dsRNA element must await sequencing of its complete genome, but the relatively low homology to RdRps of members of the *Totiviridae* might be an indication that this dsRNA element is represented by another virus family. This would not be unusual as most previous studies suggest a polyphyletic origin for fungal viruses (Koonin *et al.* 1989; Ahn & Lee 2001; Tuomivirta & Hantula 2005).

An interesting result emerging from this study was that SsRV1 and SsRV2 were detected in both *D. pinea* and *D. scrobiculata* isolates. These two viruses were first discovered in a South African isolate of *D. pinea* (Preisig *et al.* 1998). They have never previously been found in *D. scrobiculata*. The latter fungus is relatively closely related to *D. pinea* (De Wet *et al.* 2003) and for many years was known as the B morphotype of that fungus (Palmer *et al.* 1987). The two

fungi are quite different, based on ecology, morphology and phylogenetic inference (Wang *et al.* 1985; Palmer *et al.* 1987; De Wet *et al.* 2000, 2003). However, results of this study, showing that they share infections with SsRV1 and SsRV2, support the fact that they are closely related and the view that they probably evolved concurrently. The undescribed dsRNA element was almost always found in isolates of *D. scrobiculata*, but there were two intriguing exceptions. These were for two isolates of *D. pinea* from Madagascar. A broader survey is, however, needed to determine whether *D. pinea* and *D. scrobiculata* share infections with the undescribed dsRNA element.

This is the first report of triple infections with SsRV1, SsRV2 and the undescribed dsRNA element in *D. pinea* and *D. scrobiculata*. Co-infections with SsRV1 and SsRV2 were more frequently observed than triple infections. SsRV1 and SsRV2 have previously been shown to co-infect *D. pinea* (Preisig *et al.* 1998), but results of this study showed co-infections with SsRV1 and SsRV2 occurred in both *D. pinea* and *D. scrobiculata*. Single infections with either SsRV1 or SsRV2 were equally likely in both *D. pinea* and *D. scrobiculata*. Single infections with only the undescribed dsRNA element were never observed. The undescribed dsRNA element in *D. scrobiculata* is not a defective segment of SsRV1 and SsRV2 as its RdRp is vastly different to those of the SsRVs, but it could be dependent on the presence of SsRV1 and SsRV2. The sample size considered in this study was, however, insufficient to determine whether this might be the case. Multiple infections with viruses from the same and different families have previously been reported. In the canker pathogen of conifers, *G. abietina* three viruses belonging to three different virus families namely *Totiviridae*, *Partitiviridae* and *Mitovirus* were sequenced (Tuomivirta & Hantula 2005). Likewise, in the Dutch elm fungus, *Ophiostoma novo-ulmi* four mitoviruses have been sequenced (Hong *et al.* 1998). The role of these multiple infections and

the interactions between the different viruses and their fungal hosts is still unknown. This study has illustrated the complexity of the interactions between the three viruses associated with *D. pinea* and *D. scrobiculata*. These viruses also appear to be non-host specific and are easily transmitted between their different fungal hosts. This can be indicative of a more ancient origin where the viruses adapted to survive in more than one host over time.

The effects of different dsRNA elements, singly or as multiple infections, on their fungal hosts is relatively unexplored. Previous studies (Steenkamp *et al.* 1998; De Wet *et al.* 2001) have shown no phenotypic characteristics linked to the presence of dsRNA elements in *D. pinea* or *D. scrobiculata*. In contrast, Adams *et al.* (2002) observed that dsRNA-cured *D. pinea* cultures are sometimes more virulent than their dsRNA-containing parental cultures in one year and less virulent the following year. Future studies will thus concentrate on fully characterizing the dsRNA element commonly found in *D. scrobiculata* and we will then consider the possible effect of these infections on the biology of the two host fungi.

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**Table 1.** *Diplodia pinea* and *D. scrobiculata* isolates used in this study, as well as T<sub>m</sub>-values obtained after Real-Time PCR for the three distinct viruses.

| Isolates <sup>a/</sup> | Species         | Origin                     | Host                 | Collector    | T <sub>m</sub> – values <sup>b/</sup> |            |                   |
|------------------------|-----------------|----------------------------|----------------------|--------------|---------------------------------------|------------|-------------------|
|                        |                 |                            |                      |              | SsRV1                                 | SsRV2      | Undescribed dsRNA |
| CMW4254*               | <i>D. pinea</i> | Gauteng, South Africa      | <i>P. roxburghii</i> | MJ Wingfield | 88.85                                 | 87.26      | No product        |
| CMW4241                | "               | Western Cape, South Africa | <i>P. radiata</i>    | "            | 89.03                                 | 86.15      | No product        |
| CMW5848                | "               | Colombia                   | <i>P. patula</i>     | "            | 89.02                                 | 87.37      | No product        |
| CMW5849                | "               | "                          | "                    | "            | 90.29                                 | 87.70      | No product        |
| CMW5850                | "               | "                          | "                    | "            | 89.10                                 | 87.51      | No product        |
| CMW5852                | "               | "                          | "                    | "            | 89.61                                 | No product | No product        |
| CMW5853                | "               | "                          | "                    | "            | No product                            | 88.00      | No product        |
| CMW5854                | "               | "                          | "                    | "            | 89.45                                 | 87.37      | No product        |
| CMW5855*               | "               | "                          | "                    | "            | 90.19                                 | 87.50      | No product        |
| CMW5856                | "               | "                          | <i>P. patula</i>     | "            | 88.9                                  | 89.29      | No product        |
| CMW5857*               | "               | "                          | "                    | "            | 89.86                                 | 87.99      | No product        |
| CMW5858*               | "               | "                          | "                    | "            | No product                            | 87.46      | No product        |
| CMW5859                | "               | "                          | "                    | "            | 89.87                                 | 88.43      | No product        |
| CMW5860*               | "               | Madagascar                 | <i>P. patula</i>     | O Preisig    | 89.53                                 | 87.73      | 87.14             |
| CMW5861*               | "               | "                          | "                    | "            | 88.85                                 | 86.91      | No product        |
| CMW5862                | "               | "                          | "                    | "            | 89.08                                 | No product | No product        |
| CMW5863                | "               | "                          | "                    | "            | 88.50                                 | No product | No product        |
| CMW5864                | "               | "                          | "                    | "            | 88.54                                 | No product | 87.27             |
| CMW5865                | "               | "                          | "                    | "            | 90.02                                 | No product | No product        |
| CMW25385               | "               | Unknown                    | <i>P. ponderosa</i>  | GC Adams     | No product                            | 88.42      | No product        |
| CMW25386               | "               | Unknown                    | <i>P. radiata</i>    | "            | No product                            | 87.94      | No product        |
| CMW25387               | "               | Minnesota, USA             | <i>P. resinosa</i>   | "            | No product                            | 88.91      | No product        |
| CMW25388*              | "               | "                          | "                    | "            | 88.99                                 | 87.73      | No product        |
| CMW12513               | "               | France                     | <i>Larix</i> sp.     | P Chandelier | No product                            | 89.06      | No product        |
| CMW13234               | <i>D. pinea</i> | France                     | <i>Cedrus</i> sp.    | P Chandelier | 89.04                                 | 88         | No product        |





| Isolates <sup>a/</sup> | Species                | Origin          | Host                | Collector    | Tm – values <sup>b/</sup> |            |                   |
|------------------------|------------------------|-----------------|---------------------|--------------|---------------------------|------------|-------------------|
|                        |                        |                 |                     |              | SsRV1                     | SsRV2      | Undescribed dsRNA |
| CMW4900                | <i>D. scrobiculata</i> | Mexico          | <i>P. greggii</i>   | MJ Wingfield | 89.31                     | No product | No product        |
| CMW5867                | "                      | "               | "                   | "            | 89.10                     | 87.35      | 87.41             |
| CMW5868                | "                      | "               | <i>P. patula</i>    | "            | No product                | No product | No product        |
| CMW5869                | "                      | California, USA | <i>P. radiata</i>   | "            | 89.18                     | No product | No product        |
| CMW5870                | "                      | "               | "                   | "            | 89.30                     | 88.30      | 87.69             |
| CMW5871                | "                      | "               | "                   | "            | 88.91                     | 87.68      | 87.01             |
| CMW5847*               | "                      | Michigan, USA   | <i>P. banksiana</i> | M Palmer     | 89.02                     | 86.77      | 87.95             |

<sup>a/</sup>CMW refers to the Culture Collection (CMW) of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. <sup>b/</sup>Tm-values are the melting points of each product. No product means that no virus was detected. Isolates for which virus-specific products were sequenced are marked with an asterisk \*.



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**CHAPTER 6**

**CHARACTERIZATION OF A NOVEL dsRNA ELEMENT IN  
THE PINE ENDOPHYTIC FUNGUS, *DIPLODIA*  
*SCROBICULATA***

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## ABSTRACT

*Diplodia scrobiculata* and *Diplodia pinea* are endophytic fungi associated with die-back and cankers of mainly *Pinus* spp. in many parts of the world. These two fungi are closely related and have in the past been considered to represent two morphological forms (A and B morphotypes) of *D. pinea*. DsRNA elements are known to occur in both *D. scrobiculata* and *D. pinea*. Two dsRNA elements from *D. pinea*, SsRV1 and SsRV2 have previously been characterized. The aim of this study was to characterize a third dsRNA element that is most commonly associated with *D. scrobiculata* and to determine its phylogenetic relationship with other mycoviruses. The 5018 bp genome of this element was sequenced and it is referred to as *D. scrobiculata* RNA virus 1 or DsRV1. It has two open reading frames (ORFs) one of which codes for a putative polypeptide with a high homology to proteins of the vacuolar protein-sorting (VPS) machinery and the other for a RNA dependent RNA polymerase (RdRp). Phylogenetic comparisons based on the amino acid alignments of the RdRp revealed that DsRV1 is closely related to a dsRNA element isolated from *Phlebiopsis gigantea* (PgV2), and they grouped separately from virus families in which mycoviruses have previously been described. Although *D. pinea* and *D. scrobiculata* are closely related, DsRV1 does not share a high sequence homology with SsRV1 or SsRV2 and they probably have different evolutionary origins.

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## INTRODUCTION

*Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf. is a weak, opportunistic pathogen of mainly *Pinus* spp. that co-exists with the well-known pine pathogen, *D. pinea* (Desm.) Kickx, where their host ranges overlap (Palmer *et al.* 1987; Burgess *et al.* 2004b). This fungus was previously known as the B morphotype of *D. pinea* (Wang *et al.* 1985; Palmer *et al.* 1987; De Wet *et al.* 2003). Disease symptoms commonly associated with *D. pinea* and *D. scrobiculata*, in combination with various stress-inducing environmental or physical factors include die-back, cankers, collar rot and a root disease (Punithalingham & Waterston 1970; Wingfield & Knox-Davies 1980; Swart & Wingfield 1991).

*Diplodia scrobiculata* and *D. pinea* can be distinguished based on morphology, distribution, virulence and DNA sequence comparisons (Wang *et al.* 1985; Palmer *et al.* 1987; De Wet *et al.* 2000, 2002, 2003). *Diplodia scrobiculata* has a low level of virulence and a restricted distribution, while *D. pinea* can be highly virulent and it has a world-wide distribution (Blodgett & Stanosz 1997; Burgess & Wingfield 2002; Burgess *et al.* 2004a, 2004b). The genetic structure of *D. scrobiculata* populations compared to those of *D. pinea* is also different. Populations of *D. scrobiculata* are geographically isolated, with little gene flow, high allelic diversities and no multilocus genotypes shared between populations. These factors suggest a recent history of recombination and/or mutation (Burgess *et al.* 2004a). Populations of *D. pinea* show indications of a long asexual history with moderate to low gene diversities and multilocus genotypes that are shared between populations (Burgess *et al.* 2004b).

Several dsRNA elements of different size have been reported from *D. pinea* and *D. scrobiculata* (Wu *et al.* 1989; Preisig *et al.* 1998; Steenkamp *et al.* 1998; De Wet *et al.* 2001; Adams *et al.* 2002). Two of these, isolated from a South African A morphotype *D. pinea* isolate, have been

characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2) (Preisig *et al.* 1998). They are characterized by monopartite dsRNA genomes in the 5 kb size range with two ORFs. One of these ORFs codes for a capsid polypeptide (CP) and the other for a RdRp. Based on these characteristics and phylogenetic relationships, they have been shown to be closely related to viruses in the genus *Totivirus*, family *Totiviridae* (Preisig *et al.* 1998). In a recent study, a third dsRNA element was isolated from a Californian *D. scrobiculata* isolate (De Wet *et al.* 2008).

Multiple infections with different viruses are common in fungi (Buck 1986). The frequency and distribution of the three viruses associated with *D. pinea* and *D. scrobiculata* was determined using Real-time PCR with virus-specific primers (De Wet *et al.* 2008). SsRV1 and SsRV2 were found to occur in both *D. pinea* and *D. scrobiculata*, while the third dsRNA element was mainly associated with *D. scrobiculata* isolates except for two *D. pinea* isolates from Madagascar.

Interestingly, the third dsRNA element was found never to occur alone but always in combination with SsRV1 and/or SsRV2. The occurrence of multiple infections with three different viruses in these two closely related fungal species highlights the complex dynamics of the viral populations associated with *D. scrobiculata* and *D. pinea*.

Most mycoviruses are latent, causing no visible effects on their fungal hosts (Buck 1986; Ghabrial 1998). Initial studies on the dsRNA elements associated with *D. pinea* and *D. scrobiculata* showed that they have no significant effect on the virulence of these fungi (Steenkamp *et al.* 1998; De Wet *et al.* 2001). However, in a study conducted by Adams *et al.* (2002), a dsRNA-containing *D. pinea* isolate was found to be significantly less virulent than its dsRNA-free sub-culture, therefore, showing the potential of being able to attenuate virulence.

The aim of this study was to determine the sequence of the third dsRNA element associated with *D. scrobiculata*, which we refer to as *Diplodia scrobiculata* RNA virus 1 (DsRV1). A further aim was to use phylogenetic comparisons to determine the relatedness of DsRV1 to other fungal viruses.

## **MATERIALS AND METHODS**

### **Fungal isolate and dsRNA extraction**

A single conidial *D. scrobiculata* isolate (CMW5870) from California was used in this study and it is maintained in the Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, as well as the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. The fungus was grown in 250 ml Erlenmeyer flasks containing 2 % malt extract (ME) broth (Biolab Diagnostics, Midrand, South Africa), incubated at 25 °C with shaking (150 rpm) for at least two weeks or until sufficient biomass was produced for dsRNA extraction. Mycelium was harvested by centrifugation and then lyophilized. The lyophilized mycelium was ground to a fine powder using a mortar and pestle in the presence of liquid nitrogen. Trizol (Invitrogen Corporations, Carlsbad, CA, USA) and chloroform was used to extract dsRNA from the mycelium (1ml Trizol per 0.5 g mycelium). The supernatant obtained after centrifugation at 12 000 rpm at 4 °C for 10 minutes was precipitated overnight with 0.7 volumes isopropanol and 0.1 volumes sodium acetate. The dsRNA was recovered through centrifugation for 30 min at 13 000 rpm at 4 °C, washed with 70 % ethanol, dried and re-suspended in 50 µl DEPC (0.1% diethylpyrocarbonate)-treated dH<sub>2</sub>O. The isolated dsRNA was separated on a 1 % agarose (w/v) gel (Biolab Diagnostics, Midrand, South Africa) stained with ethidium bromide, using a 1 x Tris-acetic acid-EDTA (TAE) (pH 8) electrophoresis buffer. The largest dsRNA fragment (Fig. 1) was cut from the gel using a non-

UV transilluminator (DarkReader). The excised dsRNA fragment was purified using the QIAquick gel extraction kit (QIAGEN, GmbH, Germany), treated with RNase free DNase I for 2 hours at 37 °C and stored at -20 °C until further use.

### **Synthesis and cloning of cDNA using random hexamer primers**

Synthesis of cDNA from dsRNA was performed using the Roche cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). The dsRNA and random hexamer primers were denatured for 10 min at 99 °C followed by the first and second strand syntheses done following the manufacturer's instructions. The synthesized dsDNA was purified using the QIAquick gel extraction kit (QIAGEN, GmbH, Germany) and cloned using the Lucigen PCR-SMART non-proofreader cloning kit (Lucigen® Corporation, Middleton, WI, USA). Ligated plasmids were transformed into *E. coli*® chemically competent cells (Lucigen® Corporation, Middleton, WI, USA) and transformants were grown on YT-medium supplemented with kanamycin (final concentration of 30 µg/ml). A colony PCR was performed using forward CL3 and reverse SR2 primers specific to the pcrSMART™ vector. The 25 µl reaction mixture consisted of 1x PCR buffer (50 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 10 mM KCl, 5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 8), 200 µM of each dNTP, 0.2 µM of each primer and 0.25 U Fast start *Taq* polymerase (Roche Diagnostics, Basel, Switzerland). The following temperature profile was followed: 6 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C, and a final elongation step for 7 min at 72 °C. Colony PCR products were visualised on a 1 % agarose gel containing ethidium bromide using UV illumination. PCR amplified inserts were purified using the Roche PCR product purification kit (Roche Diagnostics, Basel, Switzerland) and sequenced.

### **Amplification and cloning of the complete viral genome**

The random amplified cDNA fragments were aligned according to the RdRp gene of the *Trichomonas vaginalis* virus 2 (TVV2), as they shared homology and genome-specific primers were designed. Sequences between the cDNA fragments were obtained through RT-PCR with the genome-specific primers using the Roche Titan One Tube RT-PCR system (Roche Diagnostics, Basel, Switzerland). The 50 µl reaction mixture containing 1x RT-PCR buffer (1.5 mM MgCl<sub>2</sub> and DMSO), 5 mM DTT, 0.2 mM each dNTP, 5 U RNase Inhibitor, 1 µl enzyme mix, 0.4 µM each primer and the dsRNA template. The primers and dsRNA were firstly denatured for 10 min at 99 °C and cooled on ice. The rest of the reaction mix was then added to the denatured dsRNA followed by reverse transcription for 30 minutes at 50 °C. This was followed by PCR amplification of 1 cycle at 94 °C for 2 min, 10 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 2 min, 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 2 min with a cycle elongation of 5 s per cycle and finally an elongation step of 10 min at 68 °C.

RT-PCR products were visualised on 1 % agarose gels containing ethidium bromide using UV illumination. Single band cDNA products were purified using the Roche PCR product purification kit and sequenced. Non-specific RT-PCR products were gel purified using the PCR product purification kit (Roche Diagnostics, Basel, Switzerland) and ligated overnight to the pGEM-T Easy Vector System II (Promega Corporation, Madison, WI, USA). Ligated plasmids were transformed into *Escherichia coli* JM109 cells (Promega Corporation, Madison, WI, USA) and screened for transformants on LB-medium supplemented with X-Gal (Fermentas Life Sciences, Lithuania) and IPTG (Fermentas Life Sciences, Lithuania). Colony PCR, as described in the previous section, was performed using T7 and SP6 primers. PCR amplified inserts were purified using the Roche PCR product purification kit and sequenced.



### **Determination of the distal ends of the viral genome**

TAIL-PCR (thermal asymmetric interlaced) (Liu & Whittier 1995; Nakayama *et al.*, 2001) and RLM-RACE (RNA ligase-mediated amplification of cDNA ends) (Coutts & Livieratos 2003) were used to obtain the distal ends of the viral genome. TAIL PCR entailed three consecutive PCR reactions using TAIL-cycling between high-stringency and low-stringency cycles using three nested genome-specific primers and eight degenerate primers. RLM-RACE was based on the ligation of an oligonucleotide (PC4: GCATTCGACCCGGGTT) to the dsRNA using T4 RNA ligase (Roche Diagnostics, Basel, Switzerland). This oligonucleotide was phosphorylated at the 5' end and blocked at the 3' end to prevent concatenation. First strand cDNA was then synthesized using a primer (PC5: AACCCGGGTCGTATGC) complementary to PC4 with the Fermentas First strand cDNA synthesis kit (Fermentas Life Sciences, Lithuania). This was followed by amplification of the cDNA using genome-specific primers and PC5. Products obtained were cloned using the pGEM-T Easy Vector System II, PCR amplified inserts were purified using the Roche PCR product purification kit as described previously and sequenced.

### **Isolation and amplification of genomic DNA**

The same single conidial *D. scrobiculata* isolate (CMW5870) from California from which dsRNA was extracted was grown in liquid ME medium in 1.5 ml Eppendorf tube, for one week at 25 °C. After centrifugation, the mycelial pellet was homogenized using a Retsch MM301 homogenizer (30 freq/s; 30 s), followed by the extraction of DNA using the technique described by Raeder & Broda (1985). The DNA was stored at -20 °C until further use.

ORF1- and ORF2-specific primers were tested on genomic DNA (Fig. 2a). These primers were RDF23 (5'-CCCTAACCTGCGACCTCCGTCG-3') (nt. 164) and RDR28 (5'-CCGCCATTTCTGGGGAAAGGCC-3') (nt. 1226) for ORF1 and RDF11 (5'-

CCCCGGTAGGAACGAGGTCTTCGC-3') (nt. 2180) and RDR2 (5'-CGATACCGTGCATACCGTAGAACT-3') (nt. 3309) for ORF2. As positive controls, the internally transcribed spacer (ITS) regions 1 and 2, and the 5.8S ribosomal subunit (White *et al.* 1990) and dilutions of RT-PCR products obtained from the dsRNA with the same primers, were amplified. The 25 µl reaction mixture consisted of 10x PCR buffer (50 mM Tris-HCl, 5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM KCl, 2 mM MgCl<sub>2</sub>, pH 8.3), 200 µM of each dNTP, 200 nM of each primer, 5 ng template and 0.1 U FastStart *Taq* DNA polymerase (Roche Diagnostics, Basel, Switzerland). The following temperature profile was followed: 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 52 °C and 2 min at 72°C followed by a final elongation step of 7 min at 72 °C.

PCR products were visualised on a 1% agarose gel containing ethidium bromide using UV illumination. The PCR products were then purified using the Roche High Pure PCR product purification kit (Roche Diagnostics, Basel, Switzerland) and both DNA strands were sequenced.

### **Sequencing and sequence analysis**

All sequencing was done using the ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing kit and an ABI PRISM<sup>®</sup> 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Reactions were done using protocols recommended by the manufacturers. Sequence data were processed using Chromas version 2.3 (<http://www.technelysium.com.au>) and contigs assembled using Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Alignments of overlapping contigs were done in BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsberg, CA, USA).

## Phylogenetic analysis

Translated amino acid sequences of the RdRp gene of DsRV1 were compared with 31 viruses belonging to the *Totiviridae*, *Partitiviridae*, *Hypoviridae*, *Chrysoviridae*, *Reoviridae* or *Endornavirus* (Table 1). These represent virus families in which dsRNA mycoviruses have been reported. A positive sense ssRNA virus belonging to the *Potyviridae* was used as the outgroup for the comparisons. Amino acid sequences were aligned using MAFFT version 5 (Kato *et al.* 2005). A parsimony analysis based on a strict heuristic search with a tree-bisection reconnection (TBR) branch swapping algorithm was performed and bootstrap support was determined in PAUP\* after 1000 replications. A phylogram was constructed that was rooted and edited in TreeView 1.6.6 (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

## RESULTS

### Synthesis and sequencing of cDNA from *D. scrobiculata* dsRNA

DsRNA extracted from *D. scrobiculata* mycelium and separated by gel electrophoresis revealed four segments of *ca.* 5.2 kb, 5 kb, 2 kb and 1 kb (Fig. 1). The double stranded nature of the RNA was verified by heat treatment of the native dsRNA at 99 °C to produce single stranded RNA. Amplification, using the primer pair DsRV1-F2 (5'-GGTATCGCTGGTTACCCGATCCGC-3') (nt. 3306) and DsRV1-R2 (5'-CAGATGGGGCTCAAAGGCACCTCC-3') (nt. 3458) (Fig. 2a), showed that the three smaller dsRNA fragments are deletion mutants of the largest fragment (Fig. 2b).

DNA fragments of different sizes were obtained after cDNA synthesis using denatured dsRNA of fragment 1 and random hexamer primers. These fragments were cloned and sequenced. Initial BLAST searches using the NCBI translated database (Blastx) showed homology to the RdRp of *Trichomonas vaginalis* virus 2 (TVV2).

## Genome organization of DsRV1

A total of 5018 nucleotides were assembled by overlapping contigs that were aligned according to the RdRp gene of TVV2. The complete DsRV1 sequence was cloned and sequenced at least four times to accurately determine the nucleotide positions (Genbank accession number EU547739). The dsRNA genome of DsRV1 has a GC content of 59 % and consists of two ORFs in the +3 translation frame (Fig. 2a). The existence of the two ORFs on the same dsRNA fragment was verified through RT-PCR amplification across ORFs using primers RDF20 (5'-GGAGATCACTTCGCTGTACC-3') (nt. 689) and RDR23 (5'-GGCAGCAGCCGCCTCCACGG-3') (nt. 1913) (Fig. 2a).

The first ORF (nt. 30 - 1280) encodes a putative polypeptide of 416 amino acids with a predicted molecular mass of 47.2 kDA. This polypeptide has a 60 % identity and 67 % similarity to protein complexes of the class E vacuolar protein-sorting (VPS) machinery (Q0U6X7) (Fig. 3). The context of the first methionine of DsRV1 was less favoured for translation (Kozak 1991) as it has a pyrimidine at position -3 and a purine in position +1 (CGUAUGG). It did, however, align with the amino acid sequence of a VPS protein, suggesting that it is the likely start codon of ORF1 (Fig. 3).

The second ORF (nt. 1500 – 4832) translates to 1110 amino acids coding for a RdRp with a predicted molecular mass of 122.9 kDA. It has 36 % identity and 51 % similarity to the RdRp gene of *Phlebiopsis gigantea* mycovirus 2 (PgV2) (CAJ34335), a 25 % identity and 36 % similarity to the RdRp gene of *Sphaeropsis sapinea* RNA virus 1 (SsRV1) (NP047558) and a 24 % identity and 38 % similarity to the RdRp gene of *Trichomonas vaginalis* virus 2 (TVV2) (AF127178). The RdRp gene of DsRV1 contains all eight conserved motifs (Fig. 4) found in the RdRp gene of most dsRNA viruses (Bruenn 1993). The third methionine (nt. 1500) was

considered to be the likely start codon of ORF2. It is in a more favourable context for translation initiation compared to the first (nt. 1319) and second methionine (nt. 1473) after the stop codon of ORF1, as it has a purine in position -3 and +1 (AAAAUGA) (Kozak 1991). The 219 nucleotides after the stop codon of ORF1 did not have any significant sequence homology to other known viral sequences. Furthermore, DsRV1 has a 5' UTR (untranslated region) of 29 bases and a 3' UTR of 186 bases.

### **Amplification of genomic DNA**

Despite using various reaction conditions, there was no amplification from the genomic DNA using ORF1- and ORF2-specific primers (Fig. 2a). Amplification was obtained from the genomic DNA using ITS1 and ITS4 primers as positive control, as well as from the diluted RT-PCR products using the same primers as initially used to amplify the dsRNA.

### **Phylogenetic relationships**

A most parsimonious cladogram was generated from the amino acid alignments of the RdRps from DsRV1 and 29 other viruses belonging to the *Totiviridae*, *Partitiviridae*, *Hypoviridae*, *Chrysoviridae*, *Reoviridae* and *Endornavirus* (Fig. 5). DsRV1 grouped with *Phlebiopsis gigantea* mycovirus dsRNA element 2 (PgV2), closest to *Helminthosporium victoriae* 145S virus (Hv145SV), *Penicillium chrysogenum* virus (PcV) and *Phlebiopsis gigantea* mycovirus dsRNA element 1 (PgV1). Hv145SV and PcV belong to the *Chrysoviridae*, while PgV1 and PgV2 have not yet been classified. Other than ObrV (*Operophtera brumata* reovirus) and FgV-DK21 (*Fusarium graminearum* virus DK21), all the viruses included in the phylogeny, grouped in two major clades. One of the major clades included DsRV1, PgV2, PgV1, viruses belonging to the *Chrysoviridae*, *Totiviridae*, *Hypoviridae* and those of the genus *Endornavirus*. The other clade included viruses residing in the *Partitiviridae* and the genus *Mycoreovirus*. Viruses belonging to

the three genera residing in the *Totiviridae* i.e. *Totivirus*, *Leishmaniavirus* and *Giardiavirus* grouped accordingly except the *Giardia lamblia virus* (*Giardiavirus*) that was more closely related to viruses in the *Hypoviridae* and the genus *Endornavirus* than to the other two genera (*Totivirus* and *Leishmaniavirus*) in the same family. The mycoreoviruses grouped separately from the insect reovirus, *Operophtera brumata reovirus* included in this study.

## DISCUSSION

The genome of a dsRNA element commonly associated with *D. scrobiculata* was sequenced and characterized in the study and the name *Diplodia scrobiculata* RNA virus 1 (DsRV1) has been proposed for it. DsRV1 is unencapsidated with a monopartite genome. Three smaller dsRNA segments that were isolated together with DsRV1 were shown to be deletion mutants of the virus. Phylogenetically, DsRV1 grouped most closely to a dsRNA element isolated from *Phlebiopsis gigantea* (PgV2) (GenBank accession number CAJ34335). Its next closest relatives are viruses belonging to the *Chrysoviridae* (Hv145SV and PcV) (Ghabrial *et al.* 2002; Jiang & Ghabrial 2004).

DsRV1 was isolated from a Californian *D. scrobiculata* isolate and has a genome size of 5018 bp constituting two ORFs. The first ORF codes for a putative polypeptide with relatively high sequence homology to proteins of the class E VPS machinery. The second ORF codes for a RdRp containing all eight conserved motifs found in the RdRp genes of most dsRNA viruses (Bruenn 1993). The method by which DsRV1 translates ORF2 is unknown, as the two ORFs do not overlap to enable translation to occur via ribosomal frameshifting or by internal initiation (Ghabrial 1998). The stretch of untranslated nucleotides between the two ORFs presumably has a structural function in positioning the AUG start codon of ORF2 in a suitable configuration for ribosomal access and translation initiation.

The role of the putative polypeptide encoded by ORF1 of DsRV1 could be to assist in the formation of sub-cellular compartments to protect this unencapsulated virus. Alternatively, it could play a role in virus transmission. Proteins of the VPS machinery are associated with mammalian and yeast cells and have also been reported from fungi where they sort endosomal membrane proteins to multivesicular bodies (MVB) for transport to the lysosomes where they are degraded (Reggiori & Pelham 2001; Iwaki *et al.* 2007). In retroviruses, rhabdoviruses and filoviruses, these proteins have been reported to interact with specific domains (L- or late domains) in the viral GAG-proteins to mediate viral budding or to act as adapters, linking viral L domains with the cellular VPS machinery for efficient viral particle release (Harty *et al.* 2000; Martin-Serrano *et al.* 2003). No mycoviruses have previously been reported to encode for an equivalent polypeptide.

DsRV1 probably obtained a VSP-like protein from its host and it is evolving more rapidly than its cellular homolog. This is consistent with the fact that viruses can obtain genes from their hosts (Khatchikian *et al.* 1989; McGeoch 2001) and it is known that cellular proteins sometimes assist in viral replication and transcription (Lai 1998). Host gene capture is more common in DNA viruses where it represents a mechanism to evade host immune responses (Domingo *et al.* 1998). Host gene capture has, however been reported from RNA viruses for example the ubiquitin-coding gene reported from a togavirus (Meyers *et al.* 1989) and the putative UDP glycosyltransferase gene from *Phytophthora* endornavirus (PEV1) (Hacker *et al.* 2005). In the totivirus, *Helminthosporium victoriae* 190S virus (Hv190sV), a cellular protein with sequence similarity to alcohol oxidases of methylotrophic yeasts was also found to co-purify with viral dsRNA (Soldevila *et al.* 2000; Soldevila & Ghabrial 2001).

We hypothesize that DsRV1, like viruses belonging to the *Hypoviridae* and the genus *Endornavirus*, is associated with cytoplasmic vesicles as it does not have rigid symmetrical structures encoded by inner and outer capsid proteins. Hypoviruses are enveloped in pleomorphic vesicles surrounded by rough endoplasmatic reticulum (Nuss *et al.* 2005). Viruses in the genus *Endornavirus* have unencapsidated dsRNA genomes associated with RdRp activity in cytoplasmic vesicles (Gibbs *et al.* 2005). These structural features of dsRNA's associated with vesicles are characteristic of a replicative intermediate of a ssRNA virus (Jacob-Wilk *et al.* 2006). DsRV1 and other unencapsulated dsRNA viruses therefore, probably had a ssRNA progenitor.

Based on the RdRp (ORF2), DsRV1 is phylogenetically most closely related to PgV2 (GenBank accession number CAJ34335), a dsRNA element isolated from *Phlebiopsis gigantea* that has not yet been assigned family status. ORF1 of both DsRV1 and PgV2, furthermore, encodes hypothetical proteins with no significant homology. The closest relatives to DsRV1 and PgV2 are another dsRNA element from *P. gigantea* (PgV1) and viruses belonging to the *Chrysoviridae* (Hv145SV and PcV) (Ghabrial *et al.* 2002; Jiang & Ghabrial 2004). The *Chrysoviridae* represents a family newly erected to accommodate mycoviruses with multipartite dsRNA genomes of three to four segments (Ghabrial & Castón 2005), previously considered to be part of the genus *Chrysovirus* in the *Partitiviridae* (Jiang & Ghabrial 2004). DsRV1 does have four segments but only one was shown to be functional. Based on the RdRp phylogeny and the unique genome organization of DsRV1, it appears that this virus and its relative (PgV2) occurring in *P. gigantea*, represents a new virus family.

DsRV1 shares little sequence homology with SsRV1 and SsRV2 that occur in the ascomycete fungus *D. pinea*, which is closely related to the host of DsRV1. DsRV1 is in fact more closely



related to dsRNA elements from a basidiomycete. Preisig *et al.* (1998) also reported limited sequence homology between SsRV1 and SsRV2. The existence of three unrelated viruses in two closely related fungal species suggest that they have polyphyletic and separate origins. In a recent study, De Wet *et al.* (2008) showed that DsRV1 always occurs in combination with SsRV1 and/or SsRV2.

DsRV1 is mainly found in association with *D. scrobiculata* populations that have been reported to have high allelic diversities, a history of recombination and/or mutation and potentially the existence of a cryptic sexual cycle (Burgess *et al.* 2004a). SsRV1 and SsRV2, on the other hand are mainly found in association with *D. pinea* populations that have low genetic diversities and a history of asexual recombination (Burgess *et al.* 2004b). As mycoviruses are believed to co-evolve and co-adapt with their fungal hosts (Ghabrial 1998), the genetic variation in DsRV1 could thus be the result of mutation and recombination together with its constantly evolving host (*D. scrobiculata*) to ensure adaptability to changing environments.

The ecological role of DsRV1 is unknown. In the case of SsRV1 and SsRV2, it has been shown that reduced virulence or slower growth in *D. pinea* could not be linked to the presence of these dsRNA elements (Steenkamp *et al.* 1998; De Wet *et al.* 2001). DsRV1, SsRV1 and SsRV2 occur in various combinations in their two related fungal hosts, *D. pinea* and *D. scrobiculata* without any clear pattern of association. The manner in which they interact with each other and their possible role in the biology of their pine pathogen hosts will form the basis of future studies.

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**Table 1.** Names, acronyms and accession numbers of all viruses included in the phylogenetic comparison.

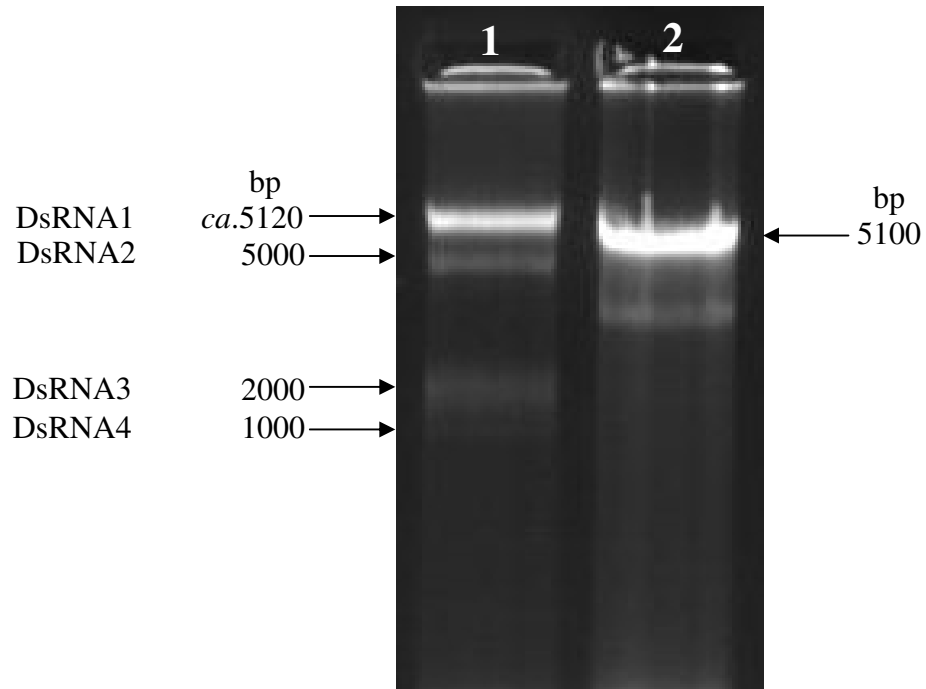
| Virus name   | Acronym      | Accession number | *Family/Genus         |
|--|--------------|------------------|-----------------------|
| <i>Diplodia scrobiculata</i> RNA virus 1             | DsRV1        | EU547739         | Unknown               |
| <i>Sphaeropsis sapinea</i> RNA virus 1               | SsRV1        | NP047558         | <i>Totiviridae</i>    |
| <i>Sphaeropsis sapinea</i> RNA virus 2               | SsRV2        | NP047560         | "                     |
| <i>Gremmeniella abietina</i> RNA virus L1            | GaV-L1       | NP624332         | "                     |
| <i>Gremmeniella abietina</i> RNA virus L2            | GaV-L2       | YP044807         | "                     |
| <i>Giardia lamblia</i> virus                         | GLV          | NP620070         | "                     |
| <i>Helminthosporium victoriae</i> 190S virus         | Hv190sV      | NP619670         | "                     |
| <i>Leishmania</i> RNA virus 1-1                      | LRV1-1       | NP043465         | "                     |
| <i>Leishmania</i> RNA virus 2-1                      | LRV2-1       | NP041191         | "                     |
| <i>Trichomonas vaginalis</i> virus 2                 | TVV2         | AF127178         | "                     |
| <i>Helicobasidium mompa</i> virus no. 17             | HmV17        | NP898833         | "                     |
| <i>Botryotinia fuckeliana</i> totivirus              | BfV          | CAM33265         | "                     |
| <i>Ophiostoma minus</i> virus                        | OmV          | CAJ34336         | "                     |
| <i>Phlebiopsis gigantea</i> dsRNA 1                  | PgV1         | CAJ34333         | Unknown               |
| <i>Phlebiopsis gigantea</i> dsRNA 2                  | PgV2         | CAJ34335         | "                     |
| <i>Ophiostoma partitivirus</i> 1                     | OPV1         | CAJ31886         | <i>Partitiviridae</i> |
| <i>Ophiostoma quercus</i> partitivirus               | OqPV         | CAJ34337         | "                     |
| <i>Aspergillus ochraceus</i> virus                   | AoV          | ABV30675         | "                     |
| Black raspberry cryptic virus                        | BRCV         | ABU55400         | "                     |
| <i>Helicobasidium mompa</i> mycovirus                | HmMV         | BAC23065         | "                     |
| <i>Vicia</i> cryptic virus                           | VCV          | ABN71234         | "                     |
| <i>Penicillium chrysogenum</i> virus                 | PcV          | YP392482         | <i>Chrysoviridae</i>  |
| <i>Helminthosporium victoriae</i> 145S virus         | Hv145sV      | YP052858         | "                     |
| <i>Cryphonectria hypovirus</i> 1                     | CHV1         | NP041091         | <i>Hypoviridae</i>    |
| <i>Cryphonectria hypovirus</i> 1-EP713               | CHV1-EP      | O04350           | "                     |
| <i>Operophtera brumata</i> reovirus                  | ObRV         | ABB17205         | <i>Reoviridae</i>     |
| Mycoreovirus-1/ <i>Cryphonectria parasitica</i> 9B21 | MYRV1/Cp9B21 | BAD51414         | "                     |
| Mycoreovirus-3/ <i>Rosellinia necatrix</i> W370      | MYRV3/RnW370 | YP392478         | "                     |
| <i>Helicobasidium mompa</i> endornavirus             | HmEV         | BAE94538         | <i>Endornavirus</i>   |
| <i>Phytophthora</i> endornavirus                     | PEV          | YP241110         | "                     |
| <i>Fusarium graminearum</i> virus-DK21               | FgV-DK21     | YP223920         | Unknown               |
| <i>Cucurbit yellows-associated virus</i>             | CYV          | CAA63099         | <i>Potyviridae</i>    |

\* Unknown refers to viruses not assigned to a specific family or genus.





**Figure 1.** A 1 % agarose gel showing the dsRNA segments isolated from *D. scrobiculata* (Lane 1) compared to SsRV1 and SsRV2 isolated from *D. pinea* (Lane 2).

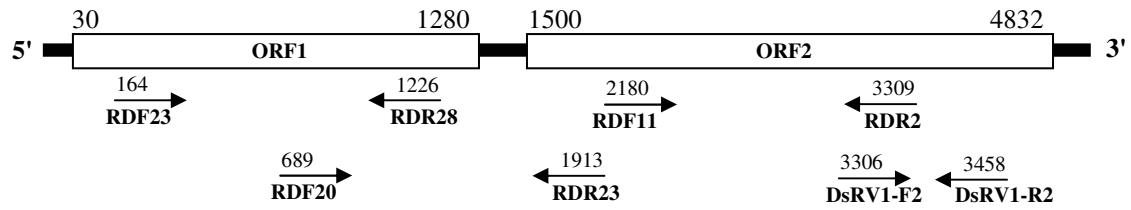




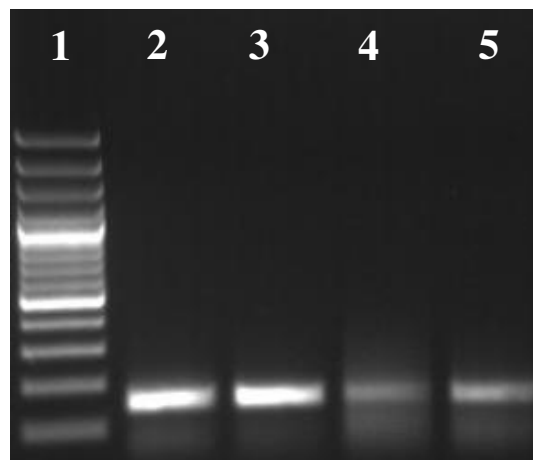
**Figure 2.** (a) A schematic representation of the genome organization of DsRV1. The white blocks represent the coding regions and the black blocks the untranslated regions. The ORF1- and ORF2-specific primers are indicated with arrows in the direction they amplify. The position of the primers on the genome is indicated above the arrow and the primer name below the arrow.

(b) A 1 % agarose gel showing RT-PCR products using the primer pair (DsRV1-F2 and DsRV1-R2) on the four dsRNA segments isolated from *D. scrobiculata*. Lane 1 = 100 bp ladder, Lane 2 = dsRNA1, Lane 3 = dsRNA2, Lane 4 = dsRNA3, Lane 5 = dsRNA4.

(a)



(b)



**Figure 3.** Amino acid alignments of the putative gene product encoded by ORF1 of DsRV1 (EU547739) and a protein belonging to the Class E vacuolar protein-sorting (VPS) machinery (Q0U6X7). Dark shading indicates identical amino acids and lighter shading indicates 60 % similar amino acids.



```

      10      20      30      40      50
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
MFRAQSNIFDDVVVKATDENLTSENWEYILDVCDKVGSSDTGAKDAVAA
-----

      60      70      80      90     100
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
IKRLAHRNANVQLYTLLELANALSQNCGIQMHKELASRSFTDAMLRLANDR
-----

      110     120     130     140     150
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
NTHQAVKAKILERMGEWSEMFSDPDLGIMEGAYMKLKTQ-----
-----MGEWTEMFASNFDLGIMEQAYMRLKTQSACDQLWFGV
-----

      160     170     180     190     200
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
-----NPNLRAPSPKPKQTQISDSDRQKEEELQMALAMSIKESKCATP
STDQRVVDPNLRPPSPKPKQTQITDSDRQKEEELQMALALSVKES-GTEP
-----

      210     220     230     240     250
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
SAAKANAPQESNAGSSSQAAPAPQPVQPQTAAATVSRVRLAFDFQPSSEPG
SAARSNPQAKAPQSSAPEQEQHQAIASGTTAATVSRVRLALYDFTPSEPG
-----

      260     270     280     290     300
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
ELQFKKGDIIAVLESVYKDWWKGLRGNIGIFPLNYVEKLQDPTREELEK
ELAFRKGDI IAVLESVYKDWWKGLRGGTIGIFPLNYVEKLQDPTKEELER
-----

      310     320     330     340     350
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
EAQTFAEVFAQIRNVEKLLALLSTNTQAGGGDGRDNEEITELYHSTLAIK
EAQMEAEVFAEIKNVEKLLALLSTSSA---DARDNEEITSLYHKIVSIR
-----

      360     370     380     390     400
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
PKLIELIGKYSQKDDFTQINEKFIKARDYESTLEASMSQPPQPSYGSR
PKLIELIGKYSQKKGKWNLLRFVWCWPLIVFQMTSRSSRTRSSRHAATK
-----

      410     420     430     440     450
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
PPYGYNAPPSPSNYTGYPSSPPQYGYGAGAPQGSAPQYPPVGANPAF
P----SLKFRRCNRQSPATVVNP-----MATAP----APRRLRLRTVAI
-----

      460     470     480     490     500
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
FMVPPAGEQRPOQTPOPGPPSDPYSLPQGRVPIGGRPQSYAPQELATAH
LLKDLLRN--SFTRLLOPQTKATDILLRMGLSPS----RWLSPHKHLLLL
-----

      510     520     530     540     550
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
YDSPVDNRHSFAGFSQPQGAPSAPOGYEYPPSQAPPGYPPQOGAPLQGP
RWLVLDQRMASSTSRSACLSVEGPHWP-----FPRKWRP
-----

      560     570     580     590     600
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
PGQONPYEQISSPPTHQPPSDPYSQPPPQVGHGYPPQOPAHAPPAPPGA
TAQWTFGRLRASKIRK-----
-----

      610     620     630
Class E VPS machinery
DsRV1 ORF1
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
SSSPAPAQGYLPYRPPGQAPSAPPVGGGGDEGFYR
-----

```

**Figure 4.** Partial amino acid alignments of the RdRp genes for a set of dsRNA viruses, showing the eight conserved motifs (marked A-H). Viruses included were DsRV1 (*Diplodia scrobiculata* RNA virus1) (EU547739), PgV1 (*Phlebiopsis gigantea* mycovirus 1) (CAJ34333), PgV2 (*Phlebiopsis gigantea* mycovirus 2) (CAJ34335), TVV2 (*Trichomonas vaginalis* virus 2) (AF127178), SsRV1 (*Sphaeropsis sapinea* RNA virus 1) (NP047558), SsRV2 (*Sphaeropsis sapinea* RNA virus 2) (NP047560), GaVL1 (*Gremmeniella abietina* RNA virus L1) (NP624332), Hv190SV (*Helminthosporium victoriae* 190S virus) (NP619670), LRV2-1 (*Leishmania* RNA virus 2-1) (NP041191), Hv145SV (*Helminthosporium victoriae* 145S virus) (YP052858), PcV (*Penicillium chrysogenum* virus) (YP392482) and FgV-DK21 (*Fusarium graminearum* virus DK21) (YP223920). All are members of the *Totiviridae* except PcV and Hv145SV belonging to the *Chrysoviridae* and DsRV1, PgV1 and PgV2 that have not been assigned to a virus family. Dark shading indicates identical amino acids and lighter shading indicates 50 % similar amino acids.





**Figure 5.** The most parsimonious phylogram generated after a phylogenetic analysis of the amino acid sequences of the RdRp genes of DsRV1 (EU547739) compared to viruses of the *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Hypoviridae*, *Reoviridae* and the genus *Endornavirus*. Viruses included were SsRV1 (*Sphaeropsis sapinea* RNA virus 1) (NP047558), SsRV2 (*Sphaeropsis sapinea* RNA virus 2) (NP047560), GaV-L1 (*Gremmeniella abietina* RNA virus L1) (NP624332), GaV-L2 (*Gremmeniella abietina* RNA virus L2) (YP044807), GLV (*Giardia lamblia* virus) (NP620070), Hv190SV (*Helminthosporium victoriae* 190S virus) (NP619670), LRV1-1 (*Leishmania* RNA virus 1-1) (NP043465), LRV2-1 (*Leishmania* RNA virus 2-1) (NP041191), TVV2 (*Trichomonas vaginalis* virus 2) (AF127178), HmV17 (*Helicobasidium mompa* virus no. 17) (NP898833), BfV (*Botryotinia fuckeliana* totivirus) (CAM33265), OmV (*Ophiostoma minus* virus) (CAJ34336), *Phlebiopsis gigantea* mycovirus 1 (PgV1) (CAJ34333), *Phlebiopsis gigantea* mycovirus 2 (PgV2) (CAJ34335), OPV1 (*Ophiostoma partitivirus* 1) (CAJ31886), OqPV (*Ophiostoma quercus* partitivirus) (CAJ34337), AoV (*Aspergillus ochraceus* virus) (ABV30675), BRCV (Black raspberry cryptic virus) (ABU55400), HmMV (*Helicobasidium mompa* mycovirus) (BAC23065), VCV (*Vicia cryptic* virus) (ABN71234), PcV (*Penicillium chrysogenum* virus) (YP392482), Hv145sV (*Helminthosporium victoriae* 145S virus) (YP052858), CHV1 (*Cryphonectria hypovirus* 1) (NP041091), CHV1-EP (*Cryphonectria hypovirus* 1-EP713) (Q04350), ObRV (*Operophtera brumata* reovirus) (ABB17205), MYRV1/Cp9B21 (Mycoreovirus-1/*Cryphonectria parasitica* 9B21) (BAD51414), MYRV3/RnW370 (Mycoreovirus-3/*Rosellinia necatrix* W370) (YP392478), HmEV (*Helicobasidium mompa* endornavirus) (BAE94538), PEV1 (*Phytophthora* endornavirus) (YP241110) and FgV-DK21 (*Fusarium graminearum* virus-DK21) (YP223920). The cucurbit yellows-associated virus (CYV) (CAA63099), a (+) ssRNA plant virus was used as outgroup.

