Understanding the global population genetics of *Diplodia pinea* and its life cycle in plantation pines

By

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Submitted in partial fulfillment of the requirements for the degree

PHILOSOPHIAE DOCTOR

In the Faculty of Natural & Agricultural Sciences, Department of Genetics,
University of Pretoria

September 2010

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Declaration

I, Wubetu Bihon Legesse declare that the thesis/dissertation, which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

September 2010

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Acknowledgments

I would like to express my deepest gratitude to my supervisors Prof. Brenda Wingfield Prof. Mike Wingfield, Prof. Bernard Slippers and Dr. Treena Burgess for your continual guidance, support and facilitation throughout my study. Without your constructive comments, advices and in-depth discussions, it would have not been possible to see this thesis done.

It appreciates the Forestry and Agricultural Biotechnology Institute (FABI), National Research Foundation (NRF)/Center of Excellence in Tree Health Biotechnology (CTHB), members of Tree Protection Cooperative Programme (TPCP), the University of Pretoria, and International Foundation for Science (IFS), for the financial support.

My lab-mates: Kersheny Naidoo, Dr. Martin Coetzee, Vivienne Clarence, Quentin Santana, Simon Martin, Matsepo Taole, Lieschen De Vos, Natalie Van Zuydam, Mesfin Bogale, Albe Van der Merwe, Mmatshepho Phasha, Phia Van Coller, Cornell Kortenhoeven and Franscois, you have been amazing in all times of my stay to creating the most clean, comfortable, enjoyable and friendly working laboratory.

I am grateful to work in FABI with FABI staffs and students who are always cooperative. I would specifically like to thank Prof. Joulanda Roux, Dr. Brett Hurley, Hardus Hatting and James Mehl who helped me in collecting samples in South Africa. I appreciate Jenny, Eva, Heidi, Rose, Martha, Helen, Renate, Galdys, Irene, Magrit Van der Nest, Juanita De Wet, Tuan Duong, Lydia and Valentina who were always ready to help in different aspects of my study.

My parents, brothers and sisters are very much appreciated for their patience and supports. Gashaw Asrat and his family, Mekonen Ayele and his family, Getachew Hiruy, Dr Alemu Gezahagn, Mesfin Tessera and Lijalem G/wahid, I thank you very much for all your encouragement and supports. My fellow Ethiopians in Pretoria: Berhanu Amsalu, Endale Gebrie, Dawit Degefu, Hiywot Menker, Azeb Berhane, Grum Bahri, Efrem Tadesse, Ahmed Hassen, Alemseged Berhe, Tsedale, Yemane, Temesgen, Dembala, Abebe and others, thank you so much for all your friendship and moral supports.

Above all, I am DEEPLY THANKFUL to my God who provided me strength and patience.

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Preface

Diplodia pinea (= *Sphaeropsis sapinea*) causes diseases on *Pinus* spp. and less commonly on other coniferous trees in many parts of the world. Its effect is mainly associated with physiological stress on the trees. Interestingly, it also has the ability to infect trees without causing symptoms and thus lives as an endophyte for part of its life cycle. The fungus is thus considered to be a latent pathogen.

While much research has been conducted on *D. pinea* during the course of the last Century, little information is available regarding the spread and colonization patterns of this pathogen. *D. pinea* is considered to reproduce asexually, because a teleomorph has never been observed. Yet, early evidence from molecular studies suggests that a cryptic sexual cycle might exist. The aims of this study were to investigate the patterns of spread of *D. pinea* and to consider the structure of gene and genotype diversity in populations ranging from a fine spatial scale within a single tree to larger geographic scale and between populations across the landscape. In addition the mode of reproduction of the pathogen was considered using data regarding the distribution of genotypic diversity and association of alleles in populations at a plantation and on a country-wide scale.

Chapter 1 presents a literature review that aims to give an overview of the general biology of *D. pinea* and to a more limited extent, the sibling species *D. scrobiculata*. The review, furthermore, considers the molecular, morphological and physiological tools that have been used to study this fungus, especially at the population level. The economic significance of the fungus and strategies pertaining to its control are also discussed.

The distributions and genetic diversity of endophytic populations of *D. pinea* in single trees, between trees and between sites were considered in **Chapter 2**. The diversity of isolates

obtained from different parts of healthy trees, including the stems, branches, and cones, were evaluated using microsatellite and VCG markers. New SSR markers were developed to increase the resolution of the microsatellite analyses.

In **Chapter 3**, the structure of genetic and genotypic diversity on larger spatial scales in the South African landscape is assessed in order to better understand patterns of spread and mode of reproduction. Samples of *D. pinea* were collected from five different plantations from KwaZulu-Natal and Mpumalanga provinces. These isolates, together with those obtained in Chapter 2, were analysed using 13 SSR markers. These data are analysed for patterns of distribution in the gene and genotype diversities, the structure between populations and the association of alleles.

Diplodia scrobiculata is a sibling species of *D. pinea* that was previously considered as a morphotype of *D. pinea* (morphotype B*). D. scrobiculata* has a more limited distribution than *D. pinea* and has not previously been observed in the Southern Hemisphere. During the extensive sampling for *D. pinea* done as part of this thesis, a small number of isolates resembling *D. scrobiculata* were discovered. The characterization of these isolates is presented in **Chapter 4**.

In **Chapter 5,** the genetic diversity and structure of *D. pinea* populations is expanded from the single tree and plantation level assessed in previous chapters, to a continental scale. Commercial forestry plantations have been established in South America, Africa and Australia during the course of the last century, with a concomitant expected introduction of *D. pinea*. In this chapter I consider whether the pattern of genetic and genotypic diversity of this pathogen is similar in different countries and whether the early evidence for recombination is more widespread than only in South Africa. For this purpose, genetic diversity is compared between populations in South Africa, Ethiopia, Australia and Argentina. The diversity of genotypes,

population structure, gene flow and linkage disequilibrium of *D. pinea* is compared amongst these introduced populations.

The mode of transmission and sources of *D. pinea* inoculum in plantations of South Africa has never been clarified. In **Chapter 6**, the possible vertical transmission of the fungus from seeds to seedlings and eventually to big trees is investigated. In addition, transmission to seedlings in open fields in the absence of mature trees, as well as naturally regenerated seedlings in close proximity to mature trees was compared.

A summary of this study and suggestions for future research are presented at the end of the thesis. As outlined above, the thesis is prepared in the form of six manuscripts, five of which are experimental. Due to the nature of the presentation, there is some unavoidable repetition in the Introduction, Materials and Methods and Reference sections of some chapters.

1

Biology and genetic diversity of the latent pathogens of Pine, *Diplodia pinea* and *D. scrobiculata* (A review)

1. Introduction

Diplodia pinea (Desm.) Kickx (=*Sphaeropsis sapinea* (Fr.:Fr.) Dyko& Sutton) is an opportunistic, latent endophytic pathogen of *Pinus* spp. and other conifers. The fungus was identified for the first time in France on dead *P. sylvestris* needles as a saprophyte. Since that time, it has been reported from most parts of the world where pines and other coniferous trees are found (Swart et al. 1985; Stanosz et al. 1997; Burgess et al. 2004a). In contrast, the sibling species *Diplodia scrobiculata* (de Wet et al. 2003) has a limited distribution in native and non-native ranges of pines. It is hypothesized that the movement of *D. pinea* around the world including the tropics and southern hemisphere has been the result of the movement of germplasm through human activities (Burgess and Wingfield 2002).

Because of its economic significance, *D. pinea,* and consequently also *D. scrobiculata*, have been studied intensively with relation to their taxonomy, morphology, distribution and control around the world. *Diplodia pinea* causes significant losses in nurseries, plantations, forests and shelterbelts (Swart and Wingfield 1991; Stanosz et al. 2005). In some countries, such as those where pines have been introduced for plantation development, *D. pinea* has had significant negative impacts on growth and productivity (Swart et al. 1985; Zwolinski et al. 1990; Wingfield et al. 2001). Plantations of *Pinus* spp. are becoming increasingly important in many parts of the world necessitating sound and integrated management of this pathogen in order to reduce losses due to disease.

During the last century, there has been much confusion concerning the taxonomy of *D. pinea*. The fungus has had many synonyms with the most commonly used in recent years being *Sphaeropsis sapinea*. One of the main sources of confusion emerged from the fact that colour, conidial septation and proliferation of conidiogenous cells were used as characters to define

Diplodia and *Sphaeropsis* (Sutton 1980; Denman et al. 2000). The emergences of molecular tools, including multigene phylogenies and microsatellite markers, have promoted a much more robust understanding of the phylogenetic relationships and diversity in these groups (de Wet et al. 2003; Crous et al. 2006). As a result, *Sphaeropsis* is today widely recognised as a synonym of *Diplodia*. Furthermore, two sibling species, *D. pinea* and *D. scrobiculata* are recognised amongst isolates that were previously described as morphotypes A and B of *D. pinea*. Within *D. pinea,* there is a morphological group, known as morphotype C that is known only in Indonesia. This needs further investigation as it might be an additional cryptic species within the complex.

This chapter reviews the taxonomy, distribution, population genetics and tools used to study *D. pinea* and *D. scrobiculata* as well as methods used to control them. Because *D. pinea* is widely distributed around the world and important where the fungus is non- native in the southern hemisphere, the review and most of the chapters of the dissertation that follow it are focussed on this species, rather than on *D. scrobiculata*. However, one of the chapters of the thesis concerns the discovery of *D. scrobiculata* in South Africa and, therefore, background to this fungus is also provided in the review.

2. Taxonomy

Diplodia pinea (=*Sphaeropsis sapinea*) apparently lacks a sexual stage in its life cycle, but has been placed in class Dothideomycetes, order Botryosphaeriales, family Botryosphaeriaceae based on phylogenetic inference (Schoch et al. 2006). The fungus has been subject to many re-classifications subsequent to its first description as *Sphaeria pinea* by Desmazieres (1842). On the basis of the morphology of conidia, which are brown in colour with a single septum, the genus was renamed as *Diplodia* (Kickx 1867). However, Sutton (1980) reviewed the name *D.*

pinea considering differences in conidial and conidiogneous cell characteristics for *Diplodia* and *Sphaeropsis,* and redescribed it as *Sphaeropsis sapinea* (Fr.) Dyko & Sutton. He considered *Sphaeropsis* to be best defined as having conidia that develop a faint septum prior to germination and conidiogneous cells that proliferate percurrently and internally, and restricted *Diplodia* to those fungi where the conidia become euseptate as they mature and where conidiogenous cells proliferate only internally.

Denman et al. (2000) argued that the characteristics applied by Sutton (1980) were not sufficient to group *Diplodia* and *Sphaeropsis* in different genera and that the genera were morphologically indistinguishable. Their view was that *Sphaeropsis* should rather be regarded as a synonym of *Diplodia* since the morphology and DNA sequence data placed it with other *Diplodia* spp. Moreover, Denman et al. (2000) suggested that conidiogenous cells in *Diplodia* can proliferate percurrently, thus contesting the major basis for discrimination between *Diplodia* and *Sphaeropsis* provided by Sutton (1980). Phylogenetic analysis using multiple gene genealogies has further resolved the taxonomic placement of *D. pinea* by clearly showing no phylogenetic distinction between *Sphaeropsis* and *Diplodia* (Burgess et al. 2001b; De Wet et al. 2003). In addition, an extensive phylogenetic analysis for members of the Botryosphaeriaceae (Crous et al. 2006) has shown that species with Diplodia-like anamorphs (including *Sphaeropsis*) most appropriately reside in *Diplodia*.

Diplodia pinea (as *Sphaeropsis sapinea*) was described as having four forms that have been referred to as the A, B, C and I morphotypes (Wang et al. 1985; Palmer et al. 1987; Swart and Wingfield 1987; Hausner et al. 1999; De Wet et al. 2000). This sub-specific division first emerged when differences in conidial and culture morphology were observed in the 1980's (Wang et al. 1985; Palmer et al. 1987). They were subsequently also supported by ecological

studies including pathogenicity tests (Wang et al. 1985; Palmer et al. 1987; De Wet et al. 2002) and those applying isozyme profiles and randomly amplified polymorphic DNA (RAPD) analyses (Palmer et al. 1987; Swart et al. 1991; Smith and Stanosz 1995; Stanosz et al. 1999; De Wet et al. 2000). Palmer et al. (1987) first showed that isolates of the A morphotype are more virulent than those of the B morphotype (Blodgett and Stanosz 1999; De Wet et al. 2002). Isolates of the C morphotype of *D. pinea* are more virulent than either of these (De Wet et al. 2002).

Mature conidia of *D. pinea* morphotype A are aseptate and occasionally single septate and have smooth walls (Wang et al. 1985). This was in contrast to those of morphotype B that has smooth conidial walls when these structures are young, but that become pitted latter and they can have up to three septa (Wang et al. 1985; Palmer et al. 1987). Wang et al. (1985) used the conidial characteristic to distinguish the A and B morphotype and for the first time introduced scanning electron microscopy (SEM) as a tool to do this. Swart et al. (1991), however, reported that the B morphotype conidia can also have smooth walls suggesting that conidia should not be used as a distinguishing feature for these forms of the fungus.

A third distinct morphotype of *D. pinea* emerged amongst cultures of the fungus from Indonesia (De Wet et al. 2000). Random amplified polymorphic DNA (RAPD) analysis was used to clearly define this form of the fungus which was subsequently designated as the "C" morphotype (De Wet et al. 2000). Isolates of C morphotype were more closely related to morphotype A, but the conidia were longer than those in both C and B morphotypes (De Wet et al. 2000, 2002).

Molecular tools have been extensively used to resolve the relatedness of the three *D. pinea* morphotypes and they have been shown to be more effective than morphological

characterization. These have included comparisons of conserved gene sequences, isozymes, random amplified polymorphic DNA (RAPD) and microsatellite markers (Palmer et al. 1987; Smith and Stanosz 1995; De Wet et al. 2000; Burgess et al 2001b). Thus isolates that had been described as representing the I morphotype of *D. pinea* (Hausner et al. 1999) because they resembled morphotype A based on culture and conidial characteristics, were later shown to represent *D. seriata* (classified at that time as *Botryosphaeria obtusa*) (Burgess et al. 2001b). The DNA sequences for the internal transcribed spacer (ITS) gene region of the rDNA were, likewise used to show that the C morphotype was similar to the A morphotype, while the A and B morphotypes differed at two sequence positions (De Wet et al 2000, 2002). Later, a detailed study using multiple gene genealogies and microsatellite markers showed that the B morphotype of *D. pinea* is only distantly related to the A and C morphotypes and hence isolates representing this form of the fungus were classified as the distinct taxon *D. scrobiculata* J. De Wet, B. Slippers & M. J. Wingfield (De Wet et al. 2003).

3. Geographical distribution

Diplodia pinea, morphotype "A" has been isolated from pines and other coniferous trees on all the continents where these trees are found (Swart et al. 1985; Palmer et al. 1987; Swart and Wingfield 1991; Stanosz et al. 1997; Mersi et al. 2007). However, morphotype "C" is known only from Indonesia were it was found causing shoot blight on *P. patula* from Indonesia (De Wet et al. 2000; Burgess et al. 2001b). *Diplodia scrobiculata* also has a limited distribution in north central USA, Mexico and southern Europe (Palmer et al. 1987; Blodgett and Stanosz 1997; De Wet et al. 2000; Burgess et al. 2004b). It has only recently been reported from the Southern Hemisphere, where it was found in South Africa as part of this dissertation (Bihon et al. 2010).

The reason for the limited distribution of the "C" morphotype and *D. scrobiculata* is unclear, especially given the wide distribution of the closely related A morphotype. This is especially strange given that the major host of *D. scrobiculata*, *P. radiata*, is widely introduced and planted in the southern hemisphere (Burgess et al. 2004b). The recent discovery of *D. scrobiculata* in the southern hemisphere, but at low levels, implies that extensive sampling and study is needed to more closely delineate the distribution of these fungi worldwide.

4. Population genetics

Intensive management of agricultural and forestry ecosystems affect the micro-organisms that occur in these environment systems. Strong selective pressures are placed on micro-organisms, many of which are pathogens, to adapt to the changing and specific conditions that are imparted by various agronomic and management protocols (McDonald and McDermott 1993; Milgroom and Fry 1997; McDonald 1997; Linde 2010). The impacts of these changes are difficult to measure and predicting what control measures will be needed to contain these altered populations is complex. Knowledge about the population dynamics, including diversity, mating systems and modes of spread, of micro-organisms does, however, allow for an understanding regarding the potential for change (Nevo 1978; McDonald and McDermott 1993; McDonald 1997). In order to develop effective control strategies, it is, therefore, important to focus on populations rather than individuals of microbes (Milgroom 2001; McDonald and Linde 2002; Linde 2010).

Burgess and Wingfield (2002) reported that pine planting stock has frequently been introduced into the southern hemisphere, especially into South Africa. These germplasm introductions have consequently resulted in high levels of genotypic diversity of *D. pinea* in the

country (Smith et al. 2000, Burgess et al. 2004a). In addition, high levels of diversity of *D. scrobiculata* have been recorded for Mexican and Californian isolates (USA) of that fungus, speculated to have been due to an overlooked sexual stage (Burgess et al. 2004b).

DNA sequence analysis of conserved gene regions, vegetative compatibility group analyses, and molecular markers including isozymes, RFLPs, RAPDs and SSRs have directly or indirectly been used to analyze the structure and genetic diversity of pathogenic fungi, including those of *D. pinea* and *D. scrobiculata*. The influence and results from the studies using these tools are discussed in the following section.

4.1. DNA sequences

Variation in DNA sequences can be used to detect genetic variation within species via amplifying several genomic regions that have polymorphisms (Taylor et al. 2006; Torriani et al. 2008). DNA sequence data from different genomic loci of *D. pinea* were not only useful to differentiate the fungus based on phylogeny from other *Diplodia* sp and morphotypes, but have also been indirectly used to analyze diversity of genotypes. For example, analysis of ITS region sequences showed that *D. scrobiculata* from Mexico is more diverse than *D. pinea* from South Africa and Indonesia (De Wet et al. 2000). The same pattern was observed for DNA sequence data of protein coding genes (BT2, CHS, EF-1α, ACT, CAL and GPD), where the phylograms showed more and deeper branches in clades containing isolates of *D. scrobiculata* than *D. pinea* (De Wet et al. 2003). In other fungi for, example *Mycosphaerella graminicola*, a greater level of sequence diversity was found in the ‘‘Old World’’ populations (Middle East and Europe) than in the ‘‘New World’’ (America and Australia) (Banke et al. 2004).

4.2. Vegetative Compatibility Groups (VCG)

Vegetative compatibility in fungi refers to the ability of isolates to form stable heterokaryons governed by a number of alleles at the vegetative compatibility loci, which are known as *het* (heterokaryon incompatibility) or *vic* (vegetative incompatibility) loci (Anagnostakis 1982; Leslie 1993; Glass et al. 2000). Compatible isolates are considered to be identical at these loci and they can undergo hyphal fusion to form heterokaryons, which is not true for genetically different isolates (Glass et al. 2000). Vegetative incompatibility occurs when individuals differ in specificity at one or more *het* or *vic* loci. Heterokaryotic fusion cells are usually compartmentalized and die due to a lytic process (Glass and Kuldau 1992; Glass et al. 2000). Strains capable of vegetative heterokaryon formation thus belong to the same vegetative compatibility group.

Vegetative compatibility group analyses have been widely used to characterise fungal population structure including that for *D. pinea*. This is particularly because the technique is relatively inexpensive and broadly applicable to fungi where there is no specific knowledge of genetic background and where specifically designed markers are not available (Leslie, 1993). The major disadvantages of using VCG analyses are that they are time consuming and sometimes difficult to interpret when closely related isolates show a weaker reaction than more distantly related isolates (Burgess et al. 2009). It is also difficult to determine allele frequencies at different vegetative incompatibility (*vic*) loci and relative relatedness of individuals cannot be determined (Leslie and Zeller 1996; Leslie 1996; Glass et al. 2000).

Using VCG analyses, Smith et al. (2000) reported that the genetic diversity for South African *D. pinea* isolates was higher than that of a Northern Sumatran population of the fungus. A later study also showed that genotypic diversity of the South African *D. pinea* population was

higher than that found in New Zealand and the USA, while genotypic diversity of an Australian population was comparatively low (Burgess et al. 2001a & b). These authors suggested that there have been multiple introductions of *D. pinea* into South Africa and that strict quarantine in Australia has limited diversity of the fungus in that country (Burgess et al. 2001a, 2004a).

4.3. Molecular markers

Markers refer to characters for which the pattern of inheritance can be followed at the morphological, biochemical or molecular (DNA markers) levels. They provide information regarding the genetics of interest in a given organism (Brown 1996; Karp et al. 1996; Ribaut et al. 2001). Molecular markers are used to evaluate the diversity of organisms based on variation in DNA sequences, mostly of non-coding intron or intergenic regions. Their main advantages over morphological markers are that DNA based markers are usually numerous, are not affected by the environment, and can be scored at virtually any stage of development of organisms (Brown 1996, Karp et al. 1996; McDonald 1997). DNA markers used to better understand the population biology of *Diplodia* include isozymes, restriction fragment length polymorphisms (RFLP's), random amplified polymorphic DNA's (RAPD's) and simple sequence repeats (SSR) or microsatellites. These markers each have advantages and disadvantages in genomic diversity analysis as is discussed below within the context of *D. pinea* research.

4.3.1. Isozymes

Isozymes are molecular forms of an enzyme, which usually have similar enzymatic properties, but different amino acid sequences (Weeden and Wendel 1989; Bonde et al. 1993). They are highly polymorphic between species and to a lesser extent within species (Glynn and Reid 1969).

Isozymes allow comparison of several alleles at a locus and they are co-dominant. However, isozyme analyses can differentiate only between isozymes that have different net charges (Burdon and Roelfs 1985; Bonde et al. 1993). Regardless of this disadvantage, isozymes have previously been used to distinguish fungal taxa and to assess the relatedness of many different fungal species including *Diplodia* spp. from pine. Palmer et al. (1987), Swart et al. (1991) and Stanosz et al. (1999), all distinguished *D. pinea* and *D. scrobiculata* using isozymes. Variability was also seen among ten South African *D. pinea* isolates with significant differences between them (Swart et al. 1991).

4.3.2. Restriction fragment length polymorphisms (RFLPs)

Restriction fragment length polymorphisms (RFLP's) are co-dominant markers that were first widely used in plant genome analysis (Jones et al. 1997; Ribaut et al. 2001). RFLP analyses can be performed using Southern blotting or it can be PCR based. The Southern-RFLP technique involves digestion of genomic DNA with sequence specific restriction endonucleases, producing a complex pattern of bands that are commonly revealed by gel electrophoresis. The separated fragments are transferred from the agarose gel to a membrane and then hybridized to labelled probes (Olive and Bean 1999).

RFLP-Southern blotting has been useful and can be used to identify larger numbers of isolates that would be difficult to identify otherwise. However, since this technique takes time and is cumbersome, it has been replaced by PCR-based locus-specific RFLP (Jones et al. 1997; Olive and Bean 1999). The PCR-RFLP technique involves amplification of a specific gene of interest followed by digestion with restriction enzymes (Power 1996; Jones et al. 1997; Olive

and Bean 1999). Visualizing bands or alleles at each RFLP locus and sequencing these alleles makes it possible to assess variation in a population (Griffiths et al 2000).

PCR-RFLP analysis of rDNA repeats has been applied in a number of cases to demonstrate the genetic diversity of microbial strains (Olive and Bean 1999). It has also been used to group *D. pinea* isolates into A, B and I types (Hausner et al. 1999). However, RFLPs have not been used subsequently to study diversity in *Diplodia* spp. from pine, because more variable and more suitable SSR markers have emerged as molecular genetics techniques have been perfected (Burgess et al. 2001b).

4.3.3. RAPDs

Random amplified polymorphic DNA (RAPD) markers are used to randomly amplify sequences in a genome (Power 1996; Jones et al. 1997; Olive and Bean 1999). The technique was widely used before more robust techniques appeared, as it requires no previous knowledge regarding the genome of an organism, is relatively easy to use, requires relatively little starting material and is inexpensive (Olive and Bean 1999). However, reproducibility between laboratories is difficult and results of the technique are thus not easy to compare between studies (Jones et al. 1997). In addition RAPDs are dominant markers (Lynch and Milligan 1993) and as such are more applicable for use in ascomycetous fungi that are haploid than in diploids or heterokaryons (McDonald 1997).

RAPDs were used to differentiate between the morphotypes of *Diplodia pinea* and to assess their population diversity. Collections of *Diplodia* isolates from pine in the north central United States, that were initially differentiated based on morphology into A (*D. pinea*) and B (*D. scrobiculata*) morphotypes, were confirmed as being distinct using RAPDs. These data also

showed that isolates of the B morphotype (= *D. scrobiculata*) were more diverse than the A morphotype (=*D. pinea*) (Smith and Stanosz 1995; Stanosz et al. 1999; De Wet et al. 2000).

4.3.4. Microsatellites

Microsatellites, also known as short tandem repeats or simple sequence repeats (SSRs), are tandem repeated sequences of varying lengths spread over the whole genome (Bruford and Wayne 1993; Griffiths et al. 2000). The loci containing microsatellites are ideal markers for studies of population genetic variation and gene flow, since they are often highly polymorphic. Simple sequence repeats are characterized by their hypervariability, co-dominant nature and abundance in the genomes of eukaryotes (Groppe et al. 1995; Griffiths et al. 2000; Lian et al. 2001). When amplified by PCR with specific flanking primers, different alleles of microsatellite-containing loci can readily be identified by their different sizes (Groppe et al. 1995). Moreover, using microsatellite markers is faster and simpler compared to AFLP and RFLP analyses, at least once the markers have been developed. As a result, microsatellites have been extensively used in taxonomy and the study of population genetics of fungi.

Microsatellite markers were shown to provide a powerful tool to define species boundaries in the *Botryosphaeriaceae* including *Diplodia* anamorphs (De Wet et al. 2003; Slippers and Wingfield 2007). Burgess et al. (2001b) developed SSR primers to study population diversity and identification of *D. pinea*. Diversity of *D. pinea* and *D. scrobiculata* isolates from different countries were evaluated using these SSR markers and the authors confirmed that both species were genetically diverse, especially in the introduced environments such as South Africa which may be because of multiple introductions and mutation (Burgess et al. 2004a and b). Microsatellite markers have also revealed the status of genetic diversity in other pathogens of

non-native plantation trees in the Southern Hemisphere including, *Ceratocystis albifundus* (Barnes et al. 2005), *Chrysoporthe cubensis* (Nakabonge et al. 2007), *Holocryphia eucalypti* (Nakabonge et al. 2008), *Teratosphaeria nubilosa* (Pérez et al. 2010).

5. Economic importance

Diplodia pinea is one of the best characterized pathogens of conifers. More than 30 species of pines and other coniferous tree species are known to be hosts of this pathogen (Palmer et al. 1985; Swart et al. 1987; Stanosz et al. 1999). Yet in many countries, the fungus is not important unless pines are stressed (Swart and Wingfield 1991; Stanosz et al. 1999; Wingfield et al. 2001). The pathogen appears to be comparatively less aggressive in its natural range than where it is grown as a non-native in plantations (Wingfield et al. 2001). *Diplodia scrobiculata* is less virulent than *D. pinea* (Palmer et al. 1987; Blodget and Stanosz 1999; De Wet et al. 2002), but interestingly, equally damaging when artificially inoculated into a wound (Palmer et al. 1987; Bihon et al. 2010).

Diplodia pinea causes a suite of disease symptoms including shoot blight or dieback, crown wilt, cankers, blue staining of cut timbers and root rot (Marks and Minko 1969; Wingfield and Knox-Davies 1980; Swart et al. 1985; Swart and Wingfield 1991; Flowers et al. 2001; Paoletti et al. 2001; Blodgett and Bonello 2003). It causes extensive damage during all growth stages of the tree (Blodgett and Stanosz 1997; Stanosz et al. 1999). In severe outbreaks, it may result in more than half of the original population of the trees needing to be removed due to severe tip blight (Flowers et al. 2001). In Swaziland more than 50 % of trees in a 600 hectare plantation were killed by this pathogen, where it caused a serious root disease in the late 1970's (Wingfield and Knox-Davies 1980).

In southern hemisphere countries such as South Africa, *D. pinea* causes significant losses to susceptible pine species in areas where there is regular hail damage (Zwolinski et al. 1990; Smith et al. 1996). It is estimated that the potential loss in pine plantations due to *D. pinea* in South Africa is about 55 % at the time of hail storms (Zwolinski et al. 1990). In the summer rainfall regions of South Africa, growers have needed to move away from planting *P. radiata,* because of its susceptibility to *D. pinea* (Lundquist 1987; Swart and Wingfield 1991).

6. Dispersal

Long distance dispersal of *D. pinea* across regions is mainly attributed to the asymptomatic persistence of the fungus within *Pinus* germplasm (Smith et al. 1996, 2000; Stanosz et al. 2005). The fungus colonizes the pith, conductive organs, bark, cones and other tissues of the tree while trees appear healthy (Smith et al. 1996; Luchi et al. 2005; Flowers et al. 2001; 2006). This is thought to have contributed to the high diversity of the pathogen in some introduced environments, for example in South Africa (Burgess and Wingfield 2002; Burgess et al. 2004a). Asymptomatic persistence of the pathogen also presents a risk of subsequent seedling mortality (Stanosz et al. 2005).

Short distance dispersal of *D. pinea* conidia after disease outbreaks is strongly related to the occurrence of rainfall, with peak conidial dispersal in association with moist wind and warmer temperature (Swart and Wingfield 1987; 1991). Conidia are released from pycnidia that have developed on dead pine tissue and litter (Fig. 1) in the presence of moisture and are disseminated by rain splash and wind. Rain splash and wind may disperse conidia relatively short distances, often on the same tree and plantations. Yet, dispersal in the absence of rain during dry periods is limited (Swart and Wingfield 1987).

Transmission of endophytic fungi can be either horizontal through infection of developing material by conidia or vertical (systemic) through seeds (Carroll 1988; Saikkonnen et al. 1998; Saikkonnen, 2007). However there is no evidence for vertical or systemic spread of *D. pinea,* where infection in trees grows into the seed, then developing seedling and eventually the tree. Vertical transmissions of endophytic fungi have reported only on those that occur on grasses and not in endophytic tree pathogens (Carroll 1988; Arnold et al. 2007).

7. Host predisposing factors

Diplodia pinea causes latent infection without visible symptoms, as described above. It then causes disease as soon as susceptible trees are exposed to biotic and/or abiotic stresses (Smith et al. 1996). Observations in the field and in laboratory experiments have shown the association between the incidence and severity of tip blight disease caused by *D. pinea* with various stress factors that can trigger host susceptibility (Swart et al. 1987; Blodgett et al. 1997; Stanosz et al. 2001). Physiological stresses caused by insect attack, water stress, pruning, hail and insufficient nutrients are the major factors that have been recorded to enhance infection of *D. pinea* (Swart et al. 1987, 1991).

Wounding created by hail damage (Zwolinski et al. 1990, 1995), pruning (Chou and MacKenzie 1988) and insect damage (Swart et al. 1987; Feci et al. 2003) that affect the attributes of host physiology can influence subsequent colonization by *D. pinea*. Wounding causes physiological stress to the tree and reduces its ability to suppress the development of latent infections by *D. pinea*. It also causes wounds to the stems and thus new infection sites. Infection of pine trees by *D. pinea* after hail has not reported from other parts of the world (Swart et al. 1991) and the importance of the pathogen in South Africa can be attributed to a combination of

common hail storms and planting of the highly susceptible species such as *P. radiata* and *P. patula*. The problem of pruning as a predisposition factor has been important in New Zealand and Australia (Swart et al. 1991; Chou and Mackenzie 1988).

Water stress plays a role in pre-disposing pine trees to diseases caused by *D. pinea* (Blodgett and Stanosz 1997, 1999; Stanosz et al. 2001). Pine seedlings and trees grown under low water potential conditions were more susceptible than those which were grown under normal conditions with adequate water (Blodgett and Stanosz 1999; Paoletti et al. 2001; Stanosz et al. 2001). Nutrient stress also increased incidence of *D. pinea* on *P. radiata* (Swart and Wingfield 1991). However, application of nitrogen fertilizer to the levels recommended for ornamental and shade trees results in decreasing resistance of red pine to *Diplodia* infection (Blodgett et al. 2005). This is because application of nitrogenous fertilizer resulted in the formation of succulent stems and lower lignin concentration in the tree that reduced tolerance (Stanosz et al. 2004; Blodgett et al. 2005). Other possibly stressful conditions such as annual precipitation and the quality of sites for planting should also be taken into account when planning pine plantation establishment (Zwolinski et al. 1990; Swart and Wingfield 1991).

Stressful conditions are not always a prerequisite for successful infection by *D. pinea* (Swart et al. 1988; Swart and Wingfield 1991). Pine shoots are susceptible to infection under optimal climatic conditions necessary for spore germination and where there is high inoculum pressure (Swart et al. 1987; Rees and Webber 1988). Saplings of three pine species sprayed with *D. pinea* spores without wounding resulted in dead-tops and wilting of seedlings (Rees and Webber 1988). These experiments indicated that when there is high inoculum pressure together with suitable environmental conditions for the spores to germinate, infection can also occur.

8. Disease management

Diplodia pinea has the ability to colonize plant tissues endophytically without visible symptoms and it is thus complicated to devise effective management strategies. Planting resistant pine species has been commonly used in combination with other silvicultural activities (Peterson 1977; Burdon et al. 1982; Swart et al. 1985; Swart and Wingfield 1991). In South Africa, Smith et al. (2002) suggested the potential for developing hybrids between susceptible *P. patula* and *P. greggii*, which is drought tolerant, adapted to high altitude and resistant to infection by *D. pinea*. However, breeding for resistance in trees under different environmental circumstances is time consuming, and does not necessarily fit with requirements for the wood for downstream processing. Consequently losses due to *D. pinea* infection remain high in South Africa (Zwolinski et al. 1990; Swart et al. 1991; Wingfield et al. 2001) although the development of less susceptible species and pine hybrids (Smith et al. 2002) is emerging as a solution to this problem. In addition to breeding, selection of pine species susceptible to diseases caused by *D. pinea* and replacing them with resistant trees has been applied. Of the pine species commonly grown in South Africa, *P. radiata* is not planted in areas where there are frequent summer hail storms and *P. patula* has been replaced by *P. greggii* in hail-prone areas (Swart et al. 1987, 1988; Swart et al. 1991; Smith et al. 2002).

Application of broad-spectrum fungicides such as Benomyl or thiophanate-methyl and bordeoux mixture can control tip blight disease caused by *D. pinea* (Peterson 1977; Palmer et al. 1986; Stanosz and smith 1996; Stanosz et al. 2005). However, their use is limited to nurseries and ornamentals because of the cost and operational problems in plantations and they are also unsafe (Swart et al. 1991). The general view is that for *D. pinea* management, it is preferable to use environmentally safe methods including the selection of sites not prone to disease, sanitation,

selection of non-susceptible species for wind breaks, managing pruning intensity and timing to avoid infection (Swart et al. 1985, 1987; Chou and Mackenzie 1988; Swart and Wingfield 1991; Stanosz et al. 2001). Hence, knowledge of environmental conditions favourable for the fungus is essential in order to ensure conditions where disease due to *D. pinea* is less likely to occur.

Biological control using dsRNA viruses in plant pathogenic fungi can be an environmentally friendly alternative to fungicides, in addition to having a lower risk of the pathogen developing resistance (Nuss 1992). For this reason, the possibility of using dsRNA virus for *D. pinea* control has been considered. These viral infections have thus been found in *D. pinea* and *D. scrobiculata* (Steenkamp et al. 1998; Preisig et al. 1998; Adams et al. 2002; De Wet et al. 2008) but unfortunately, no evidence of hypovirulence has yet been observed (Steenkamp et al. 1998).

Plants induce various defence responses against natural enemies and hence resistance is usually associated with these responses (Klessig et al. 2000). Attempts have been undertaken to study defence reactions in pine to *D. pinea* and *D. scrobiculata* infection. Blodgett and Stanosz (1998) thus reported synthesis of higher concentrations of monoterpenes and phenolics in red pine (*Pinus resinosa*) inoculated with *D. pinea* and *D. scrobiculata* compared to controls. In addition systemic induction of traumatic resin ducts has been observed in seedlings of *P. nigra* as a response of *D. scrobiculata* inoculation (Luchi et al. 2005). Similarly, accumulations of lignin and total soluble phenolics have been observed after *P. nigra* was inoculated with *D. pinea* although no correlation was seen with lesion lengths (Blodgett et al. 2005; Bonello and Blodgett 2003). The synthesis and accumulation of secondary metabolites as a response to *D. pinea* suggests that there is a possibility to develop resistant pine trees. However, differentially expressed antifungal pathogenesis related proteins against *D. pinea* and *D. scrobiculata* have not

been reported yet. These suggests a detail work is needed in this regard as well as the practical importance of those secondary metabolites reported to date.

9. Conclusions

Diplodia pinea, a pathogen that causes latent infections without visible symptoms for part of its life cycle, threatens pine production worldwide. Damage caused by this pathogen to pine trees increases with physiological and physical stresses, as well as with tree age. Water stress or drought, wounding as a result of hail and pruning and nutrient stresses are the most common factors cited as initiating disease symptoms caused by *D. pinea*. Even though *D. pinea* causes diseases mainly when trees are under stress, it also causes disease when there are favourable conditions for spores to germinate and under higher inoculum pressures. This is a problem that is particularly prevalent in nurseries and the best possible management approach currently is through the application of chemical fungicides.

Designing and implementing successful disease management strategies requires knowledge of genetic diversity and population biology. For example, in the process of screening varieties for resistance to pathogen strains, it is important to use samples that are representative of the diversity of the population. The higher the diversity of the fungus, the greater is its potential to overcome resistance and complicate breeding for resistance and other disease management practices. Diversity of *D. pinea* has been characterised using morphological parameters including colour, size, texture and septation of conidia, mycelial growth rate, vegetative compatibility (VCGs) and virulence. Moreover, molecular markers such as microsatellites are powerful and reliable tools to characterize and study diversity of *Diplodia* and

other fungal species. Their application is likely to impact on the understanding of *D. pinea* and its management in the future.

The population diversity of *D. pinea* in South Africa particularly and in other countries of the southern hemisphere is expected to be lower than that of its native range. This argument is based on the fact that the fungus has no observable sexual life cycle and it is not indigenous in the southern hemisphere. However, studies indicate that it has a high diversity in South Africa and that its diversity is in fact higher that in some native populations. This higher diversity can be explained by multiple or mass introduction from many sources or out-crossing due to the presence of cryptic sexuality in nature. It is, therefore, essential to minimize the entry of new genotypes, as well as the more virulent strains such as morphotype "C" of *D. pinea*, by strengthening the quarantine services in the country and growing more resistant pine species.

Despite years of intensive study of *D. pinea*, there are still many unanswered questions. These questions include whether a cryptic sexual stage exists, how the high levels of diversity in introduced environments are structured and distributed spatially, whether seed infections play any role in transmission and how endophytic and disease outbreak populations compare. The genetic diversity and infection processes at a small scale need to be addressed in order to better understand these critical questions of the biology of *D. pinea*. The aims of the studies presented in this thesis are therefore to study the genetic and genotypic diversity of *D. pinea* in South Africa at different spatial scales, from individual trees to localities or plantations and different provinces in the country. These data are also used to indirectly determine occurrence of genotype recombination. Comparisons of genotype diversity are also made between introduced populations from four countries and sources of *D. pinea* inoculum in South African pine plantations are investigated.

10. References

Adams GC, Wu NT, Eisenberg BE (2002) Virulence and double-stranded RNA in *Sphaeropsis sapinea*. Forest Pathology **32**: 309 – 329.

Anagnostakis SL (1982) Genetic analyses of *Endothia parasitica*: linkage data for four single genes and three vegetative compatibility types. Genetics **102**: 25 – 28.

Arnold AE, Henk DA, Ells RL, Lutzoni F, Vilgalys R (2007) Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia **99**: 185 – 206.

Banke S, Peschon A, McDonald BA (2004) Phylogenetic analysis of globally distributed *Mycosphaerella graminicola* populations based on three DNA sequence loci. Fungal Genetics and Biology **41**: 226 – 238.

Barnes I, Nakabonge G, Roux J, Wingfield BD, Wingfield MJ (2005) Comparison of populations of the wilt pathogen *Ceratocystis albifundus* in South Africa and Uganda. Plant Pathology **54**: 189 – 195.

Bihon W, Slippers B, Burgess T, Wingfield MJ, Wingfield BD (2010) *Diplodia scrobiculata* found in the southern hemisphere. Forest Pathology, doi: 10.1111/j.1439-0329.2010.00649.x.

Blodgett JT, Stanosz GR (1997) *Sphaeropsis sapinea* morphotypes differ in aggressiveness, but both infect on wounded red or jack pines. Plant Disease **81**: 143 – 147.

Blodgett JT, Stanosz GR (1998) Monoterpene and phenolic compound concentrations in water-stressed red pine inoculated with *Sphaeropsis sapinea*. Phytopathology **88**: 245 – 251.

Blodgett JT, Stanosz GR (1999) Differences in aggressiveness of *Sphaeropsis sapinea* RAPD marker group isolates on several conifers. Plant Disease **83**: 853 – 856.

Blodgett JT, Stanosz GR, Kruger EL (1997) Effects of moderate water stress on disease development by *Sphaeropsis sapinea* on red pine. Phytopathology **87**: 422 – 428.

Blodgett JT, Bonello P (2003) The aggressiveness of *Sphaeropsis sapinea* on Austrian pine varies with isolate group and site of infection. Forest Pathology **33**: 15 – 19.

Blodgett JT, Herms DA, Bonello P (2005) Effects of fertilization on red pine defence chemistry and resistance to *Sphaeropsis sapinea*. Forest Ecology and Management **208**: 373 – 382.

Bonde MR, Micales JA, Peterson GL (1993) The use of isozyme analysis for identification of plant-pathogenic fungi. Plant Disease **77**: 961 – 968.

Bonello P, Blodgett JT (2003) *Pinus nigra–Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. Physiological and Molecular Plant Pathology **63**: 249 – 261.

Brookhouser LW, Peterson GW (1971) Infection of Austrian, Scots and Ponderosa pines by *Diplodia pinea*. Phytopathology **62**: 409 – 414.

Brown JKM (1996) The choice of molecular marker methods for population genetic studies of plant pathogens. New Phytologist **133**: 183 – 195.

Bruford MW, Wayne RK (1993) Microsatellites and their applications to population genetic studies. Current Opinion in Genetics and Development **3**: 939 – 943.

Burdon JJ, Roelfs AP (1985) Isozyme and variation in asexually reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. Phytopathology **75**: 907 – 913.

Burdon RD, Currie D, Chou CKS (1982) Responses to inoculation with *Diplodia pinea* in progenies of apparently resistant trees of *Pinus radiata*. Australasian Plant Pathology **11**: 37 – 60.

Burgess T, Wingfield BD, Wingfield MJ (2001a) Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata.* Mycological Research **105**: 1331 – 1339.

Burgess T, Wingfield MJ, Wingfield BD (2001b) Simple sequence repeat (SSR) markers distinguish between morphotypes of *Sphaeropsis sapinea*. Applied Environmental Microbiology **67**: 354 – 362.

Burgess TI, Wingfield MJ (2002) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the southern hemisphere. International Forestry Review **4**: 56 – 65.

Burgess TI, Wingdield MJ, Wingfield BD (2004a) Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australasian Plant Pathology **33**: 513 – 519.

Burgess TI, Gordon TR, Wingfield MJ, Wingfield BD (2004b). Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. Mycological Research **108**: 1399 – 406.

Burgess T, Bihon W, Wingfield MJ, Wingfield BD (2009) A simple and rapid method to determine vegetative compatibility groups in fungi. INOCULUM: Newsletter of the Mycological Society of America.

Carroll G (1988) Fungal Endophytes in Stems and Leaves: From Latent Pathogen to Mutualistic Symbiont. Ecology **69**: 2 – 9.

Chou CKS, Mackenzie M (1988) Effect of pruning intensity and season on *Diplodia pinea* infection of *Pinus radiata* stems through pruning wounds. European Journal of Forest Pathology **18**: 437 – 444.

Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Philips AJL, Alves A, Burgess T, Barber P, Groenewald JZ (2006) Phylogenetic lineages in the *Botryosphaeriaceae*. Studies in Mycology **55**: 235 – 253.

Denman S, Crous PW, Taylor JW, Kang JC, Pascoe I, Wingfield MJ (2000) An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. Studies in Mycology **45**: 129 – 140.

De Wet J, Wingfield MJ, Coutinho TA, Wingfield BD (2000) Characterization of *Sphaeropsis sapinea* isolates from South Africa, Mexico and Indonesia. Plant Disease **84**: 151 – 156.

De Wet J, Wingfield MJ, Coutinho TA, Wingfield BD (2002) Characterization of the 'C' morphotype of the pine pathogen *Sphaeropsis sapinea*. Forest Ecology and Management **161**: 181 – 188.

De Wet J, Burgess T, Slippers T, Preisig O, Wingfield BD, Wingfield MJ (2003) Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. Mycological Research **107**: 557 – 566.

De Wet J, Preisig O, Wingfield BD, Wingfield MJ (2008) Patterns of multiple virus infections in the conifer pathogenic fungi, *Diplodia pinea* and *Diplodia scrobiculata*. Journal of Phytopathology **156**: 725 – 731.

Feci E, Smith D, Stanosz DR (2003) Association of *Sphaeropsis sapinea* with insect-damaged red pine shoots and cones. Forest Pathology **33**: 7 – 13.

Flowers J, Nuckles E, Hartman J, Vaillancourt L (2001) Latent infection of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. Plant Disease **85**: 1107 – 1112.

Flowers, Hartman, Vaillancourt (2006) Histology of *Diplodia pinea* in diseased and latently infected *Pinus nigra* shoots. Forest Pathology **36**: 447 – 459.

Glass NL, Kuldau GA (1992) Mating type and vegetative incompatibility in filamentous ascomycetes. Annual Review of Phytopathology **30**: 201 – 204.

Glass NL, Jacobson DJ, Shiu PKT (2000) The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. Annual Review of Genetics **34**: 165 – 168.

Glynn AN, Reid J (1969) Electrophoretic patterns of soluble fungal proteins and their possible use as taxonomic criteria in the genus *Fusarium*. Canadian Journal of Botany **47**: 1823 – 1831.

Griffiths AJF, Miller JH, Suzuki DT, Lewontin RC, Gelbart WM (2000) An introduction to genetic analysis 7th edition. Freeman and Company, New York.

Groppe K, Sanders I, Wiemken A, Boller T (1995) A Microsatellite marker for studying the Ecology and diversity of fungal endophytes (*Epichloe* spp.) in grasses. Applied and Environmental Microbiology **61**: 3943 – 3949.

Hausner G, Hopkin AA, Davis CN, Reid J (1999) Variation in culture and rDNA among isolates of *Sphaeropsis sapinea* from Ontario and Manitoba. Canadian Journal of Plant Pathology **21**: 256 – 264.

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, van DeWiel C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E, Malcevschi A, Marmiroli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazque A, Karp A (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding **3**: 381 – 390.

Karp A, Seberg O, Buiatti M, (1996) Molecular Techniques in the Assessment of Botanical Diversity. Annals of Botany **78**: 143 – 149.

Klessig D, Durner J, Noad R, Navarre D, Wendehenne D, Kumar D, Zhou J, Shah J, Zhang S, Kachroo P, Frifa Y, Pontier D, Lam E, Silva H (2000) Nitric oxide and salicylic acid signalling in plant defense. Proceedings of the National Academy of Science, USA **97**: 8849 – 8855.

Leslie JF (1993) Fungal vegetative compatibility. Annual Review of Phytopathology **31**: 127 – 150.

Leslie JF (1996) Fungal vegetative compatibility-promises and prospects. Phytoparasitica **24**: 3 – 6.

Lian C, Zhou Z, Hogetsu T (2001) A simple method for developing microsatellite markers using amplified fragments of inter-simple sequence repeat (ISSR). Journal of Plant Research **114**: 381 – 385.

Linde CC (2010) Population genetic analyses of plant pathogens: new challenges and opportunities. Australasian Plant Pathology **39**: 23 – 28.

Luchi N, Ma R, Capretti P, Bonello P (2005) Systemic induction of traumatic resin ducts and resin flow in Austrian pine by wounding and inoculation with *Sphaeropsis sapinea* and *Diplodia scrobiculata*. Planta **221**: 75 – 84.

Lundquist JE (1987) A history of five forest diseases in South Africa. South African Forestry Journal **140**: 51 – 59.

Marks GC, Minko G (1969) The pathogenicity of *Diplodia pinea* to *Pinus radiata* D. Don. Australian Journal of Botany **17:** 1 – 12.

McDonald BC (1997) The population genetics of fungi: tools and techniques. Phytopathology **87**: 448 – 453.

McDonald BA, Linde C (2002) Pathogen population genetics, evolutionary potential and durable resistance. Annual Review of Phytopathology **40**: 349 – 379.

McDonald BC, McDermott JM (1993) Population Genetics of Plant Pathogenic Fungi. BioScience **43**: 311 – 319.

Maresi G, Luchi N, Pinzani P, Pazzagli M, Capretti P (2007) Detection of *Diplodia pinea* in asymptomatic pine shoots and its relation to the Normalized Insolation index. Forest Pathology **37**: 272 – 280.

Milgroom MG (2001) The synthesis of genetics and epidemiology: Contributions of population biology in plant pathology. Journal of Plant Pathology **83**: 57 – 62.

Milgroom MG, Fry WE (1997) Contributions of population genetics to plant disease epidemiology and management. Advances in Botanical Research **24**: 1 – 30.

Nakabongea G, Gryzenhouta M, Wingfield BD, Wingfield MJ, Roux J (2007) Genetic diversity of *Chrysoporthe cubensis* in eastern and southern Africa. South African Journal of Science 103: 261 – 264.

Nakabonge G, Burgess T, Gryzenhout M, Wingfield BD, Wingfield MJ, Roux J (2008) Population structure of the fungal pathogen *Holocryphia eucalypti* in Australia and South Africa. Australasian Plant Pathology **37**: 154 – 161.

Nevo E (1978) Genetic variation in natural populations: Patterns and theory. Theoretical Population Biology **13**: 121 – 177.

Nuss, DL (1992) Biological control of chestnut blight: An example of virus-mediated attenuation of fungal pathogenesis. Microbiology Review **56**: 561 – 576.

Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. Journal of Clinical microbiology **37**: 1661 – 1669.

Palmer MA, Nicholls TH (1985) Shoot blight and collar rot of *Pinus resinosa* caused by *Sphaeropsis sapinea* in forest tree nurseries. Plant Dissease **69**: 739 – 740.

Palmer MA, Nicholls TH, Croghan CF (1986) Fungicidal control of shoot blight caused by *Spharopsis sapinea* on red pine nursery seedlings. Plant Disease **70**: 194 – 199.

Palmer MA, Stewart EL, Wingfield MJ (1987) Variation among isolates of *Sphaeropsis sapinea* in the north central United States. Phytopathology **77**: 944 – 948.

Paoletti E, Danti R, Strati S, 2001. Pre- and post-inoculation water stress affects *Sphaeropsis sapinea* canker length in *Pinus halepensis* seedlings. Forest Pathology **31**: 209 – 218.

Pérez G, Slippers B, Wingfield BD, Huntera GC, Wingfield MJ (2010) Micro- and macrospatial scale analyses illustrates mixed mating strategies and extensive gene flow in populations of an invasive haploid pathogen. Molecular Ecology **19**: 1801 – 1813.

Peterson GW (1977) Infection, epidemiology and control of *Diplodia* blight of Austrian, Ponderosa and Scots pines. Phytopathology **67**: 511 – 514.

Power EGM (1996) RAPD typing in microbiology, a technical review. Journal of Hospital Infection **34**: 247 – 265.

Preisig O, Wingfield BD, Wingfield MJ (1998) Coinfection of a fungal pathogen by two distinct double-stranded RNA viruses. Virology **252**: 399 – 406.

Rees AA, Webber JF (1988) Pathogenicity of *Sphaeropsis sapinea* to seed, seedlings and saplings of some central American pines. Transactions of the British Mycological Society **91**: 273 – 277.

Ribaut JM, William, Khairallah, Worland, Hoisington (2001) Genetic basis of physiological traits. *In*: Reynolds, et al (eds). Application of physiology in plant breeding, CIMMYT 29 – 47.

Saikkonnen K (2007) Forest structure and fungal endophytes, Fungal Biology Reviews **21**: 67 – 74.

Saikkonnen K, Faeth SH, Helander ML, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. Annual Review of Ecology and Systematics **29**: 319 – 343.

Schoch CL, Shoemaker RA, Spatafora JW, Crous PW (2006) A multigene phylogeny of the Dothideomycetes using four nuclear loci. Mycologia **98**: 1041 – 1052.

Slippers B, Wingfield MJ (2007) *Botryosphaeriaceae* as endophytes and latent pathogens of woody plants: Diversity, ecology and impact. Fungal Biology Reviews **21**: 90 – 106.

Smith DR, Stanosz GR (1995) Confirmation of two distinct populations of *Sphaeropsis sapinea* in the north central United States using RAPDs. Phytopathology **85**: 699 – 704.

Smith H, Wingfield MJ, Crous PW, Coutinho TA (1996) *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus spp*. and Eucalyptus spp. in South Africa. South African Journal of Botany **62**: 86 – 88.

Smith H, Wingfield MJ, de Wet J, Coutinho TA (2000) Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. Plant Disease **84**: 139 – 142.

Smith H, Wingfield MJ, Coutinho TA (2002) The role of latent *Sphaeropsis sapinea* infections in post-hail associated die-back of *Pinus patula*. Forest Ecology and Management **164**: 177 – 184.

Stanosz GR, Smith DR (1996) Evaluation of fungicides for control of *Sphearopsis* shoot blight or red pine nursery seedlings. Canadian Journal of Forest Research **26**: 492 – 497.

Stanosz GR, Smith DR, Guthmiller MA, Stanosz JC (1997) Persistence of *Sphaeropsis sapinea* on or in asymptomatic shoots of red and jack pines. *Mycologia* **89**: 525 – 530.

Stanosz GR, Swart W, Smith D (1999) RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. Mycological Research **103**: 1193 – 1202.

Stanosz GR, Blodgett JT, Smith DR, Kruger EL (2001) Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. New Phytologist **149**: 531 – 538.

Stanosz GR, Trobaugh J, Guthmiller M A, Stanosz JC (2004) *Sphaeropsis* shoot blight and altered nutrition in red pine plantations treated with paper mill waste sludge. Forest Pathology **34**: 245 – 253.

Stanosz GR, Smith R, Albers JS (2005) Surveys for asymptomatic persistence of *Sphaeropsis sapinea* on or in stems of red pine seedlings from seven Great Lakes region nurseries. Forest Pathology **35**: 233 – 244.

Steenkamp ET, Wingfield BD, Swart WJ, Wingfield MJ (1998) Double-stranded RNA and associated virulence in South African isolates of *Sphaeropsis sapinea*. Canadian Journal of Botany **76**: 1412 – 1417.

Sutton BC (1980) The Coelomycetes. Commonwealth Mycological Institute, Kew, U.K., 696p

Swart WJ, Knox-Davies PS, Wingfield MJ (1985) *Sphaeropsis sapinea*, with special reference to its occurrence on *Pinus* spp. in South Africa. South African Forestry Journal **35**: 1 – 8.

Swart WJ, Wingfield MJ (1987) Conidial dispersal of *Sphaeropsis sapinea* in three climatic regions of South Africa. Plant Disease **71**: 1038 – 1040.

Swart WJ, Wingfield MJ, Knox-Davies PS (1987) Factors associated with *Sphaeropsis sapinea* infection of pine trees in South Africa. Phytophylactica **19**: 505 – 510.

Swart WJ, Wingfield MJ, Knox-Davies PS (1988) Relative susceptibilities to *Sphaeropsis sapinea* of six *Pinus* spp. cultivated in South Africa. European Journal of Forest Pathology **18**: 184 – 189.

Swart WJ, Wingfield MJ (1991) Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. Plant Disease **75**: 761 – 766.

Swart WJ, Wingfield MJ, Palmer MA, Blanchette RA (1991) Variation among South African isolates of *Sphaeropsis sapinea*. Phytopathology **81**: 489 – 493.

Taylor JW, Turner E, Pringle A, Dettman J, Johannesson H (2006) Fungal species: thoughts on their recognition, maintenance and selection. In: Fungal in the Environment, eds. Gadd GM, Watkinson SC, Dyer PS, Cambridge University Press 313 – 339.

Torriani SFF, Goodwin SB, Kema GHJ, Pangilinan JL, McDonald BA (2008) Intraspecific comparison and annotation of two complete mitochondrial genome sequences from the plant pathogenic fungus *Mycosphaerella graminicola*. Fungal Genetics and Biology **45:** 628 – 637.

Wang CG, Blanchette RA, Jackson WA, Palmer MA (1985) Differences in conidial morphology among isolates of *Sphaeropsis sapinea*. Plant Disease **69**: 838 – 841.

Weeden NF, Wendel JF (1989) Genetics of plant isozymes. Page 46 – 72 *in*: Isozymes in plant biology. Soltis DE ed, Chapman and Hall Ltd publisher, UK.

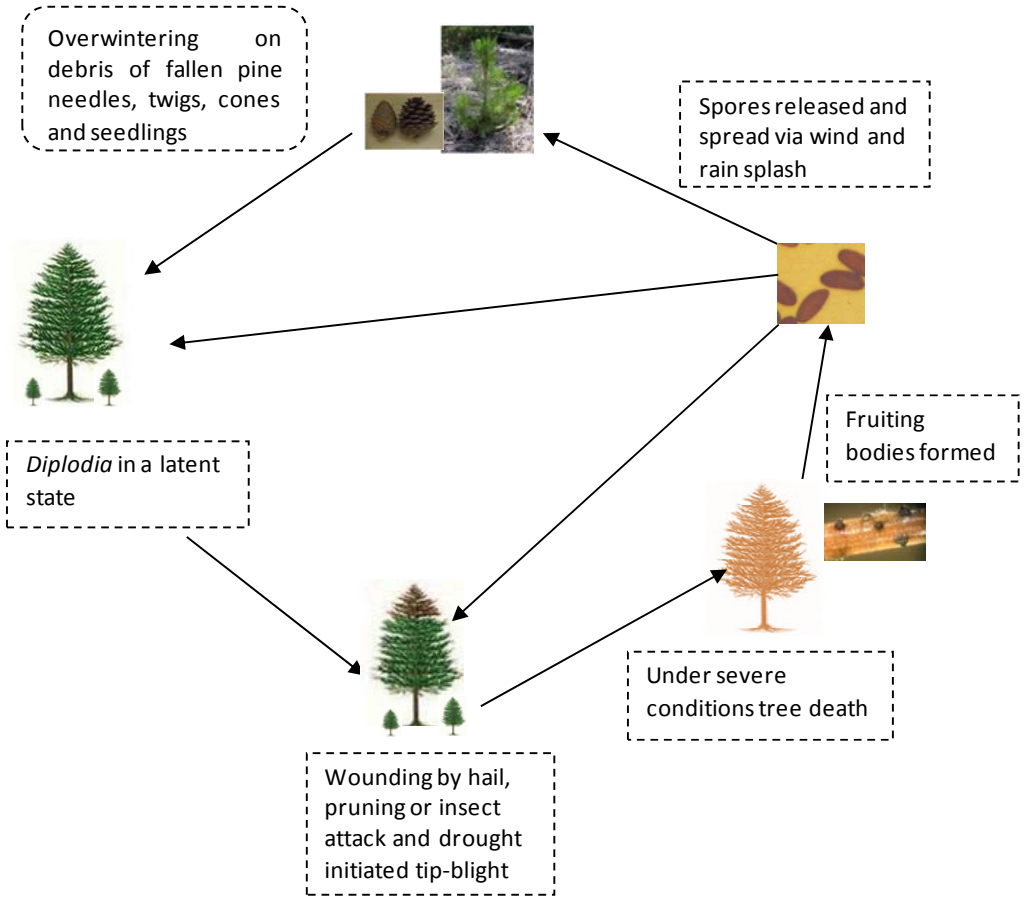
Wingfield MJ, Knox-Davies PS (1980) Association of *Diplodia pinea* with a root disease of pines in South Africa. Plant Disease **64**: 221 – 223.

Wingfield MJ, Slippers B, Roux J, Wingfield BD (2001) Worldwide movement of exotic forest Fungi, especially in the tropics and the southern hemisphere. BioScience **51**: 134 – 140.

Zwolinski JB, Swart WJ, Wingfield MJ (1990) Intensity of dieback induced by *Sphaeropsis sapinea* in relation to site conditions. European Journal of Forest Pathology **20**: 167 – 174.

Zwolinski, Swart, Wingfield (1995) Association of *Sphaeropsis sapinea* with insect infestation following hail damage of *P. radiata*. Forest Ecology and Management **72**: 293 – 298.

Figure 1. Disease cycle of *Diplodia pinea* in pine trees. An image of pine tree was found with permission from **www.etchingsetc.net**.



www.etchingsetc.net/images/pinetree_huge.jpg

Table 1. Timeline and techniques used in studying diversity in *D. pinea and D. scrobiculata*

Techniques	Key Discovery	Source
Morphology of conidia, cell wall, virulence and mycelia growth	- Observed the presence of more than two morphotypes of *Diplodia* in different countries	Stanosz et al. 1999, Swart et al. 1991, Palmar et al. 1987, Wang et al. 1985
Isozymes	- Distinguished morphotypes of *Diplodia pinea* (Morphotype A and B)	Palmer et al. 1987, Swart et al. 1991, Stanosz et al. 1999.
RAPD	- Diplodia differentiated into A and B morphotypes	- Smith and Stanosz 1995 - Stanosz et al. 1999 - De Wet et al. 2000
Amplification of ITS gene region	- *D. pinea* is closely related to *D. seriata* (=*Botryosphaeria obtusa*)	- Jacobs and Rehner 1998
RFLP	- Grouped DNA isolates into A, B and I morphotypes	-Hausner et al. 1999
Vegetative compatibility groups (VCG)	- Genotypic diversity of *D. pinea* populations was higher in South Africa than in northern Sumatra. - Higher diversity in South Africa *Diplodia* populations followed by New Zealand and USA	- Smith et al. 2000, - Burgess et al. 2001a.
Microsatellite markers (SSRs)	- Differentiating the four morphotypes of *D. pinea* into A, B, C and I - The I morphotype is confirmed to be *D. seriata* not *D. pinea* - Diversity of *D. pinea* is high	- Burgess et al. 2001b, - De Wet et al. 2003
ITS gene region	- A and C morphotypes are more related to each other than the B morphotype	- De Wet et al. 2002,
Protein coding genes (Bt2, CHS, EF-1α, ACT, CAL and GPD)	- Combined phylogram of protein coding genes confirmed that the B morphogype is a separate taxon, *D. scrobiculata*	- De Wet et al. 2003

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Distribution of *Diplodia pinea* and its genotypic diversity within asymptomatic *Pinus patula* trees

In press as:
Bihon W, Burgess T, Slippers B, Wingfield MJ, Wingfield BD (2010) Distribution of *Diplodia pinea* and its genotypic diversity within asymptomatic *Pinus patula* trees. Australasian Plant Pathology.

Abstract

Diplodia pinea (= *Sphaeropsis sapinea*) is an endophytic fungus and opportunistic canker pathogen of *Pinus* spp. The diversity of this fungus has been studied at broad geographic scales, but little is known regarding its population structure at smaller spatial scales such as within a single tree. This is despite the importance that diversity in a single tree might hold for understanding the biology of the fungus, especially the role of the endophytic or asymptomatic phase in disease development. Moreover there was not information regarding the distribution of the fungus within healthy trees and its persistence. The genotypic diversity of these isolates was investigated using microsatellite markers. Five polymorphic markers were developed and these were used together with eight previously developed markers and vegetative compatibility tests to study the genotypic diversity of *D. pinea* isolates. In this study, *D. pinea* was isolated for the first time in the well structured stems of healthy *P. patula* trees along with branches and cones. From a total of 44 isolates collected from five trees, 39 microsatellite haplotypes and 32 vegetative compatibility groups (VCG's) were identified. The results indicate high genotypic diversity of *D. pinea* within individual asymptomatic trees which will lead to disease outbreak when trees are physiologically stressed.

Introduction

Diplodia pinea (Desm.) J. Kickx) is an important pathogen of *Pinus* spp. causing shoot die-back, stem cankers, seedling mortality and under stress conditions such as hail damage, tree mortality (Swart et al. 1987; Blodgett et al. 1997; Burgess and Wingfield 2001; Stanosz et al. 2001). *Diplodia pinea* can be an endophyte in asymptomatic *Pinus* tissue (Smith et al. 1996; Burgess et al. 2001a; Stanosz et al. 2005; Maresi et al. 2007) and is commonly encountered as a saprophyte on cone bracts and coarse woody debris (Smith et al. 1996; Flowers et al. 2001; Santini et al. 2008).

Diplodia pinea is an opportunistic pathogen that causes disease when trees are subjected to biotic or abiotic stress. Hail damage (Zwolinski et al. 1990) and drought (Blodgett et al. 1997; Desprez-Loustau et al. 2006) commonly predispose trees resulting in the onset of disease. *P. radiata* is one of the most susceptible species with losses of up to 55% reported in plantations following hail damage in South Africa (Zwolinski et al. 1990). As a consequence *P. radiata* has been excluded from South African plantations in regions receiving summer rainfall and frequent hailstorms (Lundquist 1987; Swart and Wingfield 1991). The only known method of reducing the impact of this disease is to reduce the stress in plantations by early thinning and by planting disease tolerant pine species (Swart and Wingfield 1991; Burgess and Wingfield 2001).

Understanding the genetic diversity of a pathogen is increasingly recognized as an important part of successful disease management (McDonald and McDermott 1993). In this regard, the population diversity of *D. pinea* has been studied previously at large geographic scales, such as in countries and globally (Smith et al. 2000; Burgess et al. 2001a; Burgess et al. 2004a). In general, these studies have revealed high levels of diversity for the pathogen, even in some environments where the pathogen has been introduced. For example, Burgess et al. (2004a)

observed higher diversity for *D. pinea* in exotic *Pinus* plantations in South Africa than in three populations collected from native trees in the suspected native range of the fungus from the northern hemisphere. However, there has been no detailed study focussed on diversity of *D. pinea* at finer spatial scales, such as in and amongst closely spaced individual asymptomatic trees. The genetic diversity, distribution and abundance of endophytic *D. pinea* within an individual healthy tree are unknown. Is the tree colonised by a single clone or is it diverse due to multiple infections over longer periods of time? Is the fungus distributed evenly throughout the tree or is it localised? Studies of this kind would contribute to the understanding of the biology and control of the fungus.

Vegetative compatibility groups (VCGs) and microsatellite markers are frequently used techniques to characterize and study genotypic diversity of fungi species. VCGs are a simple and inexpensive method of determining genetic diversity (Milgroom and Cortesi 1999; Burgess et al. 2009). Vegetative compatibility is governed by a number of alleles at the vegetative compatibility loci. Compatible isolates are identical at these loci and represented by the same VCG (Leslie 1993). Moreover, microsatellite markers provide a robust method to study the genetic diversity of fungi and other organisms. This is because they are highly polymorphic and relatively inexpensive to use once they have been developed (Zane et al. 2002). Microsatellite markers have previously been developed to study populations of *Diplodia* spp. on pine (Burgess et al. 2001b). While these markers showed high allelic diversity in populations of *D. scrobiculata* (Burgess et al. 2004b) the allelic diversity in populations of *D. pinea* was relatively low with most markers having a single dominant allele (Burgess et al. 2004a).

The objective of this study was to determine the abundance, distribution and genetic diversity of endophytic *D. pinea* isolates infecting individual trees. The diversity of isolates was

assessed using microsatellite markers and VCGs. The original set of eight microsatellite markers (Burgess et al., 2001b) was also expanded in this study by developing an additional five markers using genome sequencer 20 (GS20), which has recently been shown to be an efficient method to screen microsatellite enriched libraries (Santana et al. 2009).

Materials and Methods

Distribution and abundance

Three apparently healthy *P. patula* trees (Tree 1, 2, 3) with 12 to 15 years of age at Balgown, in the KwaZulu-Natal midlands and two trees (Tree 4 and 5) of the same species and age at Sabie, in the Mpumalanga province, South Africa were selected and felled. Seven stem discs of approximately 3 to 4 cm thick were collected at equal distances from the bottom to the tops of the trees. In addition, five primary branches and their sub-branches were collected at different points of the crowns of the trees. Eleven cones were also collected from two trees at Sabie and one tree at Balgown. The samples were stored at 4 ^{o}C and isolations were undertaken within two days.

Four pieces of wood (approximately 2.5 mm^2) were aseptically cut from each of the seven stem discs (28 samples per tree) and placed on 2.0 % MEA (2 % m/v Biolab malt extract, 1.5 % m/v Biolab Agar) containing 0.04 % streptomycin. For the branch samples, five pieces (10 cm long) per branch (25 per tree) were cut and dipped into 70 % EtOH for 3-5 minutes, followed by 3.5 % NaOCl and 70 % EtOH for 1 minute and 4 washes of 1 minute in sterile distilled water before blotting dry on tissue paper (Burgess et al. 2006). Sections (approximately 5 mm thick) were cut from each branch sample and these were split in half. Each piece was placed onto the agar surface in Petri dishes containing 2 % MEA. Cone bracts were dipped in ethanol, flamed

and cut into sections as described by Smith et al. (2000) and placed onto the surface of MEA in Petri dishes.

The Petri dishes were incubated under a continuous light at 25 ^{o}C, for 4 to 6 days, after which isolates with white and fluffy mycelium typical of *D. pinea* were sub-cultured into 2 % WA (2 % m/v Biolab agar) containing two autoclaved pine needles to stimulate the production of pycnidia. After two to three weeks, pycnidia were collected and placed in 1.5 ml Eppendorf tubes with 50 µl of distilled H_2O. Pycnidia were vortexed and plated on MEA overnight after which single germinating conidia were identified and single conidial cultures produced. The abundance and distribution of the fungus in stem discs, branches and cones were therefore determined. Isolates are maintained in the culture collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), Pretoria University (Table 1).

Development of microsatellite markers

Microsatellite markers were produced using a modification of the method described by Santana et al. (2009). Microsatellite rich regions of *D. pinea* isolate CMW 4245 were randomly amplified using seven inter-simple sequence repeat (ISSR) primer sets; 5'DDB$(CCA)_5$, 5'DHB$(CGA)_5$, 5'YHY$(GT)_5$G, 5'HVH$(GTG)_5$, 5'NDB$(CA)_7$C, 5'NDV$(CT)_8$, and 5'HBDB$(GACA)_4$ and all of their possible combinations (Burgess et al., 2001b). The ISSR-PCR reactions and conditions were the same as described by Van der Nest et al. (2000), but by varying the annealing temperatures between 45 ºC and 60 ºC. PCR products that resulted in clear bands of different size on agarose gels were pooled, precipitated using 70 % EtOH, and sequenced using a Roche Genome Sequencer 20 (GS20).

One thousand eight hundred and seventy contigs ranging from 45 to 716 bp were produced from more than 10 000 reads. Reads were assembled using Vector NTI 10.3.0 computer program and larger contigs containing microsatellite regions in the centre of the fragment sequence were further analysed. Primers were designed flanking the microsatellite rich regions using Primer3 (http://frodo.wi.mit.edu) and confirmed manually (Table 2).

DNA extraction, PCR amplification and separation of SSR loci

Cultures were grown on MEA in Petri dishes for two weeks and mycelium was scraped from the surface of agar plates for DNA extraction. The mycelium was ground using tungsten beads (3mm) (Qiagen, Hilden, Germany) at a speed of 5 m/s for 20 seconds in warm CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) following the manufacturer's instructions in a FastPrep FP120 homogenizer (Southern Cross Biotechnology). This maceration step was repeated 4 times prior to DNA extraction. The concentration and quality of DNA was estimated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

The 13 microsatellite loci were amplified for all *D. pinea* isolates following the method of Burgess et al. (2004a) with annealing temperatures given in Table 3. All SSR-PCR products were multiplexed and run in a single lane. An amount of 1 µl of these multiplexed PCR products was separated on an ABI Prism 3100 Genetic analyzer. The mobilies of SSR products were compared to those of internal size standards (LIZ-500) and allele sizes were estimated by GeneScan 2.1 and GeneMapper 3.7 computer software (Applied Biosytems).

Gene and genetic diversity

For each of the loci, individual alleles were assigned a different letter. For each isolate, a data matrix of 13 multistate characters (one for each locus) was compiled (eg. AABDCBADACCB). The frequency of each allele at each locus for the entire and clone corrected populations was calculated. Allele diversity was determined using the program POPGENE (Yeh *et al*. 1999) and the equation $H = 1 - \sum x^2_k$, where x_k is the frequency of the k^{th} allele (Nei 1973). Maximum percentage genotypic diversities were not estimated since isolate numbers were low per tree and it was clear that there were high diversities from the number of SSR haplotypes and VCGs observed. UPGMA dendrogram was constructed using individual allele matrix of mean character differences in PAUP version 4.0 (Swofford 2002) to understand relations of genotypes within and between trees.

Vegetative compatibility groups

In order to support the population diversity study using SSR markers, VCG were determined for all 44 isolates. Oat Meal Agar (OMA) was prepared as described by Smith et al. (2000). Six isolates were placed on a Petri dish containing OMA in a manner such that all isolates could be paired with each other in all possible combinations as well as with themselves as internal controls (Burgess et al. 2009). All isolates were paired in all possible combinations. The cultures were incubated at 25 ˚C in the dark for four to five days until barrage lines were obvious indicating incompatibility or different VCG's.

Results

Distribution and abundance

From the 276 isolations attempted in this study, 44 *D. pinea* isolates were obtained. Of these isolates only 14 were obtained from 140 isolations from stem disks, giving an average isolation success of 10 %. These isolates were from asymptomatic wood, not from the bark. Isolations from branches were more successful with 26 isolates obtained from 125 isolations, which is an isolation success of 21 % (Table 1). Four isolates were obtained from cones. This indicated that the fungus is available in all the organs tested even though it was not evenly distributed. Nei's genetic diversity index ranged from $H = 0.174$ to 0.274 per each tree and from 0.000 to 0.402 per each locus (Table 3).

Development of microsatellite markers

Fourteen primer pairs were designed from the DNA sequence and then tested for their ability to amplify single PCR products and whether the loci that were amplified were polymorphic. From these primer pairs, five were found to be polymorphic while the remainder of the primers were not and either did not result in amplification or resulted in multiple banding patterns (Table 2). These SSR loci (SS12 to SS16) had different fragment sizes (bp) to those developed previously (Burgess et al. 2001b) (Table 2). Each of the five new markers produced 2-4 alleles among the 44 *D. pinea* isolates. The size ranges of alleles produced were from 62 to 172 base pairs and the allelic diversity ranged from 0.089 to 0.361 (Table 3).

Segregation of SSR alleles

Thirteen SSR loci were amplified for 44 isolates of *D. pinea* isolated from 5 trees (Table 4). SSR loci rendered 36 alleles among the 44 *D. pinea* isolates used in this study with a total of 39 haplotypes (Table 1 and 3). Thirteen isolates were obtained from Tree 1 and were represented by 12 different haplotypes with 25 alleles and nine polymorphic loci. The least number of isolates, six, were obtained from Tree 2 and were all of different haplotypes with 20 alleles and seven polymorphic loci (Table 3). The percentage of polymorphic loci within a tree ranged between 46 % and 69 %. Of the 36 alleles, nine were found in all five trees, four were found in at least four trees and nine were found in at least three trees (Table 3). There were only six private alleles distributed among three of the five trees. Very few haplotypes were duplicated within a tree and no haplotypes were duplicated between trees (Table 1). Genetic relatedness between isolates within a tree and isolates between sites were evaluated using UPGMA dendrogram. Figure 1 show isolates from tree one, two and three were more similar to each other. Isolates from tree four and five were grouped in the same clade further from other isolates.

Vegetative Compatibility Groups

The 44 *D. pinea* isolates from the five sampled trees represented by 32 different VCGs (Table 1). The 13 isolates obtained from Tree 1 were represented by eight VCGs. The six isolates obtained from Tree 2, the seven isolates from Tree 3, the 11 isolates from Tree 4 and the seven isolates from Tree 5 were represented by 4, 6, 10 and 6 VCG's, respectively. Most of the isolates representing the same VCGs were from within the same tree and from branches, but there were also VCGs shared between isolates from stem and branch or branch and cone. VCG's were shared between Tree 1, 2 and 3 which were harvested within a plantation at Balgown.

Overall, there were fewer VCG's than microsatellite haplotypes for the 44 isolates considered. For example, 13 isolates from Tree 1 represented by 11 microsatellite haplotypes and 9 VCGs. Some isolates having the same VCG had different microsatellite haplotypes (Table 1). In the same tree, four isolates which were represented by one VCG (VCG 1) was shared by 3 microsatellite haplotypes (Table 1). Two of these haplotypes differed at only one locus, but the third haplotype differed from the other two at 5 loci. VCG 3, 4 and 5 were found in more than one tree, but with different microsatellite profiles in each tree. VCG's 1, 6 and 7 have the same microsatellite hapoloypes.

Discussion

Results of this study showed that *D. pinea* can be isolated from all parts of asymptomatic established trees, including asymptomatic wood from the stems, although with low isolation success. All other studies in which the distribution of *D. pinea* in asymptomatic material has been examined have concentrated on shoots, needles and cones (Smith et al. 2000; Flowers et al. 2003). To the best of our knowledge, the fungus has not been previously isolated from the resinous asymptomatic wood in the stems of established trees. This is also the first study to consider the incidence, distribution and diversity of *D. pinea* in mature asymptomatic wood of trees. Both microsatellite markers and VCGs showed that *D. pinea* isolates existing within asymptomatic mature trees have a high level of genetic diversity.

Isolation of *D. pinea* was possible in all the tissues examined with different rates of distribution and abundance. Isolation success from asymptomatic branches averaged at 21 % across the five trees. These isolation rates were comparable with other studies (Flowers et al. 2001; Flowers et al. 2003; Stanosz et al. 2005). The lower rate of isolation of *D. pinea* in

asymptomatic trees compared with dieback trees can be due to the localization of the fungi in a specific position within the tissue. Flowers et al. (2003), by halving terminal buds and bark discs from asymptomatic shoots, demonstrated the distribution of *D. pinea* is discontinuous, as it was not always possible to isolate from both halves of a terminal bud and bark disks. This explains the relatively low recovery of *D. pinea* from healthy tissues. Using direct polymerase chain reaction (PCR) from plant tissues provided more positive detection of *D. pinea* latent infection than isolation on growth media (Maresi et al. 2007).

Previous studies on detection of *D. pinea* in asymptomatic host tissue have either concentrated on young seedlings, or when samples were from mature trees, only shoots were examined (Stanosz et al. 1997; Flowers et al. 2003; Maresi et al. 2007). The fungus has also commonly been found as a saprophyte in cone bracts and debris (Swart and Wingfield 1991; Santini et al. 2008) and from blue stained wood after harvesting (Vanneste et al. 2002). This is the first study to consider the presence of *D. pinea* in healthy wood of mature trees although its abundance was low (10 %) compared to isolation from branches (21 %).

Gene and genotypic diversity was high within individual trees and between trees examined in this study. Several VCG's were represented by more than one microsatellite haplotype, with the exception of Tree 4 where three isolates assigned to two different VCG'S had the same microsatellite haplotypes. In addition, while some microsatellite haplotypes found in a single VCG differed at only 1-2 loci, others differed at many loci. Similar differences in groupings based on VC types as opposed to SSR haplotypes have been observed previously (Milgroom et al. 2008; Breuillin et al. 2006). Breuillin *et al*. (2006) observed not only more SSR haplotypes than VCGs in *Cryphonectria parasitica,* but also in one population more VCGs than microsatellite haplotypes. Likewise, Milgroom et al. (2008) observed more VCGs than sequence

characterized amplified region (SCAR) markers in the chestnut blight fungus. These and our results emphasise that the genetic and phenotypic markers are not always fully congruent and have different level of polymorphisms due to mutation. Here VCG's are controlled by an unknown number of *vic* loci and the interaction of these loci groups isolates into different phenotypes (Leslie 1993). Nonetheless, the results for both the microsatellite markers and VCG's in this study showed that there was a high level of genetic diversity for *D. pinea* isolates within mature pine trees and sites.

Regardless of the agreement between the two methods, estimates of diversity using both microsatellite haplotypes and VCG's were high. Isolates were also clustered according to site they originated and trees similar to *D. pinea* populations in a larger macro-spatial scale in South Africa which were structured based on geographical locations (unpublished data). This is consistent with the previously observed high level of genotypic diversity for the fungus in South Africa (Smith et al. 2000; Burgess et al. 2001a; Burgess et al. 2004a) and other endophytic fungal species such as *Rhabdocline parkeri* on Douglas fir (McCutcheon and Carroll 1993). While previous studies have considered isolates collected at a broad spatial scale in South Africa, it is clear from this study that individual trees can harbour many genotypes of *D. pinea.* This implies that endophytic colonisation is not the result of a single infection or multiple infections by the same *D. pinea* individual at one stage. High diversity of genotypes within asymptomatic *Pinus* trees probably results from multiple infections by different genotypes of *D. pinea* throughout the development of the tree and persists within the tree for longer time. Multiple infections of single needles by different individuals are common on Norway spruce where lots of distinct *Lophodermium piceae* isolates were obtained from a single needle (Muller et al., 2001). Maximum infection and diversity are reached when trees age and have increased foliage

(McCutcheon and Carroll 1993; Gamboa and Bayman 2001). Moreover, frequent introduction and mutations of *D. pinea* genotypes could be the reason for higher diversity (Burgess et al. 2004a). Persistence and higher diversity of *D. pinea* as an endophyte implied that there is a danger of a disease outbreak when trees faced physiological stresses (Stanosz et al. 1997; Smith et al. 2002). Moreover, it will be difficult for breeding for resistance and to implement control measures.

In general *D. pinea* the fungus was found in all parts of the asymptomatic *P. patula* tree and both microsatellite and VCG markers revealed the presence of many genotypes. Isolation of different genotypes of the fungus deep inside the stem also indicated that infection had occurred at earlier stages of the tree and persisted throughout the growing stages. A recent study of another opportunistic endophyte, *Neofusicoccum australe* has shown that the same VCG's were isolated from cankers on diseased trees and endophytically from asymptomatic trees of *Agonis flexuosa* (Dakin et al. 2010). Theoretically, outbreaks of disease caused by *D. pinea* can therefore rapidly develop from endophytic infections when pine trees are subjected to physical or physiological stress.

References

Blodgett JT., Kruger EL, Stanosz GR (1997) Effects of moderate water stress on disease development by *Sphaeropsis sapinea* on red pine. Phytopathology **87**: 422 – 428.

Breuillin F, Dutech C, Robin C (2006) Genetic diversity of the chestnut blight fungus *Cryphonectria parasitica* in four French populations assessed by microsatellite markers. Mycological Research **110**: 288 – 296.

Burgess T, Wingfield MJ (2001) Exotic pine forestry in the Southern Hemisphere: A brief history of establishment and quarantine practices. South African Forestry Journal **192**: 79 – 84.

Burgess T, Wingfield BD, Wingfield MJ (2001a) Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata.* Mycological Research **105**: 1331 – 1339.

Burgess T, Wingfield MJ, Wingfield BD (2001b) Simple sequence repeat markers distinguished among morphotypes of *Sphaeropsis sapinea*. Applied and Environmental Microbiology **67**: 354 – 362.

Burgess T, Wingfield MJ, Wingfield BD (2004a) Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australasian Plant Pathology **33**: 513 – 519.

Burgess T, Gordon TR, Wingfield MJ, Wingfield BD (2004b) Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. Mycological Research **108**: 1399 – 1406.

Burgess TI, Sakalidis ML, Hardy GE (2006) Gene flow of the canker pathogen *Botryosphaeria australis* between *Eucalyptus globulus* plantations and native eucalypt forests in Western Australia. Austral Ecology **31**: 559 – 566.

Burgess TI, Bihon W, Wingfield MJ, Wingfield BD (2009) A simple and rapid method to determine vegetative compatibility groups in fungi. *Inoculum,* Mycological Society of America **60**: 1 – 2.

Dakin N, White D, Hardy G, Burgess TI (2010) The opportunistic pathogen, *Neofusicoccum australe*, is responsible for crown dieback of peppermint (Agonis flexuosa) in Western Australia. Australasian Plant Pathology **39**: 1 – 5.

Desprez-Loustau ML, Marcais B, Nageleisen LM, Piou' D, Vannini A (2006) Interactive effects of drought and pathogens in forest trees. Annals of Forest Science **63**: 597 – 612.

Flowers J, Nuckles E, Hartman J, Vaillancourt L (2001) Latent infections of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. Plant Disease **85**: 1107 – 1112.

Flowers J, Hartman J, Vaillancourt L (2003) Detection of latent *Sphaeropsis sapinea* infections in Austrian pine tissues using nested-polymerase chain reaction. Phytopathology **93**: 1471 – 1477.

Gamboa MA, Bayman P (2001) Communities of Endophytic Fungi in Leaves of a Tropical Timber Tree *(Guarea guidonia:* Meliaceae). Blotroplca **33**: 352 – 360.

Leslie JF (1993) Fungal vegetative compatibility. Annual Review of Phytopathology **31**: 127 – 150.

Lundquist JE (1987) A history of five forest diseases in South Africa. South African Forestry Journal **140**: 51 – 59.

Maresi G, Luchi N, Pinzani P, Pazzagli M, Capretti P (2007) Detection of *Diplodia pinea* in asymptomatic pine shoots and its relation to the Normalized Isolation index. Forest Pathology **37**: 272 – 280.

McCutcheon TL, Carroll GC (1993) Genotypic diversity in populations of a fungal endophyte from douglas fir. Mycologia **85:** 180 – 186.

McDonald BA, McDermott JM (1993) Population genetics of plant pathogenic fungi: Electrophoresis markers give unprecedented precision to analysis of genetic structure of populations. BioScience **43**: 311 – 319.

Milgroom MG, Cortesi P (1999) Analysis of population structure of the chestnut blight fungus based on vegetative incompatibility genotypes. Proceedings of the National Academy of Sciences, USA **96**: 10518 – 10523.

Milgroom MG, Sotirovski K, Spica D, Davis JE, Brewer MT, Milev M, Cortesi P (2008) Clonal population structure of the chestnut blight fungus in expanding ranges in south Eastern Europe. Molecular Ecology **17**: 4446 – 4458.

Müller MM, Valjakka R, Suokko A, Hantula J (2001) Diversity of endophytic fungi of single Norway spruce needles and their role as pioneer decomposers. Molecular Ecology **10**: 1801 – 1810.

Nei M (1973) Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences USA **70**: 3321 – 3323.

Santana QC, Coetzee MPA, Steenkamp ET, Mlonyeni OX, Hammond GNA, Wingfield MJ, Wingfield BD (2009) Microsatellite discovery by deep sequencing of enriched genomic libraries. Biotechniques **46**: 217 – 223.

Santini A, Pepori A, Ghelardini L, Capretti P (2008) Persistence of some pine pathogens in coarse woody debris and cones in a *Pinus pinea* forest. Forest Ecology and Management **256**: 502 – 506.

Smith H, Wingfield MJ, Crous PW, Coutinho TA (1996) *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. South African Journal of Botany **62**: 86 – 88.

Smith H, Wingfield MJ, de Wet J, Coutinho TA (2000) Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. Plant Disease **84**: 139 – 142.

Smith H, Wingfied MJ, Couinho TA (2002) The role of latent *Sphaeropsis sapinea* infectins in post-hail associated die-back of *Pinus paula*. Forest Ecology and Managemet **164**: 177 – 184.

Stanosz GR, Smith DR, Guthmiller MA, Stanosz JC (1997) Persistence of *Sphaeropsis sapinea* on or in asymptomatic shoots of red and jack pines. Mycologia **89**: 525 – 530.

Stanosz GR, Blodgett JT, Kruger EL (2001) Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. New Phytologist **149**: 531 – 538.

Stanosz GR, Smith DR, Albers JS (2005) Surveys for asymptomatic persistence of *Sphaeropsis sapinea* on or in stems of red pine seedlings from seven Great Lakes region nurseries. Forest Pathology **35**: 233 – 244.

Swart WJ, Wingfield MJ, Knox-Davies PS (1987) Factors associated with *Sphaeropsis sapinea* infection of pine trees in South Africa. Phytophylactica **19**: 505 – 510.

Swart WJ, Wingfield MJ (1991) Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. Plant Disease **75**: 761 – 766.

Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony (* and other Methods), Version 4. Sunderland, MA: Sinauer Associates.

Van der Nest MA, Steenkamp ET, Wingfield BD, Wingfield MJ (2000) Development of simple sequence repeats (SSR) markers in *Eucalyptus* from amplified inter-simple sequence repeats (ISSR). Plant Breeding **119**: 433 – 436.

Vanneste JL, Robert AH, Stuart JK, Roberta LF, Patrick TH (2002) Biological control of sapstain fungi with natural products and biological control agents: review of the work carried out in New Zealand. Mycological Research **106**: 228 – 232.

Yeh FC, Yang RC, Boyle T (1999) PopGene version 1.31 (Microsoft windows based freeware for population genetic analysis. Alberta).

Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. Molecular Ecology **11**: 1 – 16.

Zwolinski JB, Swart WJ, Wingfield MJ (1990) Intensity of die-back induced by *Sphaeropsis sapinea* in relation to site conditions. European Journal of Forest Pathology **20**: 167 – 174.

Table 1. Source, microsatellite haplotype and VCG group for 44 isolates of *D. pinea* isolated from five *P. patula* trees. Profiles generated from the amplification of 13 microsatellite loci and vegetative compatibility group (VCG) of all the isolates obtained from asymptomatic *P. patula* trees in South Africa.

Tree No.	Isolate number	Isolated from	Microsatellite haplotypes[1]	VCG group
Tree 1	CMW29161	Stem	BACAAABBCABAB	**3**
	CMW29162	Stem	BAAAAABACAAAB#	**5**
	CMW29163	Stem	BACAAAABCBBAB	8
	CMW29164	Stem	BAAAAABACAAAB#	**5**
	CMW29146	Branch	CAAAAABACAACB	**1**
	CMW29147	Branch	CACAAAABCBAAB	9
	CMW29148	Branch	CACAAAAACBCAB	10
	CMW29149	Branch	CAAAAABADABCB	11
	CMW29150	Branch	BACAAAAACABAB*	**1**
	CMW29151	Branch	BACAAAAACABAB*	**1**
	CMW29152	Branch	BACAAAAACBBCB	**1**
	CMW29153	Branch	BACAAAAACBAAB	12
	CMW29171	Cone	BACAAABACABBA	13
Tree 2	CMW29165	Stem	BAAAACBACABAB	**5**
	CMW29166	Stem	BBAAACBACAACB	**3**
	CMW29154	Branch	BACAAACBCABCB	**2**
	CMW29155	Branch	CACAAAAAAAACB	**2**
	CMW29156	Branch	BACAABBBCBAAB	**2**
	CMW29172	Cone	BACAAABABABCC	**4**
Tree 3	CMW29168	Stem	BACAAABACABCB	14
	CMW29169	Stem	BACAAAAACABCB	**4**
	CMW29170	Stem	BACAAABADABCB	**15**
	CMW29157	Branch	BACAAABACABCB	**3**
	CMW29158	Branch	BACAAAAACAAAB	16
	CMW29159	Branch	BACAAABACABBB	17
	CMW29160	Branch	BACAAABACAACB	18
Tree 4	CMW29450	Stem	CACAABCBCAABD	19
	CMW29451	Stem	AADAABCBCAABD	20
	CMW29440	Branch	CACAABCBCAACD§	**6**
	CMW29441	Branch	CACAABCBCAACD§	**6**
	CMW29442	Branch	CABAABBCDAACD	21
	CMW29444	Branch	AACAABBBCAACD	22
	CMW29445	Branch	CACAABCBCAACD§	23
	CMW29446	Branch	BACAABBBCABCD	24
	CMW29448	Branch	AACAABCBCAACD	25
	CMW29452	Cone	CADAABCBCAACD	26
	CMW29453	Cone	CACAABCBCABCD	27
Tree 5	CMW29458	Stem	AADAABABCAACD	28
	CMW29459	Stem	CADAABABBAACD	29
	CMW29460	Stem	CACAABBBBAACD	30
	CMW29454	Branch	CACAABCBBABBD	31
	CMW29455	Branch	CADAABBBBAABD¥	**7**
	CMW29456	Branch	CADAABBBBAABD¥	**7**
	CMW29457	Branch	AADAABBBDAABD	32

Profiles with the same symbols (#, *, § and ¥) are identical haplotypes and isolates within the same VCGs are indicated by the same numbers.

Table 2. Characteristics of polymorphic new SSR markers

Primer Name	Locus	Sequence (5' to 3')	Atm (°C)	Core repeat motifes	Fragment Length (bp)
WB1-a WB1-b	SS12	**PET**-ACC ACC ACC ACC GTC AAG GAA CGC CAT CGT CGT CAC	62	$(ACC)_9AC$	107
WB2-a WB2-b	SS13	**FAM**-GGC GTG TGT GAT GAG ATG AG GTC CTT TGT GTG TTG GGT TG	55	$(CGAGA)_4$ CGAGC $(CGAGA)_6$	180
WB4-a WB4-b	SS14	**NED**-CAC CAC CAC CAA CAC CTT G CGT GTT GGA AGC GAC GAC	58	$(CTT)_5$ $(CCT)_9$	149
WB7-a WB7-b	SS15	**NED**-GAA TCA CTG GCC GGT TTG GAG TCC AGC CTT TCC TCC TC	55	$(GGA)_5$ AGA $(GGA)_4$ AGA $(GGA)_3$	99
WB8-a WB8-b	SS16	**VIC**-GGG GAA AAG ACG TGT TGT TGT CAG CAT CGT CGT CCC ATT AG	55	$(GA)_{11}$	99

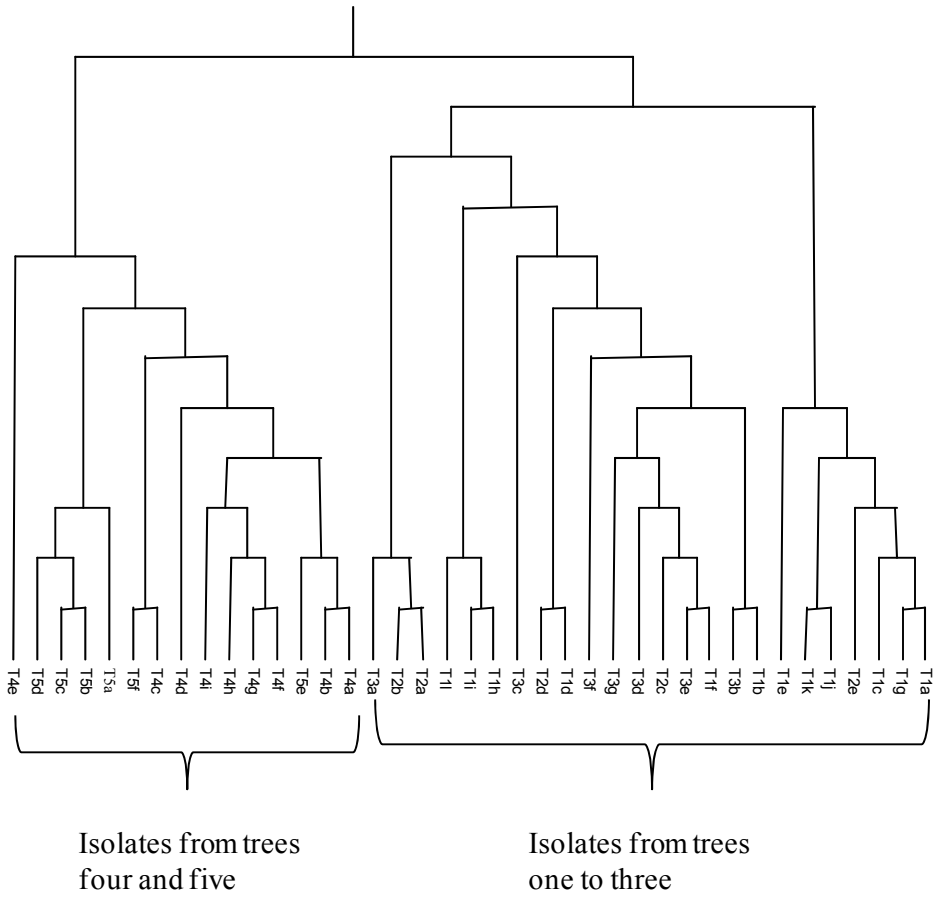
The forward primers were labelled with a phosphoramidite fluorescent dye indicated as FAM, NED, PET and VIC. ( ) Parenthesis under the core repeat motifs column indicate repeated nucleotides, subscript numbers indicates the number of repeats.

Table 3. Allele size (bp) and frequency at 13 SSR loci for *D. pinea* isolates from five *Pinus patula* trees used in this study

Locus	Alleles	Tree1	Tree2	Tree3	Tree4	Tree5	*H*
SS1	377	…	…	…	0.273	0.286	
	408	0.615	1.000	1.000	0.091	…	
	409	0.384	…	…	0.636	0.714	**0.279**
SS2	193	1.000	1.000	0.857	1.000	1.000	
	195	…	…	0.143	…	..	**0.049**
SS5	499	0.154	0.500	0.143	…	…	
	500	…	…	…	0.091	…	
	501	0.846	0.500	0.857	0.727	0.286	
	502	…	…	…	0.182	0.714	**0.368**
SS7	382	1.000	1.000	1.000	1.000	1.000	**0.000**
SS8	279	1.000	1.000	1.000	1.000	1.000	**0.000**
SS9	256	0.923	0.833	0.857	…	…	
	258	0.077	…	…	1.000	1.000	
	260	…	0.167	0.143	…	…	**0.132**
SS10	279	0.615	0.167	0.143	…	…	
	313	0.308	0.833	0.857	0.273	0.571	
	315	0.077	…	…	0.727	0.429	**0.402**
SS11	171	0.769	0.667	1.000	…	…	
	172	0.231	0.333	…	0.909	1.000	
	190	…	…	…	0.091	…	**0.192**
SS12	98	0.077	…	…	…	…	
	111	…	…	0.143	…	0.714	
	112	0.846	1.000	0.286	1.000	0.143	
	115	0.077	…	0.143	…	0.143	**0.261**
SS13	156	0.615	0.833	1.000	1.000	1.000	
	172	0.385	0.167	…	…	…	**0.150**
SS14	159	0.462	0.167	0.286	0.818	0.857	
	160	0.462	0.833	0.714	0.182	0.143	
	170	0.077	…	…	…	…	**0.359**
SS15	62	0.539	0.833	…	…	…	
	68	…	0.167	0.143	0.182	0.571	
	70	0.462	…	0.857	0.818	0.429	**0.361**
SS16	98	…	…	0.143	…	…	
	100	1.000	1.000	0.714	…	…	
	101	…	…	0.143	…	…	
	107	…	…	…	1.000	1.000	**0.089**
No. of isolates		***13***	***6***	***7***	***11***	***7***	
No. of haplotypes		***12***	***5***	***7***	***9***	***6***	
No. alleles		***25***	***20***	***23***	***21***	***20***	
No. unique alleles		***2***	***0***	***3***	***1***	***0***	
polymorphic loci		***9***	***7***	***8***	***7***	***6***	
H		***0.274***	***0.180***	***0.195***	***0.174***	***0.198***	

Observed allelic diversity (*H*) (Nei 1973) for each tree is given in the last line and for each locus in bold in the final column.

Figure 1. Relatedness of isolates evaluated using UPGMA phylogram trees. The sign T1a stands for Tree "1" isolate "a" or one and the same applies for others.



3

Population genetic analysis of South African *Diplodia pinea* populations reveals diverse sources of infection and cryptic recombination

Abstract

Understanding the population diversity and dynamics of plant pathogens such as *D. pinea* is an essential component of tree health management. While there have been a number of studies on the population structure of *D. pinea* globally, there has not been a detailed study of the South African populations aimed at elucidating basic biological questions such as reproduction and infection. This study considers the population diversity and structure of *D. pinea* in South Africa at different spatial scales from single trees to plantations, as well as comparing infections on healthy and diseased trees. A total of 236 isolates were characterized using thirteen microsatellite markers. Analysis of these markers confirmed previous results that *D. pinea* has a high level of genetic and genotypic diversity in South Africa, with the latter values ranging from 6 % to 68 % for the different plantations. The data also reflect a fungus with randomly associated alleles in populations at local plantation scales and for the population as a whole. These results suggest that recombination is occurring in *D. pinea* and that it most likely has cryptic sexual state. The study also reveals the sources of endophytic infection and stresss related disease out-breaks as diverse infections tha have occurred over a long time period. In contrast, wound-associated die-back appears to be caused by clones of the pathogen occurring in narrow time frames.

Introduction

A number of pine species were introduced into South Africa from Europe in the early 1700's and to the area known as the Western Cape Province (Lundquist 1987; Burgess and Wingfield 2001). Since that time, pines have been extensively planted in the KwaZulu-Natal, Mpumalanga and Northern Province, as well as the Eastern and Western Cape Provinces. Concomitant with the expansion of pine plantations from south to north along the east coast, there have been reports of disease caused by the pathogen *Diplodia pinea* (Desm.) Kickx *(=Sphaeropsis sapinea*). Thus, Diplodia shoot blight and die-back, the disease attributable to this fungus, was first reported in 1909 from the Eastern Cape, then in the 1927 in the Northern Province (Lundquist 1987) and subsequently throughout the country (Swart and Wingfield 1991).

Diplodia pinea is a haploid, opportunistic plant pathogen of mostly *Pinus* spp., but also occasionally found on other coniferous trees. It has been reported as causing significant losses to pine stands in many parts of the world (Burgess and Wingfield 2001, 2002). However, it owes its notoriety to the extensive damage that it causes after hail damage in South Africa (Swart et al. 1985; Swart and Wingfield 1991).

Diplodia species are latent pathogens, causing disease when trees are subjected to environmental or other stress conditions (Swart and Wingfield 1991; Smith et al. 1996; Stanosz et al. 1997). The predisposing factors favouring infection by *D. pinea* include wounding through hail and pruning, water stress and other environmental extremes (Swart and Wingfield 1991; Smith et al. 2002; Blodgett and Bonello 2003). In South Africa, substantial loss of timber has resulted from disease caused by *D. pinea* following hail damage. *Pinus radiata* is particularly susceptible and has been abandoned as a plantation species in areas receiving summer rainfall where hail is common (Lundquist 1987; Zwolinski et al. 1990; Swart and Wingfield 1991).

Previously, the population diversity of South African *D. pinea* (morphotype A) isolates from seed cones has been studied using Vegetative Compatibility Groups (VCG's) (Smith et al. 2000). Subsequently, SSR markers and VCGs were used to characterize the diversity of 26 and 54 isolates of this species from South Africa, respectively (Burgess et al. 2001; Burgess et al. 2004). Because *D. Pinea* is an introduced athogen in South Africa, it was surprising that these studies showed a higher genetic diversity in *D. pinea* populations in South Africa than those from elsewhere in the world. However, the sample sizes used in the SSR analyses were insufficient to establish whether there was any structure to the South African population. While VCGs were useful in these studies to understand genetic diversity, it was not possible to consider gene flow and relative relationships between populations (Glass et al. 2000). Most recently, Bihon et al (2010a) used 13 microsatellite markers to show that a high level of diversity was also evident in individual, healthy, mature trees.

In populations of an organism where a sexual state has not been found, it is difficult to explain the presence of high genotypic diversity (Kohli and Kohn 1998). Populations that reproduce only asexually are expected to exhibit a higher degree of clonality, with few genotypes at higher frequencies (Chen and McDonald 1996; Kohli and Kohn 1998). The explanation for the observed high levels of genotypic diversity, despite expected exclusive asexual reproduction of *D. pinea* in South Africa, was that there had been multiple introductions from a wide variety of sources, consistent with forestry practice in the country (Smith et al. 2000; Burgess et al 2004). The possibility that the genotypic diversity could also result from cryptic sexual reproduction has, however, not been tested. In this regard, genetic recombination can be estimated directly by showing the presence of sexual structures or indirectly by measuring non-random associations among loci and using genealogical approaches (Moore et al. 2009).

The aim of this study was to address three key questions regarding the genotypic diversity of *D. pinea* in South Africa. Firstly, to determine how the diversity of the fungus is distributed spatially, within a single plantation and between geographically separated plantations. A second aim was to consider where the fungus reproduces exclusively asexually or whether and undetected, cryptic sexual cycle might also occur. Finally, we considered whether there is a distinction between the population diversity of isolates associated with disease outbreaks and those collected as endophytes from healthy pine tissue.

Materials and Methods

Sample collection and isolation

Sampling of asymptomatic and symptomatic (dieback) *P. patula* trees was conducted in the two major pine-growing provinces or regions of South Africa, namely KwaZulu-Natal and Mpumalanga (Fig. 1). Samples were collected from branches on 3 to 5 year-old trees as well as branches, stems and cones on mature trees older than 12 years of age. Samples from Balgown were all from asymptomatic trees and those from the Boston plantation were from die-back after water stress. One segment of a plantation at Seven Oaks (KwaZulu-Natal province) had suffered hail damage before sampling and die-back symptoms were obvious on most of the trees. Samples at this site were thus collected from die-back symptoms after hail damage. Others from the Mpumalanga province were collected from a mixture of die-back and asymptomatic trees. The samples were maintained at 4 ^{0}C in the laboratory and isolations were made within three days after collection.

Isolations were made from stems and branches as described previously by Bihon et al. (2010a). Cultures were incubated under continuous light at 25 ^{o}C. After 4 to 6 days of

incubation, cultures with a white and fluffy mycelium typical of *D. pinea* were sub-cultured onto 2 % Water Agar (2 % m/v Biolab agar) with two autoclaved pine needles on the agar surface to stimulate the production of pycnidia. After two to three weeks, single spore isolates or isolates from hyphal tips were made and each of the cultures is maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

DNA Extraction, PCR amplification and separation of SSR loci

Cultures were grown on MEA in Petri dishes for two weeks and mycelium scraped from the surface of the plates for DNA extraction as described by de Wet et al. (2003). Thirteen SSR loci were amplified for all isolates as described previously (Bihon et al. 2010a & b). Thirteen fluorescently labelled SSR-PCR products were multiplexed and one micro-litre of these multiplexed PCR products was separated on ABI Prism 3100 Genetic analyzer. The amplicon peaks were determined based on the four fluorescent dyes used and the sizes of the DNA fragments. The mobilities of SSR products were compared to those of internal size standards (LIZ-500) and allele sizes were estimated by GeneScan 2.1 and GeneMapper 3.7 computer software (Applied Biosytems).

Gene and genotypic diversity

For each of the loci, individual alleles were assigned a different letter. For each isolate, a data matrix of 13 multistate characters (one for each locus) was compiled (eg. AABDCGDDABDDE). The frequency of each allele at each locus for the entire and clone-corrected populations was calculated, and allele diversity determined using the program POPGENE (Yeh *et al*. 1999) and the equation $H = 1 - \sum x^2_K$, where x_k is the frequency of the

k^{th} allele (Nei 1973). Chi-square test was conducted to evaluate the differences in the frequencies of alleles at each locus after removing the duplicated multilocus haplotypes (clone correction). Genotypic diversity (G) was estimated using the equation $G = {}^{1}\!\Big/\!\sum pi^{2}$ where *Pi* is the observed frequency of the i^{th} phenotype (Stoddart and Taylor 1988).

Population differentiation and clustering

Clone corrected populations were analyzed using the program Multilocus (Agapow and Burt 2000), with an estimate of Wright's, F_{ST} as Θ = Q-q/1-q to calculate population differentiation theta (Θ) where Q is the probability that two alleles from the same population are the same and q is the probability that two alleles from different population are the same (Weir 1997). When Θ = 0, allele frequencies between populations are equal and thus these populations are interpreted as being identical. However, when Θ = 1, frequencies of alleles are unique to a population and the populations are isolated from each other sharing no common alleles. The level of difference between the observed value and 1000 times randomized data sets were determined by the probability value at $P < 0.05$. The null-hypothesis that there is no population differentiation was rejected when significant difference between populations at $P < 0.05$ was observed. Population differentiation or genetic distance and approximate geographic distances were compared between populations in order to consider whether there was a correlation between genetic diversity and physical distance.

Population structure was inferred and assigned in STRUCTURE 2.2 that clusters individuals into *K* distinct populations (clusters) and permits mixed ancestry (Pritchard et al. 2000). The program was run for 100,000 replicates of Monte Carlo Markov Chain (MCMC)

analysis after an initial burn-in of 20,000 for *K* ranging from 1 to 15 at 20 iterations. A Cluster identity of >75 % was used to assign clusters.

Index of Association

Association of alleles for a clone-corrected *D. pinea* population was inferred by calculating the Index of Association (I_A) and rBarD (r_d) using the program Multilocus (Agapow & Burt 2000). The observed values of I_A and r_d were compared to 1000 times randomized data sets at a $P < 0.05$ level of significance. When the observed I_A and r_d value was significantly different from the randomized data sets at ($P < 0.05$), the null hypothesis that the alleles are randomly associated and the population is expected to freely undergo recombination, was rejected. Such a result indicates linkage between the loci, which is unlikely in this case, or a clonally reproducing population. In contrast, if the observed I_A and r_d fell within the distribution range, the null hypothesis of random association of alleles would be supported.

Analysis of molecular variance (AMOVA)

AMOVA, using 9999 permutations, was conducted to differentiate the sources of variation between and within populations, as well as between regions or provinces, using the software GeneAlEx version 6.2 (Peakall and Smose 2006). The null hypothesis was that variation within populations, between populations and among regions is equally responsible for the total genetic diversity in South African *D. pinea* strains.

Results

Sample collection and isolation

A total of 236 *D. pinea* isolates were obtained from seven locations (Fig. 1). Fifty-nine isolates were collected from Seven Oaks pine plantation (KwaZulu-Natal province) where most trees had been damaged by a hail storm and die-back was evident. In addition, isolates were collected from die-back on trees after drought stress at Boston (56) and from asymptomatic trees at Balgown (26) and Vryheid (13). In Mpumalanga province, isolates were obtained from samples collected in Sabie (47), Ngodwana (28), and Lothair (9), representing a mixture of die-back symptoms and asymptomatic tissues.

Gene and genotypic diversity

The 13 SSR markers produced a total of 43 alleles among 236 individuals with a minimum of 18 alleles (Ngodwana) and a maximum of 29 alleles (Boston). Isolates from Balgown had 28 alleles and those from Sabie 25 alleles. Most of the loci were polymorphic within plantations (Fig. 2). The percentage of polymorphic loci ranged from 30.8 % in Ngodwana to 92.3 % in the Boston collections. There were three unique alleles in Balgown, three in Seven-Oaks, two in Lothair-Vryheid and one each in Boston, Sabie and Ngodwana (Fig. 2). Only 9 of the 43 alleles were shared between all the plantations (Table 1). The thirteen loci each contained two to five alleles when considering all the isolates. Two alleles from each locus were dominant within populations, while the rest were rarely found.

There was high genotype diversity in most of the South African *D. pinea* populations sampled and genotype diversity differed significantly between these populations (Table 1). The percentage diversity ranged from 6 % at Seven-Oaks to 68 % at Balgown. The lowest genotype

diversity was obtained for the population acquired from hail-damaged and diseased trees in the Seven Oaks plantation, while the highest genotype diversity was obtained for a population from asymptomatic trees at Balgown. Isolates from Boston that had been collected from die-back symptoms following drought stress showed 43.7 % diversity. In total, all the isolates were represented by 122 haplotypes, of which 36.7 % were detected only once (Table 1). From the clone corrected populations, Chi-square values indicated that there were highly significant differences ($P < 0.001$) for gene diversity at most SSR loci between populations, with the exception of locus SS8 which was not significantly different ($P < 0.05$) (Table 2). The Lothair and Vryheid populations were subsequently considered collectively because the gene and genotype diversities were not significantly different at any of the SSR loci and the population differentiation (Θ) was not significantly different between them.

Population differentiation

Population differentiation (Θ) values differed significantly ($P < 0.05$) between the six South African populations of *D. pinea* (Table 3). Moreover, there was no correlation between the genetic and geographic distances with $r^2 = 0.07$ (Fig. 3). High levels of genetic distance were found even between the most closely situated populations (e.g. Sabie and Ngodwana, $\Theta = 0.631$), but there were also data points with average genetic distances at greater physical distances (e.g. Sabie and Boston, $\Theta = 0.212$) (Table 3).

All isolates of *D. pinea* from South Africa resided in six clusters when analyzed using the program STRUCTURE 2.2 and *LnK* had a maximum of K = 6 (Fig. 4a & b). This implied that entire population represents six sub-populations. These six groups also reflect the geographic origins of most of the isolates. However, there were some isolates residing in a population that

did not match their geographic origin. For example, the Boston population of isolates was separated into two clusters, one of which included isolates from the Lothair-Vryheid population.

Index of Association

In the assessment of random mating for the entire South African *D. pinea* population, the Index of Association (I_A) and rbarD ($\bar{r}D$) values were 0.373 and 0.031, respectively, and they all fell within the values obtained from 1000 randomized datasets (P = 0.973 for all isolates combined) (Fig. 5). The I_A and $\bar{r}D$ for each of the six population were also not significantly different from those of the randomized datasets (Table 4), suggesting that the null hypothesis there is recombination of genotypes is supported.

Molecular variance

Genetic variation for *D. pinea* isolates was partitioned within and among populations and provinces using analysis of molecular variance (AMOVA). The result indicated that 55 % of the variation was due to that among populations and 45 % due to variation within populations. There was no contribution to total genetic variation resulting from variation among regions (Table 5).

Discussion

The results of this study revealed high levels of genetic and genotypic diversity among six spatially separated populations of *D. pinea* in South Africa with little gene flow between these populations. The results also suggest that the high level of genotypic diversity is, at least in part, due to the presence of cryptic sex or other forms of recombination amongst alleles, and possibly also new alleles arising through mutation. Interestingly, levels of diversity were significantly

lower in a population of *D. pinea* associated with disease after hail damage, compared to a population derived from endophytic infections on healthy trees.

The total number of alleles, unique alleles and percent polymorphic loci reflect a high level of gene diversity for *D. pinea* in South Africa. Nei's (1973) mean gene diversity (*H*) for all of populations was also high, ranging from 0.194 to 0.443. Similarly, there was high genotypic diversity in most of the populations and overall in the South Africa *D. pinea* population, ranging from 6 % to 68 %. This is surprising for an introduced pathogen that is believed to reproduce exclusively asexually (McDonald and McDermott 1993; Milgroom 1996; Taylor et al. 1999). These high levels of gene and genotypic diversity in *D. pinea* populations are consistent with those found previously (Smith et al. 2000; Burgess et al. 2001; Burgess et al. 2004). For example, Burgess et al. (2001) reported 71 % genotypic diversity in South African populations. Similar studies using VCG and SSR markers also suggested very high levels of diversity for *D. pinea* in South Africa (Smith et al. 2000; Burgess et al. 2004). These results raise the question as to how such high diversity could have arisen in the non-mating populations of *D. pinea*.

Mutation could have played a role in increasing genetic diversity in *D. pinea* populations in South Africa over the past century. However, this source of variation would be expected to be small in terms of the total diversity observed and relatively short period of time that the fungus has been present in South Africa (McDonald and McDermott 1993). Potential evidence for mutation is derived from the private or unique alleles found in the geographically defined populations of *D. pinea*. These alleles might also represent remnants of diversity introduced since the establishment of plantations into these regions. Genetic drift and selection would have been expected to eliminate many such unique alleles from the populations over time (Halliburton

2004), unless they confer a fitness benefit to the fungus (FitzSimmons et al. 1997; Milgroom and Cortesi 1999; Burgess et al. 2004).

In all the local populations considered in this study, as well as the South African population as a whole, alleles at the different loci were randomly associated. This result is considered evidence of recombination as has been shown in other fungi (Geiser et al. 1994; Arie et al. 2000; Groenewald et al. 2008; Pérez et al. 2010). No sexual structures have, however, been observed in *D. pinea*, despite the fact that it has been studied intensively for more than a century (Swart and Wingfield 1991). Hence, *D. pinea* either has a rare, cryptic sexual state or it has an alternative form of asexual recombination, such as via parasexuality (Milgroom 1996; Taylor et al. 1999). These would be reasonable alternative explanations for the high levels of genotypic diversity observed in South Africa, and elsewhere, in this apparently asexual (Burgess et al. 2004) fungus.

A higher level of genetic variation was observed in endophytic populations of *D. pinea,* compared to populations obtained from die-back symptoms after hail damage. Isolates with the highest level of genotype diversity (68.4 %) were from asymptomatic *P. patula* trees at Balgown, while the lowest levels of diversity were observed for isolates obtained from die-back trees after a hail storm (e.g. in Seven Oaks at 5.8 %). These results suggest endophytic communities of *D. pinea* result from numerous individual infections over the life-time of trees. Furthermore, this diverse endophytic community is involved in causing disease when plants are under stress in the absence of wounding. However, when wide-scale concurrent wounding occurs such as during hail storms, then infections leading to disease would arise from a much more uniform population. This would, most likely be via asexual conidia that happen to be produced and spread at the same time. Consistent with our results, Dakin et al. (2010) reported that all isolates of *Neofusicoccum*

australe from asymptomatic *Agonis flexuosa* were in different vegetative compatibility groups (VCGs), while 26 isolates from a cankered tree included eleven VCG's. This would suggest that wounding is involved in the infection process because it is provides an easy entry for fungi that are unable to infect intact tissues (Flowers et al. 2006). An alternative explanation for the lower diversity of the fungus after hail damage or in cankers could be competition and selection among the endophytic strains. Strains that are relatively active at the time when trees are stressed (Smith et al 2002) would grow faster and take over large portions of the tree than strains that are in an opposite manner.

Genetic diversity was significantly differentiated between all populations. STRUCTURE analyses indicated that, isolates resided in six distinct populations, which were mostly consistent with the geographic origins of the isolates. Furthermore, AMOVA analysis showed that 55 % of the variation was distributed amongst populations. These data suggest that little gene flow occurs between populations in plantations, even those that are geographically relatively close to each other (approximately 65 km). This low rate of gene flow between populations might be explained by the fact that the conidia of *D. pinea* are spread mainly by rain splash or via wind (Swart et al. 1987; Swart and Wingfield 1991) and hence have limited potential for long distance dispersal. This main mode of spread was inferred from the fact that in months of the year where there was no rainfall, virtually no conidia were collected inside plantations (Swart et al. 1987). There was, however, some evidence of movement of genotypes between populations, such as those from Boston and Lothair and Vryheid. These exceptions most likely reflect occasional long distance dispersal due to human intervention by moving infected wood, cones or live plants.

This study has shown that new genotypes of *D. pinea* are generated via cryptic recombination that results in higher diversity in South African populations. Furthermore, the

study has provided new insights into the role of endophytic infections and wounds in disease outbreaks. The exact mechanism giving rise to recombination in *D. pinea* will require intensive further study. We suggest that there is a need to support the evidence of natural recombination by characterizing genes associated with mating and presence of sexual structure in nature.

References

Agapow PM, Burt A (2000) 'Multilocus 1.2' (Department of Biology, Imperial College: Ascot, UK).

Arie T, Kaneko I, Yoshida T, Noguchi M, Nomura Y, Yamaguchi I (2000) Mating-type genes from asexual phytopathogenic ascomycets *Fusarium oxysprum* and *Alternaria alternata*. Molecular Plant-Microbe Interactions **13**: 1330 – 1339.

Bihon W, Burgess T, Slippers B, Wingfield MJ, Wingfield BD (2010a) Distribution of *Diplodia pinea* and its genotypic diversity within asymptomatic *Pinus patula* trees. Australasian Plant Pathology (*in press*).

Bihon W, Slippers B, Burgess T, Wingfield MJ, Wingfield BD (2010b) Sources of *Diplodia pinea* endophytic infections in *Pinus patula* and *P. radiata* seedlings in South Africa. Forest Pathology (*in press*).

Blodgett JT, Bonello P (2003) The aggressiveness of *Sphaeropsis sapinea* on Austrian pine varies with isolate group and site of infection. Forest Pathology **33**: 15 – 19.

Burgess T, Wingfield MJ (2001) Exotic pine forestry in the Southern Hemisphere: A brief history of establishment and quarantine practices. South African Forestry Journal **192**: 79 – 84.

Burgess T, Wingfield BD, Wingfield MJ (2001) Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata.* Mycological Research **105**: 1331 – 1339.

Burgess T, Wingfield MJ (2002) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the southern hemisphere. International Forestry Review **4**: 56 – 65.

Burgess TI, Wingfield MJ, Wingfield BD (2004) Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australasian Plant Pathology **33**: 513 – 519.

Chen RS, McDonald BA (1996) Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. Genetics **142:** 1119 – 1127**.**

Dakin N, White D, Hardy GE, Burgess TI (2010) The opportunistic pathogen, *Neofusicoccum australe*, is responsible for crown dieback of peppermint (*Agonis flexuosa*) in Western Australia. Australasian Plant Pathology **39**: 202 – 206.

De Wet J, Burgess T, Slippers B, Preisig O, Wingfield BD, Wingfield MJ (2003) Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. Mycological Research **107**: 557 – 566.

FitzSimmons NN, Moritz C, Limpus CJ, Pope L, Prince R (1997) Geographic structure of mitochondrial and nuclear gene polymorphisms in Australian green turtle populations and male-biased gene flow. Genetics **147**: 1843 – 1854.

Flowers JL, Hartman JR, Vaillancour LJ (2006) Histology of *Diplodia pinea* in diseased and latently infected *Pinus nigra* shoots. Forest Pathology **36**: 447 – 459.

Geiser DM, Arnold ML, Timberlake WE (1994) Sexual origins of British *Aspergillus nidulans* isolates. Proceedings of the National Academy of Sciences, USA **91**: 2349 – 2352.

Glass NL, Jacobson DJ, Shiu PKT (2000) The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. Annual Review of Genetics **34**: 165 – 168.

Groenewald M, Linde CC, Groenewald JZ and Crous PW (2008) Indirect evidence for sexual reproduction in *Cercospora beticola* populations from sugar beet. Plant Pathology **57**: 25 – 32.

Halliburton R (2004) Introduction to population genetics. Pearson Education, Inc., USA.

Kohli Y, Kohn LM (1998) Random association among alleles in clonal populations of *Sclerotinia sclerotiorum*. Fungal Genetics and Biology **23**: 139 – 149.

Lundquist JE (1987) A history of five forest diseases in South Africa. South African Forestry Journal **140**: 51 – 59.

McDonald BA, McDermott JM (1993) Population Genetics of Plant Pathogenic Fungi: Electrophoretic markers give unprecedented precision to analyses of genetic structure of populations. BioScience **43**: 311 – 319.

Milgroom MG (1996) Recombination and multilocus structure of fungal populations. Annual Review of Phytopathology **34**: 457 – 477.

Milgroom MG, Cortesi P (1999) Analysis of population structure of the chestnut blight fungus based on vegetative incompatibility genotypes. Proceeding of the National Academy of Sciences, USA **96**: 10518 – 10523.

Moore GG, Singh R, Horn BW, Carbone I (2009) Recombination and lineage-specific gene loss in the aflatoxin gene cluser of *Aspergillus flavus*. Molecular Ecology **18**: 4870 – 4887.

Nei M (1973) Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences USA **70**: 3321 – 3323.

Peakall R, Smouse PE (2006) GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes **6**: 288 – 295.

Pérez G, Slippers B, Wingfield BD, Huntera GC, Wingfield MJ (2010) Micro- and macrospatial scale analyses illustrates mixed mating strategies and extensive gene flow in populations of an invasive haploid pathogen. Molecular Ecology **19**: 1801 – 1813.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of Population Structure Using Multilocus Genotype Data. Genetics **155**: 945 – 959.

Smith H, Wingfield MJ, Cours PW, Coutinho TA (1996) *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in Pinus Spp. and Eucalyptus spp. in South Africa. South African Journal of Botany **62**: 86 – 88.

Smith H, Wingfield MJ, de Wet J, Coutinho TA (2000) Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. Plant Disease **84**, 139 – 142.

Smith H, Wingfield MJ, Coutinho TA (2002) The role of latent *Sphaeropsis sapinea* infections in post-hail associated die-back of *Pinus patula*. Forest Ecology and management **164**: 177 – 184.

Stanosz GR, Smith DR, Guthmiller MA, Stanosz JC (1997) Persistence of *Sphaeropsis sapinea* on or in asymptomatic red and jack pines. Mycologia **89**: 525 – 530.

Stoddart JA, Taylor JF (1988) Genotype diversity: Estimation and prediction in samples. Genetics **118**: 705 – 711.

Swart WJ, KInox-Davis PS, Wingfield MJ (1985) *Sphaeiopsis sapinea,* with special reference to its occurrence on *Pinus* spp. in South Africa. South Africa Forestry Journal **35**: 1 – 8.

Swart WJ, Wingfield MJ, Knox-Davies PS (1987) Conidial dispersal of *Sphaeropsis sapinea* in three climatic regions of South Africa. Plant Disease **71**: 1038 – 1040.

Swart WJ, Wingfield MJ (1991) Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. Plant Disease **75**: 761 – 766.

Taylor JW, Jacobson DJ, Fisher MC (1999) The evolution of asexual fungi: Reproduction, speciation and classification. Annual Review of Phytopathology **37**: 197 – 246.

Weir BS (1997) Genetic data analysis II. Sinauer Associates Inc.: Sunderland, MA.

Yeh FC, Yang RC, Boyle T (1999) POPEGENE version 1.31 (Microsoft windows based freeware for population genetic analysis, Alberta).

Zwolinski JB, Swart WJ, Wingfield MJ (1990) Economic impact of a post-hail outbreak of dieback induced by *Sphaeropsis sapinea*. European Journal of Forest Pathology **20**: 405 – 411.

Figure 1. Locations where samples were collected

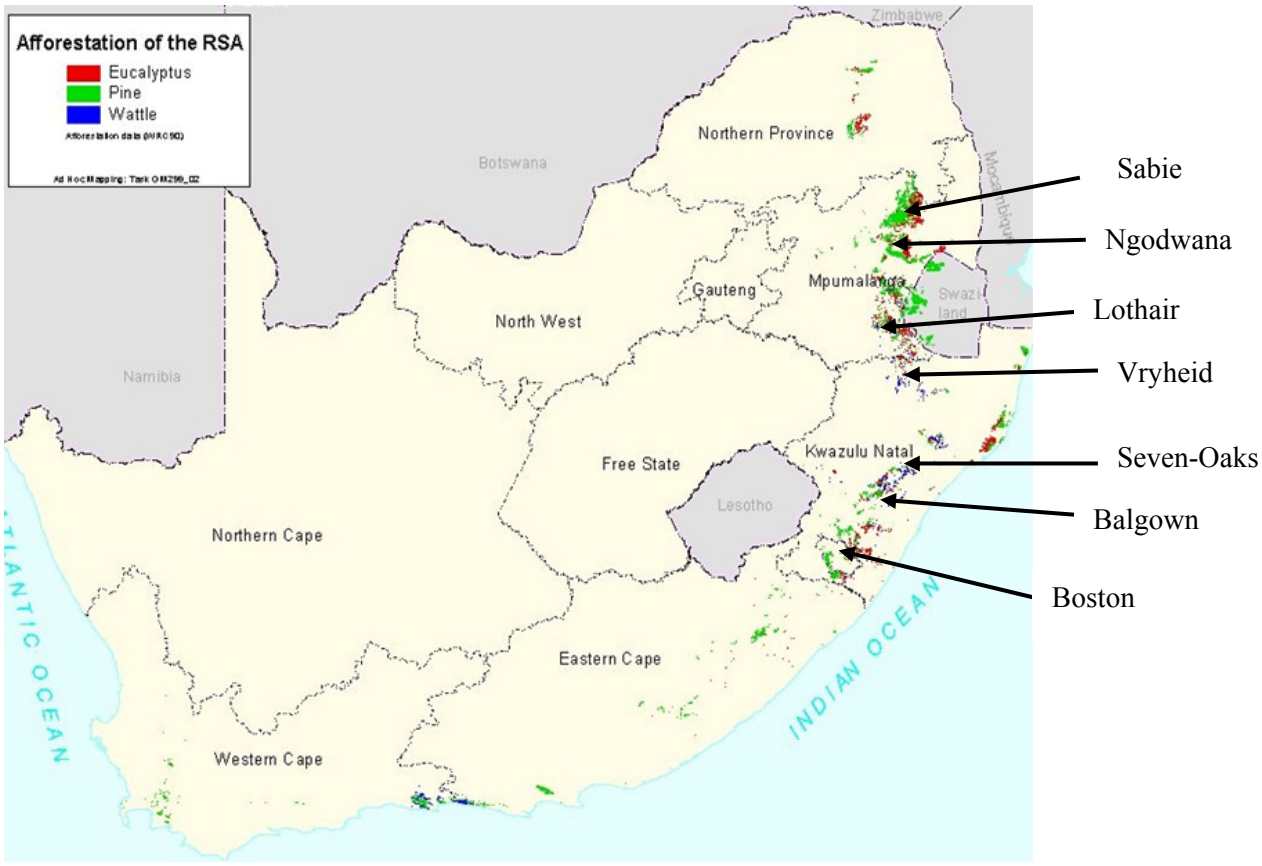


Figure 2. Differences in genetic structure including number of alleles, polymorphic loci and unique alleles for isolates from five plantations. Lot-Vry represents the population Lothair and Vryheid.

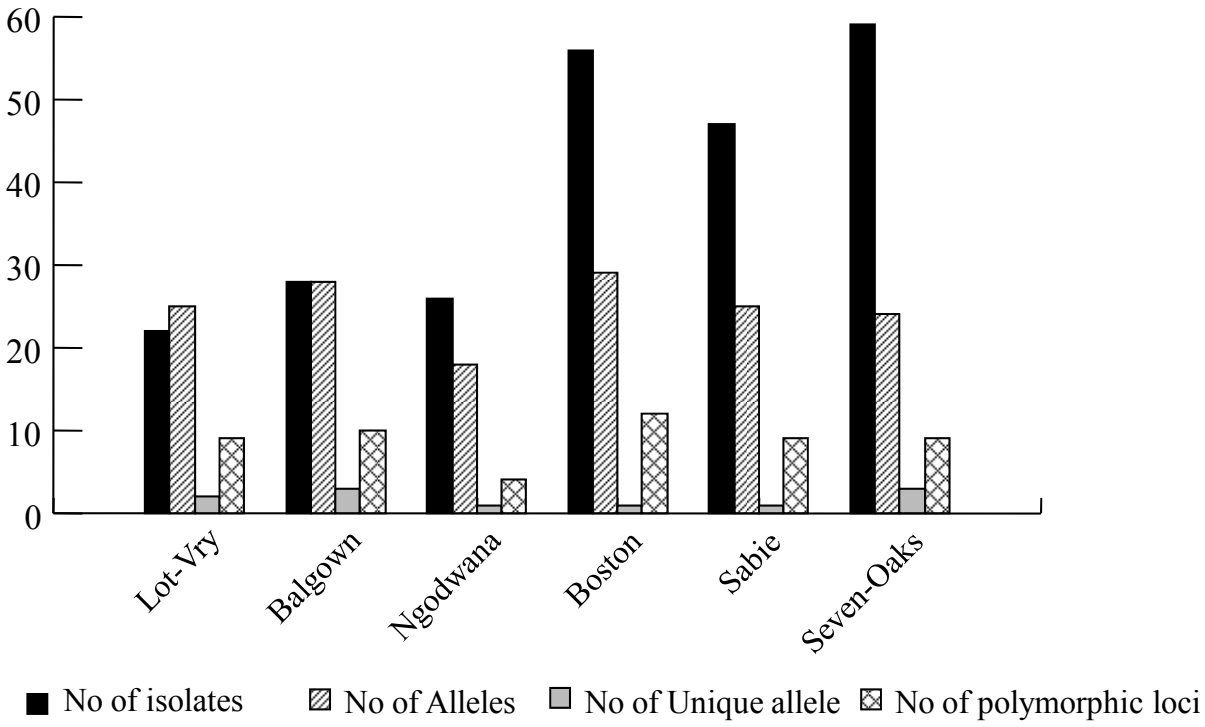


Figure 3. Correlation between genetic (θ) and geographic distances

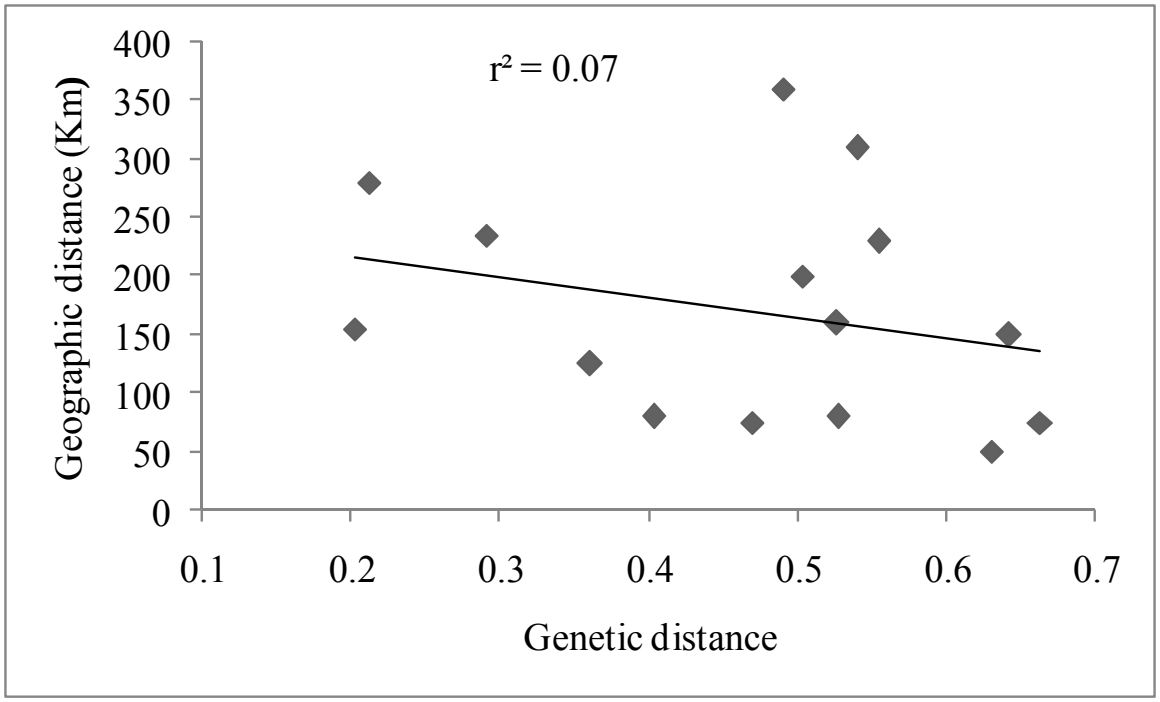
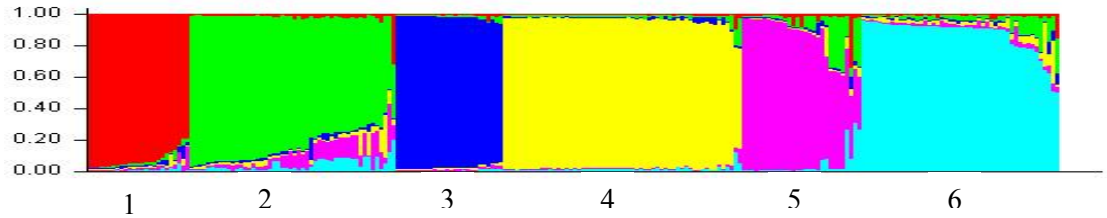


Figure 4. Affiliation of individual genotypes of *D. pinea* as assessed using Structure 2.2 and separated into six discrete vertical bars that are organized by sampling groups (a) and different K vs LnK values (b). Differences in colour within a vertical bar (a) indicate a multi-population affiliation of an individual genotype. The higher K vs LnK values represent the most likely number of clusters of individuals (b).



Different colours indicated different populations: 1 = Balgown, 2 = Boston, Lot-Vry, 3 = Ngodwana, 4 = 7-Oaks, 5 = Boston and 6 = Sabie

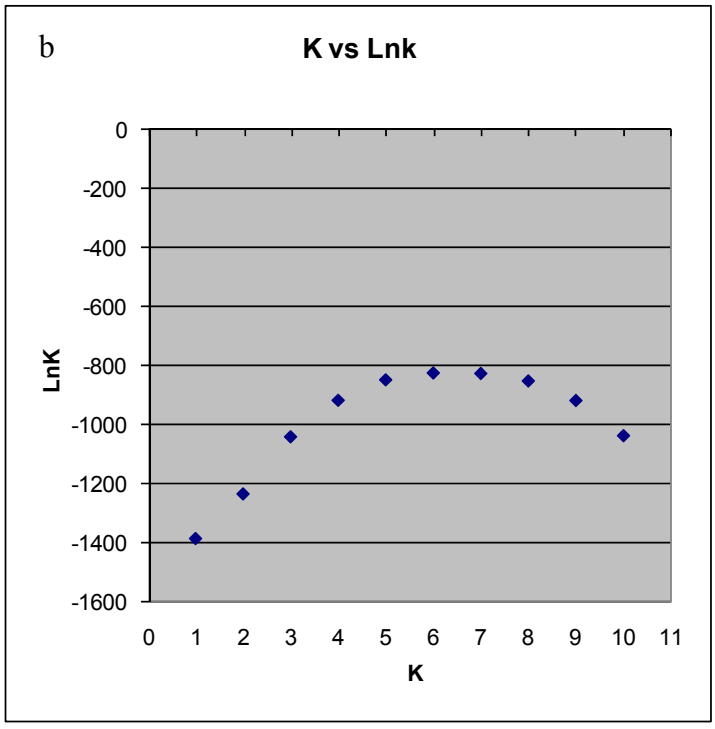


Figure 5. Index of Association (I_A) of clone corrected populations of *D. pinea* in South Africa. Arrows indicated where the observed I_A found from the 1000 times randomized data sets. P indicated the probability level we accept the differences. When $P < 0.05$, the H_o was rejected.

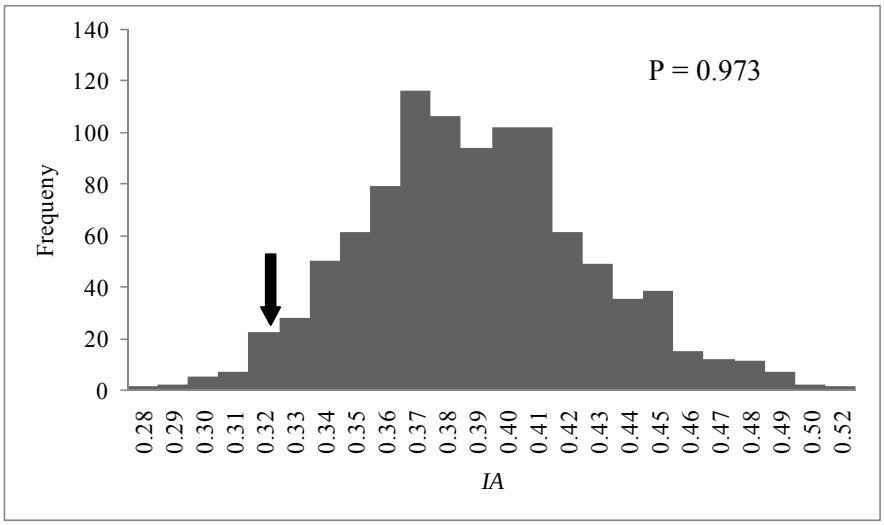


Table 1. Allele frequencies and genotype diversity by population

Locus	*Allele*	*Balgown*	*Boston*	*7-Oaks*	*Sabie*	*Ngodwana*	*Lot-Vry*
SS1	A	..	..	..	0.191	..	..
	B	0.577	0.036	..	..	..	..
	C	0.423	0.964	1.000	0.809	1.000	0.727
	D	..	..	..	..	..	0.273
SS2	A	0.962	0.839	0.017	0.979	..	0.727
	B	0.038	0.161	0.983	0.021	1.000	0.227
	C	..	..	..	..	..	0.045
SS5	A	0.231	0.268	..	..	..	0.091
	B	..	0.268	..	0.021	..	0.045
	C	0.769	0.250	1.000	0.723	..	0.773
	D	..	0.214	..	0.255	1.000	0.091
SS7	A	..	..	0.034	..	..	..
	B	..	0.232	..	0.106	..	0.045
	C	1.000	0.768	0.966	0.894	1.000	0.955
SS8	A	1.000	0.821	0.966	1.000	1.000	1.000
	B	..	0.179	0.034	..	..	..
SS9	A	1.000	0.857	..	0.128	1.000	0.864
	B	..	0.143	1.000	0.872	..	0.136
SS10	A	0.154	0.214	0.136	0.064	..	0.455
	B	0.769	0.643	0.864	0.489	0.692	0.545
	C	0.077	0.143	..	0.447	0.308	..
SS11	A	..	..	0.017	..	..	0.091
	B	0.923	0.018	0.203	..	1.000	..
	C	0.077	0.982	0.780	1.000	..	0.909
SS12	A	0.038	..	0.017	..	..	..
	B	..	..	0.017	..	..	..
	C	0.038	0.018	0.966	0.255	..	..
	D	0.846	0.982	..	0.723	0.846	1.000
	E	0.077	..	..	0.021	0.038	..
	F	..	..	..	..	0.115	..
SS13	A	0.808	1.000	0.797	1.000	0.192	1.000
	B	0.192	..	0.203	..	0.808	..
SS14	A	..	..	0.017	..	..	..
	B	0.385	0.964	0.983	0.936	1.000	0.955
	C	0.615	0.018	..	0.064	..	0.045
	D	..	0.018	..	..	..	..
SS15	A	0.231	..	..	..	..	..
	B	0.077	0.089	0.153	0.383	0.423	0.136
	C	0.692	0.911	0.847	0.617	0.577	0.864
SS16	A	0.038	..	..	..	..	..
	B	0.923	0.179	..	..	..	..
	C	0.038	..	..	..	..	..
	D	..	0.821	1.000	1.000	1.000	1.000
G		*17.789*	*24.500*	*3.436*	*24.275*	*6.145*	*11.52*
*G**		*68.421*	*43.750*	*5.824*	*51.648*	*23.636*	*52.380*
MLH		*21*	*34*	*20*	*34*	*10*	*15*

G = Genotype diversity (Stoddart & Taylor 1988) and G* is percentage maximum diversity = (G/N)*100, and *H* = Observed allelic diversity (Nei 1973) for each population, MLH = Multilocus Haplotypes.

Table 2. Gene diversities (*H*) and contingency chi-square tests for differences in allele frequencies for 13 SSR loci across clone corrected populations of *D. pinea*.

Locus	*Gene diversity (H)*						*Chi-Sq*	*df*
	Boston	Lot-Vry	Balgown	Sabie	Nogdwana	7-Oaks		
SS1	0.057	0.320	0.499	0.337	0.000	0.000	138.14***	15
SS2	0.291	0.320	0.091	0.057	0.000	0.095	87.107***	5
SS5	0.741	0.516	0.363	0.372	0.000	0.000	164.646***	15
SS7	0.360	0.124	0.000	0.208	0.000	0.180	132.66***	10
SS8	0.208	0.000	0.000	0.000	0.000	0.180	9.738NS	5
SS9	0.327	0.320	0.091	0.251	0.000	0.180	102.39***	15
SS10	0.535	0.480	0.390	0.576	0.500	0.420	37.476***	10
SS11	0.057	0.124	0.172	0.000	0.000	0.455	114.88***	15
SS12	0.057	0.000	0.331	0.455	0.340	0.095	106.12***	20
SS13	0.000	0.000	0.363	0.000	0.320	0.455	57.12***	5
SS14	0.113	0.124	0.472	0.161	0.000	0.095	61.37***	15
SS15	0.291	0.320	0.490	0.457	0.500	0.420	36.91***	10
SS16	0.251	0.000	0.177	0.000	0.000	0.000	110.11***	15
Mean	0.438	0.338	0.443	0.365	0.194	0.314		

*Significant difference at P < 0.05, ** Significant difference at P < 0.01, *** highly significant difference at P < 0.001, NS = non-significant difference at P < 0.05, *df* = degree of freedom = (Number of alleles – 1) * (Number of populations – 1).

Table 3. Pair-wise comparisons of six population differentiations (Θ) among *D. pinea* populations from different locations in South Africa

	Balgown	*Boston*	*7-Oaks*	*Sabie*	*Ngodwana*	*Lot-Vry*
Balgown	--	0.404***	0.527***	0.491***	0.541***	0.291***
Boston		--	0.528***	0.212***	0.555***	0.203***
7-Oaks			--	0.504***	0.643***	0.470***
Sabie				--	0.631***	0.361*
Ngodwana					--	0.664***

* indicates significant difference at P < 0.05 and *** indicated highly significant different at P < 0.01.

Table 4. Observed I_A and $\bar{r}_d$ values of each populations

Population	I_A	$\bar{r}_d$	*P*
7-Oaks	-0.111	-0.012	0.727
Boston	-0.012	-0.001	0.525
Balgown	0.00002	0.0000002	0.468
Sabie	-0.063	-0.008	0.684
Ngodwana	-0.259	-0.087	1.000
Lot-Vry	-0.213	-0.027	0.837

Table 5. Analysis of molecular variance of South African *D. pinea* populations hierarchically partitioned.

Source of Variation	*df*	*SS*	*MS*	*Est. Var.*	*% Variation*	*PhiPT*	*p-Value*
Among Regions	1	21.003	21.003	0.000	0%		
Among Pops	4	230.530	57.632	1.516	55%		
Within Pops	230	280.544	1.220	1.220	45%		
Total	235	532.076				0.481	0.01

df = degree of freedom, SS = Sum of square, MS = Mean square and Est. Var. = Estimated variance

4

Diplodia scrobiculata found in the southern hemisphere

Published as:
Bihon W, Slippers B, Burgess T, Wingfield MJ, Wingfield BD (2010) *Diplodia scrobiculata* found in the southern hemisphere. Forest Pathology, doi: 10.1111/j.1439-0329.2010.00649.x

Abstract

Diplodia scrobiculata, a latent pathogen of *Pinus* spp. and other conifers with a limited distribution in the United States, Mexico and southern Europe, has not previously been reported in the southern hemisphere. This is unlike its close relative *D. pinea* that is found in most parts of the world where pines are native or have been introduced. During an intensive *D. pinea* survey conducted in *Pinus patula* plantations in eastern parts of South Africa, a small number of isolates atypical of *D. pinea* were found. Morphological studies and DNA sequence comparisons showed that these isolates represent *D. scrobiculata*. Microsatellite analyses suggest that the South African isolates of *D. scrobiculata* might have originated from California. Pathogenicity tests showed that some of the *D. scrobiculata* isolates were as pathogenic as those of *D. pinea* on *Pinus radiata* and *Pinus elliottii*.

Introduction

Diplodia pinea (Desm.) Kickx. is one of the best known pathogens of *Pinus* and other conifers worldwide. It causes significant economic losses especially in association with biotic and abiotic stresses such as hail damage, pruning wounds, drought, insect damage and extreme temperatures (Swart and Wingfield 1991; Smith et al. 2002; Blodgett and Bonello 2003). Previously, four morphotypes of *D. pinea* (A, B, C and I) were recognised (Wang et al. 1985; Palmer et al. 1987; Smith and Stanosz 1995; Hausner et al. 1999; De Wet et al. 2000; Burgess et al. 2001b). Analyses using sequences for multiple nuclear loci and polymorphic microsatellite markers led the description of the B morphotype isolates as the discrete species, *D. scrobiculata* (De Wet et al. 2003). The I morphotype was found to represent *D. seriata* and the C morphotype isolates were phylogenetically identical to *D. pinea*, but represent a virulent form of the pathogen known only from Indonesia (De Wet et al. 2000; Burgess et al. 2001b).

Diplodia pinea has been detected in virtually every country of the world where pines are grown as non-natives (Burgess et al. 2001a, 2002, 2004a). Conversely, *D. scrobiculata* has a limited distribution and host range and it is known only from Mexico, California, north-central USA and southern Europe (Morelet and Chandelier 1993; Blodgett and Stanosz 1997; Burgess et al. 2004b; Lazzizera et al. 2008; Muñoz et al. 2008). In these areas, the two species are often found together on pine and other coniferous tree species (Palmer et al. 1987). *Pinus radiata*, the predominant plantation species in many regions in the southern hemisphere, is a host of *D. scrobiculata* in its native range (Burgess et al. 2004b), as is *P. patula* where it is native in Mexico (Burgess et al. 2004a). Thus, it is enigmatic that *D. scrobiculata* has not moved internationally in a similar manner to *D. pinea*.

During the course of a recent intensive sampling of *P. patula* in order to undertake population genetic studies of *D. pinea* in South Africa, a small number of isolates having a distinct morphology were detected. The aim of this study was to identify these isolates using morphological characteristics and DNA sequence analysis.

Materials and methods

Sampling and Isolation

Approximately 580 samples were taken from stems, branches (asymptomatic or with die-back symptoms) and cones of *P. patula* trees in plantations occurring in the KwaZulu-Natal and Mpumalanga provinces of South Africa. The samples from mature trees and 2 - 5 year old saplings were taken to the laboratory, stored at 4 ^{o}C and isolations were undertaken within two days after sampling.

Tissue samples were surface disinfected using a modification of the method of Smith et al. (2002) by dipping them into 70 % EtOH for 3-5 minutes followed by 3.5 % NaOCl and 70 % EtOH for 1 minute and 4 washes of 1 minute duration in sterile distilled water. Single conidia were removed from pycnidia produced on pine needles in agar and single spore isolates were produced as described by De Wet et al. (2003). Single conidial cultures were established on 2 % malt extract agar, MEA (2 % m/v biolab malt extract and 1.5 % m/v biolab Agar) and are maintained in the culture collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Morphological and cultural characteristics

Mycelial morphology and cultural characteristics resulted in the identification of 183 isolates with morphology similar to that of *Diplodia* spp. The majority of these resembled *D. pinea*, which was the target of the survey. Six of these isolates were, however, different from the others. In order to determine the identity of these six isolates, the characteristics of their mycelial growth, conidial size and morphology were examined in detail.

Six isolates identified as *D. pinea* were randomly selected and the six isolates representing the unknown fungus were grown on MEA for mycelium growth comparison. From each of the isolates, about 5 mm diameter plugs were aseptically transferred from the actively growing margins of cultures to the surface of 2 % MEA in 90 mm Petri dishes and incubated at 25 ^{0}C with three replicate plates for each isolate. Radial colony growth was measured from the centre to the edge of the plate every day starting three days after initiating the growth studies and until the mycelium reached the edges of the Petri dishes. The mean cumulative growth of each isolates at each time point was calculated and these were compared with each other. Since the growth rates of six *D. pinea* isolates were similar, their average was compared against those of the individual rates of the unidentified isolates.

Conidial sizes and morphology for the six isolates of the unknown fungus were examined after they had been induced to sporulate on 2 % water agar (Biolab) containing sterilized pine needles and incubated in 25 ^{0}C under conditions of continuous light. The lengths and widths of 10 conidia per isolate were measured and the averages computed. The surface morphology and presence or absence of septa in the conidia was assessed using a light microscope.

DNA extraction, PCR amplification and phylogenetic analysis

Cultures of the six unknown and six *D. pinea* isolates were grown on MEA in Petri dishes for two weeks and mycelium scraped from the surface of the agar for DNA extraction. The mycelium was ground in the presence of tungsten beads (3 mm) (Qiagen, Hilden, Germany) in warm CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) using FastPrep FP120 homogenizer (Southern Cross Biotechnology) at 5 m/s for 20 seconds. This maceration was repeated 4 times before DNA was isolated.

The internal transcribed spacer regions (ITS) of the rDNA operon were amplified using primers ITS1 (5′TCC GTA GGT GAA CCT GCG GG) and ITS4 (5′GCT GCG TTC TTC ATC GAT GC′) (White et al. 1990). In addition, part of the translation elongation factor (EF1-α) gene region was amplified using primers EF1-728 (5'CAT CGA GAA GTT CGA GAA GG) and EF1-986R (5'TAC TTG AAG GAA CCC TTA CC) (Carbone and Kohn 1999) with PCR conditions and reactions as described previously for *D. pinea* (De Wet et al. 2003). Sequencing was conducted with the same primers and reactions were run on an ABI PRISM™ 3100 Auto-sequencer (Applied BioSystems, Foster City, California, USA).

The sequences for the six unknown isolates and six *D. pinea* isolates were aligned with those of known isolates of *D. pinea* and *D. scrobiculata* available on GenBank (Table 1). Alignment was done using MAFFT multiple sequence alignment for amino acid and nucleic acid (http://tim-pani.genome.ad.jp/%7emafft/server/) (Katoh et al. 2002). Datasets for the aligned sequences of the ITS and EF1-α gene regions were subjected to partition homogeneity tests in PAUP version 4.0 (Swofford 2002) to determine whether the datasets could be combined. Characters were unweighted and unordered; gaps were treated as a fifth character (new state). The most parsimonious trees were obtained using heuristic searches with random stepwise

addition in 100 replicates, using the tree bisection-reconnection branch-swapping option, and with the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved.

Identification based on microsatellite loci

Five previously developed microsatellite loci (SS1, SS2, SS7, SS8 and SS9) (Burgess et al. 2001b) were amplified for six isolates of the unknown fungus. All SSR-PCR products for the isolates were multiplexed and separated on ABI Prism 3100 Genetic analyzer (Applied Biosystems). The mobility of SSR products were compared to those of internal size standards (LIZ-500) and allele sizes were estimated using GeneScan 2.1 and GeneMapper 3.7 computer software (Applied Biosytems). The allele sizes and frequencies were compared with those for *D. scrobiculata* populations from California, Mexico and north-central America (Burgess et al. 2004b).

Pathogenicity tests

Pathogenicity tests were conducted on 18-month-old *P. radiata* and *P. elliottii* plants using the six unknown and six *D. pinea* isolates. Pieces of bark, approximately 25 mm^2 were removed at about 20 cm from the apices of the plants using a sterile scalpel. A plug of MEA covered with actively growing mycelium of the test fungi was placed into the stem wounds and wrapped with Parafilm to protect the inoculation sites from desiccation. Three *P. radiata* and three *P. elliottii* seedlings were used for each isolate inoculated and an equal number of plants were inoculated with sterile MEA plugs to serve as controls.

Lesion lengths were measured two weeks after inoculation. The mean lesion lengths associated with the six *D. pinea* isolates and the six isolates of the unknown fungus were analysed using ANOVA in GenStat Discovery Edition 3 (Rothamsted Experimental Station, UK). Isolations were made from the lesions on MEA to determine whether the lesions had resulted from the inoculated fungi and identifications of the re-isolated fungi were made based on morphology.

Results

Sampling and isolation

The majority of the 183 isolates obtained during this study that resembled *Diplodia* spp. originated from the branch samples, and 14 isolates were from asymptomatic *P. patula* stems. Of the six isolates with a morphology differing from the others, four were from samples collected in a plantation in Sabie (Mpumalanga Province), and the remaining two were from the Balgown and Boston plantations (KwaZulu-Natal Province). Two of the six isolates having a distinct morphology were isolated from the internal parts of the stems of adult asymptomatic trees and the others were from branches of 3 to 4 year-old *P. patula* trees showing symptoms of die-back.

Morphological comparisons

The six isolates of the unknown fungus grew more slowly than those known to represent *D. pinea*. After 6 days, all colonies of the *D. pinea* isolates had abundant aerial mycelium and had covered the surface of the Petri dishes, while those of the unknown fungus took up to eight days to cover the surface of the plates and the mycelium was appressed to the surface of the medium (Fig 1.). The conidia of the unknown fungus were brownish in colour, with up to three septa and

pitted surfaces. The dimensions of these conidia ranged between 34.0 – (36.4) – 39.6 µm in length and 11.1 – (13.8) – 16.6 µm in width (averages in brackets).

Phylogenetic analyses

BLAST analysis showed that the sequences for the six unidentified isolates were similar to NCBI reference *D. scrobiculata* collections with a nucleotide sequence similarity of 98 % to 99 %. Moreover, the combined analysis of ITS and EF-1α DNA sequences indicated that these six isolates clustered together with sequences of *D. scrobiculata* (Fig. 2). The other isolates clustered with *D. pinea* (Fig. 2).

Microsatellite marker analyses

Of the five SSR loci, SS1 and SS2 were monomorphic and the remaining three were polymorphic for the six isolates of the unknown fungus. The polymorphic loci had two to three alleles per locus to give a total of 9 alleles across the 5 loci. Five of these alleles were identical to alleles previously found to be unique in Californian populations of *D. scrobiculata* at loci SS1, SS2, SS7 and SS8. One allele from locus SS8 was the same as that in populations of *D. scrobiculata* from California and Mexico. The two alleles at locus SS9 from the South African population have not been observed previously in any other population (Table 2).

Pathogenicity tests

All of the *D. pinea* and the six isolates identified as *D. scrobiculata* resulted in lesions that were significantly larger than those of the control treatment (Fig. 3). ANOVA showed that the lesions associated with the *D. pinea* isolates (41.51 ± 1.74 mm) were significantly longer ($P < 0.05$) than

the lesions associated with *D. scrobiculata* (31.54 ± 1.7 mm) regardless of the pine species inoculated. However, some of the *D. scrobiculata* isolates (e.g CMW30223) produced lesions that were equal in length to those associated with *D. pinea* on both pine species (Fig. 3a & b). *Diplodia scrobiculata* isolate CMW30227 produced the smallest lesions on both of the *Pinus* spp. On *P. radiata,* lesions caused by *D. scrobiculata* varied significantly between the different isolates, but there were no significant differences between the lesions caused by *D. pinea* isolates on this pine species. *Diplodia pinea* inoculated onto *P. radiata* resulted in longer lesions than those on *P. elliottii* (Fig 3a & b). Lesions produced by both *D. scrobiculata* and *D. pinea* varied on *P. elliottii* (Fig 3b) indicating this *Pinus* sp. is more tolerant to infection than *P. radiata*.

Discussion

Morphology and DNA sequences in this study provided robust evidence that *D. scrobiculata* is present in South Africa. This is the first time that the fungus has been found in plantations of non-native pines in the southern hemisphere. The discovery is intriguing given the fact that the closely related fungus *D. pinea* is one of the most common fungi found on pines in this area (Smith et al. 2000).

Only one percent of isolates originating from the *D. pinea* survey were found to represent *D. scrobiculata* implying that the fungus is very rare and apparently a poor competitor in the South African environment. *Diplodia pinea,* which is generally more competitive in northern hemisphere (Burgess et al. 2004b), could have already dominated the niche in South Africa before the arrival of *D. scrobiculata*. This is likely, because *Pinus* spp. were originally introduced from Europe where *D. pinea* is the dominant species (Burgess et al. 2004a). The

faster growth and superior competitive ability of *D. pinea* could favour its ability to compete for resources as compared with its close relative, *D. scrobiculata*.

The rare occurrence of *D. scrobiculata* might explain why the fungus has not been found in previous studies on *D. pinea* in South Africa, Australia and New Zealand (Smith et al. 2000; Burgess et al. 2001a, 2004a). Peripherally, *D. scrobiculata* and *D. pinea* have a very similar morphology and they were treated as the same fungus for many years. Thus, isolates of *D. scrobiculata* in past studies in the southern hemisphere might also have been overlooked and treated as those of *D. pinea*.

Allele sizes generated from five SSR microsatellite markers suggest that the isolates of *D. scrobiculata* from South Africa are more similar to isolates from California, and different to those from Mexico and the north-central USA. The isolates from California that were most similar to South African isolates were isolated from the Cambria area (Burgess et al. 2004b) from which *P. radiata* germplasm was imported in 1959 to Europe, Australia and New Zealand (Burgess and Wingfield 2002). It is also known that *P. radiata* seed has been imported into South Africa from California which could explain the common alleles in isolates from the two locations.

Diplodia scrobiculata is a pine pathogen although previous pathogenicity studies have shown it to be less virulent than *D. pinea* (Palmer et al. 1987; Blodgett and Stanosz 1997, 1999; De Wet et al. 2000). Interestingly, this study suggested that, it may be possible some isolates of the former fungus are as virulent as those of *D. pinea.* Difference in virulence, however, depends on the methods of inoculation and *Pinus* spp. For example, when these two fungi were inoculated onto shoots of *P. banksiana* they were equally pathogenic but *D. pinea* was more virulent on *P. resinosa* (Palmer et al. 1987). The current work and previous studies have also

shown that there are differences in the virulence of strains of these two fungi and further studies should be undertaken to better understand the relative virulence of these two fungi.

This study represents the first report of *D. scrobiculata* from the southern hemisphere. This pathogen was most probably introduced into South Africa with germplasm of *P. radiata* from California. *D. scrobiculata* appears to compete less successfully with *D. pinea* and is present in South Africa at a much lower frequency than *D. pinea*. However, contrary to previous reports some of the genotypes of *D. scrobiculata* present in South Africa may be as virulent as those of *D. pinea* on pine species grown in the country, and as with *D. pinea*, care should be taken in order to avoid importation of additional genotypes of this pathogen.

References

Blodgett JT, Stanosz GR (1997) *Sphaeropsis sapinea* morphotypes differ in aggressiveness but both infect non-wounded red or jack pines. Plant Disease **81**: 143 – 147.

Blodgett JT, Stanosz GR (1999) Differences in Aggressiveness of *Sphaeropsis sapinea* RAPD Marker Group Isolates on Several Conifers. Plant Disease **83**: 853 – 856.

Blodgett JT, Bonello P (2003) The aggressiveness of *Sphaeropsis sapinea* on Austrian pine varies with isolate group and site of infection. Forest Pathology **33**: 15 – 19.

Burgess T, Wingfield BD, Wingfield MJ (2001a) Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata.* Mycological Research **105**: 1331 – 1339.

Burgess T, Wingfield MJ, Wingfield BD (2001b) Simple sequence repeat markers distinguished among morphotypes of *Sphaeropsis sapinea*. Applied Environmental Microbiology **67**: 354 – 362.

Burgess T, Wingfield MJ (2002) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the southern hemisphere. International Forestry Review **4**: 56 – 65.

Burgess T, Wingfield MJ, Wingfield BD (2004a) Global distribution of *D. pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australasian plant pathology **33**: 513 – 519.

Burgess T, Gordon TR, Wingfield MJ, Wingfield BD (2004b) Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. Mycological Research **108**: 1399 – 1406.

Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycets. Mycologia **91**: 553 – 556.

De Wet J, Wingfield MJ, Coutinho TA, Wingfield BD (2000) Characterization of *Sphaeropsis sapinea* isolates from South Africa, Mexico, and Indonesia. Plant Disease **84**: 151 – 156.

De Wet J, Slippers B, Preisig O, Wingfield BD, Wingfield MJ (2003) Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. Mycological Research **107**: 557 – 566.

Hausner G, Hopkin, AA, Davis CN, Reid J (1999) Variation in culture and rDNA among isolates of *Sphaeropsis sapinea* from Ontario and Manitoba. Canadian Journal of Plant Pathology **21**: 256 – 264.

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research **30**: 3059 – 3066.

Lazzizera C, Frisullo S, Alves A, Lopes J, Phillips AJL (2008) Phylogeny and morphology of *Diplodia* species on olives in southern Italy and description of *Diplodia olivarum* sp. nov. Fungal Diversity **31**: 63 – 71.

Morelet M, Chandelier P (1993) Sur un cas de variabilite chez *Sphaeropsis sapinea*. European Journal of Forest Pathology **23**: 317 – 320.

Muñoz Z, Moret A, Garcés S (2008) The use of *Verticillium dahliae* and *Diplodia scrobiculata* to induce resistance in *Pinus halepensis* against *Diplodia pinea* infection. European Journal of Forest Pathology **120**: 331 – 337.

Palmer MA, Stewart EL, Wingfield MJ (1987) Variation among isolates of *Sphaeropsis sapinea* in the north central United States. Phytopathology **77**: 944 – 948.

Smith DR, Stanosz GR (1995) Confirmation of two distinct populations of *Sphaeropsis sapinea* in the north central United States using RAPDs. Phytopathology **85**: 699 – 704.

Smith H, Wingfield MJ, De Wet J, Coutinho TA (2000) Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. Plant Disease **84**: 139 – 142.

Smith H, Wingfield MJ, Coutinho TA (2002) The role of latent *Sphaeropsis sapinea* infections in post-hail associated die-back of *Pinus patula*. Forest Ecology and Management **164**: 177 – 184.

Swart WJ, Wingfield MJ (1991) Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. Plant Disease **75:** 761 – 766.

Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Wang CG, Blanchette RA, Jackson WA, Palmer MA (1985) Differences in conidial morphology among isolates of *Sphaeropsis sapinea*. Plant Disease **69**: 838 – 841.

White TJ, Bruns TD, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal genes for phylogenetics, p. 315 – 322. *In*: Innis MA, Gelfand DH, Sinisky JJ, White TJ (ed.) PCR protocols: a guide to methods and applications. Academic Press, San Diego.

Table 1. Lists of isolates used in the study

Isolate code	Species	GenBank Accession No	
		ITS	EF1-α
CMW7776	*D. mutila*	AY972106	DQ280420
CMW190	*D. pinea*	-	AY624251
CMW4876	*D. pinea*	AY253294	AY624252
CAP166	*D. pinea*	EU392284	EU392261
CAP168	*D. pinea*	EU392285	EU392262
CAP169	*D. pinea*	EU392286	EU392263
CMW29483[1]	*D. pinea*	HM100283	HM100274
CMW29144[1]	*D. pinea*	HM100284	HM100275
CMW29323[1]	*D. pinea*	HM100285	HM100276
CBS113423	*D. scrobiculata*	DQ458900	DQ458885
CBS109944	*D. scrobiculata*	DQ458899	DQ458884
CMW189	*D. scrobiculata*	AY253292	AY624253
CMW4900	*D. scrobiculata*	AF264907	AY624255
CMW5870	*D. scrobiculata*	-	AY625254
CMW30222[1]	*D. scrobiculata*	HM100277	HM100268
CMW30223[1]	*D. scrobiculata*	HN100278	HM100269
CMW30224[1]	*D. scrobiculata*	HM100279	HM100270
CMW30225[1]	*D. scrobiculata*	HM100280	HM100271
CMW30226[1]	*D. scrobiculata*	HM100281	HM100272
CMW30227[1]	*D. scrobiculata*	HM100282	HM100273

[1]Isolated in this study.
ITS, internal transcribed spacer; EF-1α, elongation factor.

Figure 1. Comparisons of mycelial growth of six isolates of the unknown *Diplodia* species with the average growth for six *D. pinea* isolates (•) at 25 ^{0}C for eight days commencing on day three. Each growth curve for the solates of the unknown *Diplodia* sp. represents an average of three measurements. Measurements were taken from the centre of isolates to the edge of the plate.

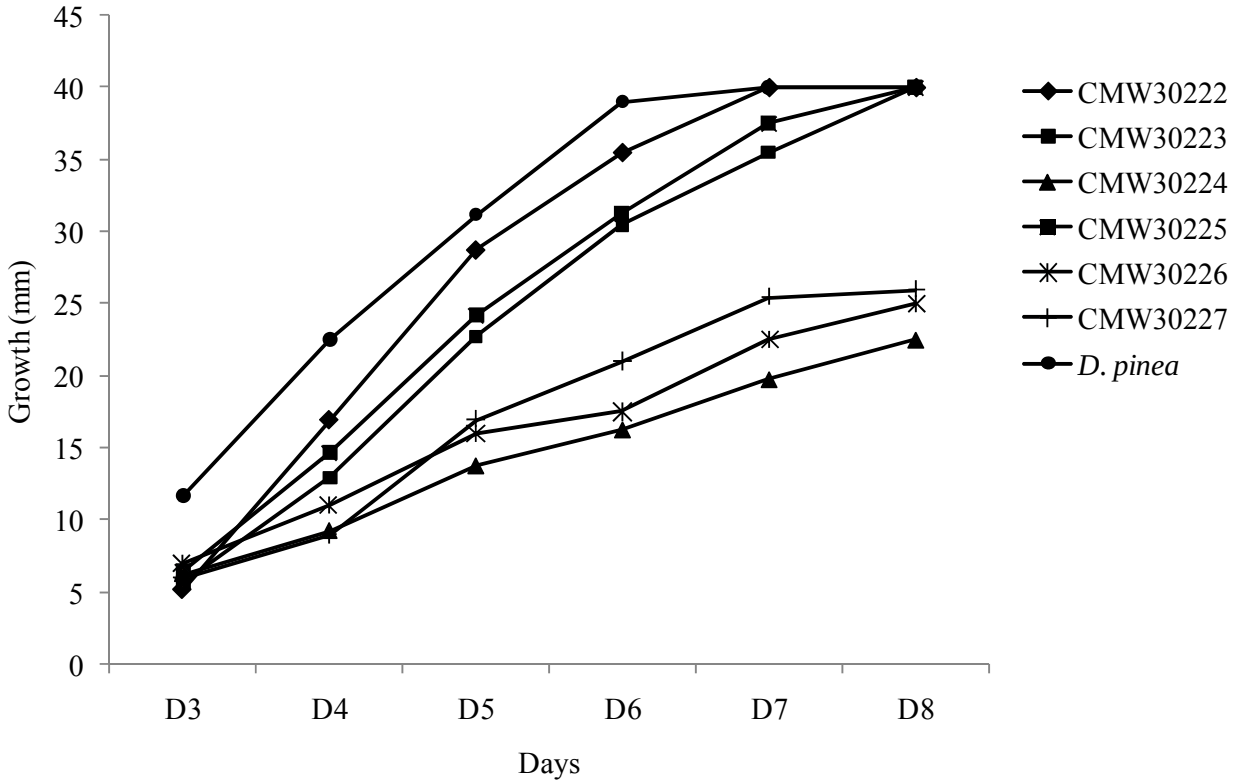


Figure 2. Phylogenetic tree constructed from the combined ITS rDNA and elongation factor (EF-1α) sequences for six isolates of an unknown *Diplodia* sp. presented in bold.

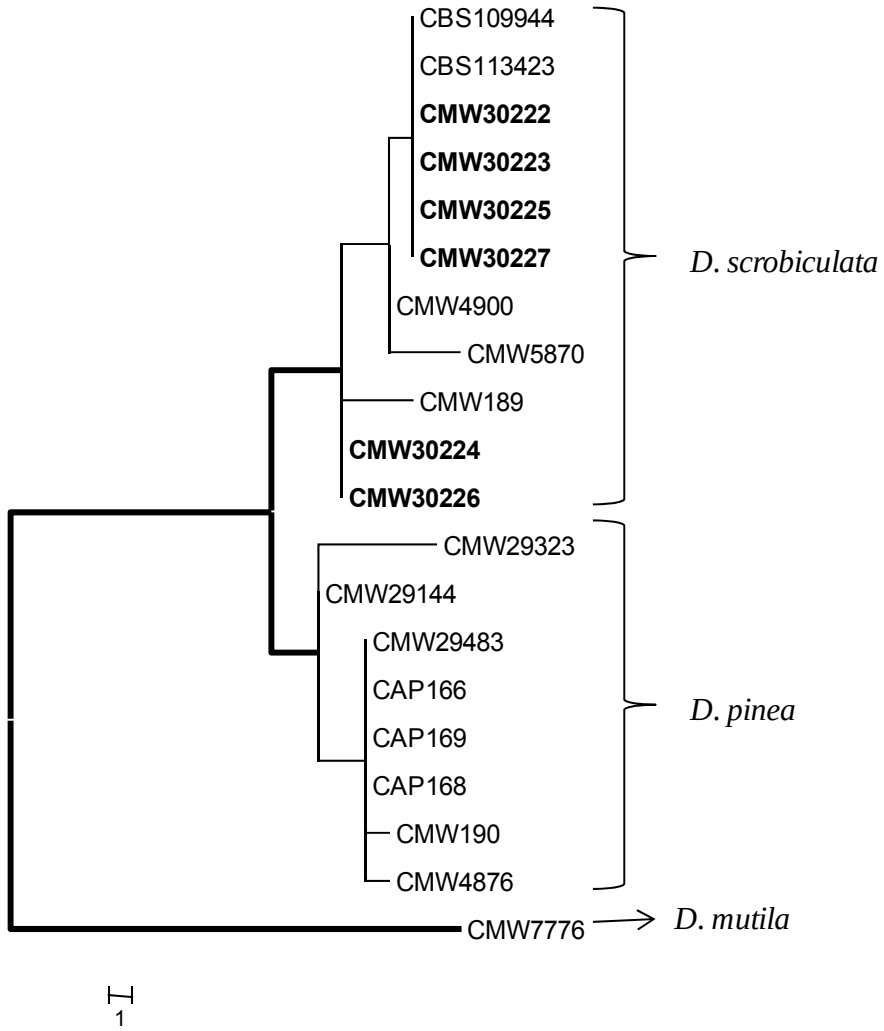


Figure 3. Mean lesion length (mm) resulting from inoculations with six isolates for six isolates of *D. pinea* (shaded) and the unknown *Diplodia* sp. (un-shaded) on (a) *P. radiata* and (b) *P. elliottii* seedlings. Vertical bars show ± S.E and the same letters within a graph or *Pinus* spp. indicated there were no significant lesion length differences at $P \leq 0.05$.

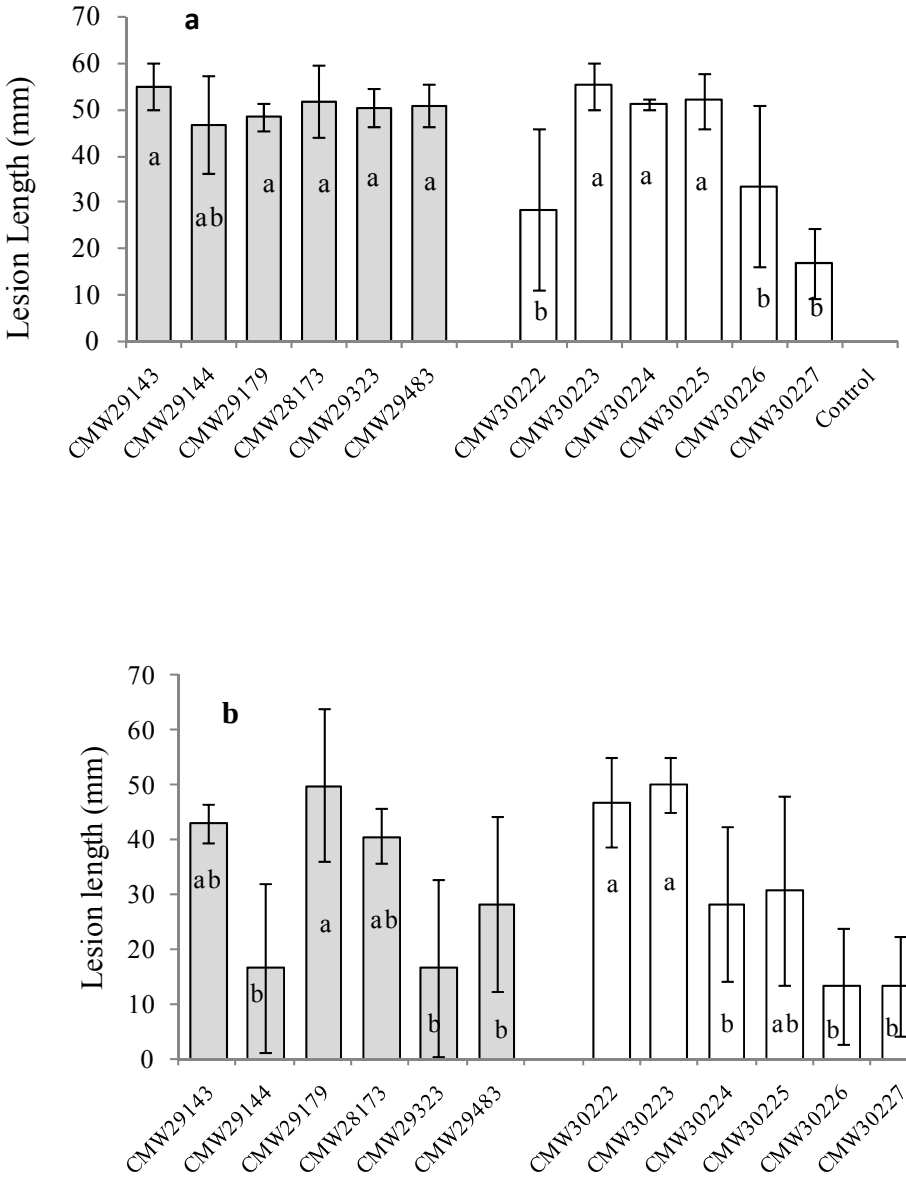


Table 2. Comparisons of allele sizes (bp) and frequencies of South African (RSA) *D. scrobiculata* isolates with those of populations from California (Cal), Mexico (Mex) and north-central America (NCA) at 5 loci. Data for Cal, Mex and NCA were taken from BURGESS et al. (2004b). Shaded region indicated that alleles of the RSA population correspond to those in other populations. Unique alleles in the RSA population are shaded and in bold.

Locus	Allele	Cal	Mex	NCA	RSA
SS1	326	-	0.050	0.050	-
	361	-	0.250	0.950	-
	342	-	0.050	-	-
	444	0.055	-	-	1.000
	468	0.945	0.250	-	-
	508	-	0.400	-	-
SS2	200	0.222	-	-	1.000
	204	-	0.250	-	-
	206	0.778	0.750	1.000	-
SS7	383	0.667	-	-	0.833
	387	0.056	-	-	-
	394	0.277	-	-	0.167
	396	-	0.050	-	-
	401	-	-	0.650	-
	404	-	-	0.300	-
	409	-	-	0.050	-
	411	-	0.500	-	-
	415	-	0.250	-	-
	419	-	0.125	-	-
	423	-	0.050	-	-
SS8	279	-	-	-	**0.167**
	283	0.111	-	-	0.500
	288	0.111	0.850	-	0.333
	293	-	0.100	0.150	-
	295	-	-	0.500	-
	298	-	-	0.100	-
	301	-	-	0.050	-
	305	-	0.050	0.100	-
	317	0.556	-	-	-
	322	0.056	-	-	-
	333	0.167	-	-	-
SS9	236	1.000	-	0.050	-
	237	-	-	0.950	-
	238	-	1.000	-	-
	242	-	-	-	**0.833**
	254	-	-	-	**0.167**

5

High levels of genetic diversity and cryptic recombination are widespread in introduced *Diplodia pinea* populations

Abstract

Introduced populations typically have reduced diversity compared to those that are native. It is, therefore, unusual that introduced populations of *Diplodia pinea* have been shown to have high levels of genetic diversity, even surpassing diversity in certain native regions. This is thought to be due to multiple introductions over time or the existence of a cryptic sexual cycle. In this study, we consider whether populations of *D. pinea* in Southern Hemisphere countries have similar patterns of diversity, share some level of genetic identity and how they might be influenced by sexual recombination. A total of 173 isolates from Argentina, Australia, Ethiopia and South Africa were characterized using 12 microsatellite markers. The results show that all these populations have high gene and genotype diversities, with the Australian population having the lowest diversity. Very few private alleles were found, suggesting that isolates for different countries might share a source of introduction. However, based on allele distribution and frequency, each of the populations appeared to be evolving independently. The results showed that in all but the Australian population, alleles are randomly associated, suggesting that widespread sexual recombination has influenced population structure.

Introduction

Diplodia pinea is a well-known pathogen of coniferous trees including more than 30 *Pinus* spp. worldwide (Swart et al. 1985; Palmer et al. 1987). It was first reported in France in 1842 on diseased *P. sylvestris* and has subsequently been found in most countries where *Pinus spp.* are grown (Swart and Wingfield 1991; Stanosz et al. 1999; Burgess et al. 2001a; Feci et al. 2003). *Diplodia pinea* is thought to have been moved around the world with pine plantig stock most probably in its endophytic form, seeds or seed lots contaminated with debris (Wingfield et al. 2001, Burgess and Wingfield 2002, Bihon et al. 2010b). The introduced populations of *D. pinea* have been characterized in only a few instances (Smith et al. 2000; Burgess et al. 2001a & b, 2004). Consequently the history and possible connection between these introductions is poorly understood.

Knowledge of population dynamics of plant pathogens, including genetic diversity and modes of reproduction, allows for an understanding regarding the potential for evolutionary change (Nevo 1978; McDonald and McDermott 1993; McDonald 1997). Patterns of pathogen dispersal around the world and also within regions can be inferred from studying geographic distribution, structure and population diversity (Milgroom and Fry 1997; McDonald and Linde 2002). These can also indirectly contribute to the development of sound management strategies such as through the selection of resistant genotypes (McDonald and McDermott 1993; Burgess and Wingfield 2002; McDonald and Linde 2002).

Fungal plant pathogens are expected to be genetically more diverse in their native environments than where they are introduced (McDonald and McDermott 1993). This is due to genetic bottlenecks (Linde 2010) associated with the limited diversity of an introduction (Milgroom et al. 2008; Goss et al. 2009; Linde 2010). This is especially evident in asexual fungi

or in sexually reproducing fungi where the absence of one mating type precludes genetic recombination (Goss et al. 2009). In contrast to this expectation, previous studies have shown that *D. pinea* is more diverse in its introduced range in South Africa than in a population from the USA, which is thought to be part of the native range of the pathogen (Burgess et al. 2004). This diversity is hypothesized to have originated from numerous introductions over the past two centuries, and linked to the fact that germplasm including seed has been regularly introduced into the country (Smith et al. 2000; Burgess and Wingfield 2002; Burgess et al. 2004). In contrast, Burgess et al. (2001a, 2004) found a much lower diversity in Australia compared with South Africa, and hypothesised that this was due to stricter quarantine restrictions and considerably fewer imports of plant material (Burgess and Wingfield 2002). To further complicate this issue, a recent study has, however, suggested that this diversity could be linked to a cryptic sexual cycle (Chapter 3).

While South African populations of *D. pinea* have been studied in detail, and Australian *D. pinea* populations to a lesser degree, no information is available regarding other introduced populations. The aim of this study was to consider whether the high levels of diversity and evidence for recombination is unique to the South African populations of *D. pinea* or whether this is true for other areas. To answer these questions, microsatellite data were used to compare *D. pinea* populations from Australia, South Africa, Ethiopia and Argentina.

Materials and Methods

Isolation and isolates

A total of 173 *D. pinea* isolates from four countries Australia, South Africa, Ethiopia and Argentina were used in this study (Table 1). Isolates from Western Australia (N = 28) and Argentina (N = 32) were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Isolates from South Africa (N = 56) were collected in the Boston area of the KwaZulu-Natal province. South African isolates from Boston were used because samples were collected from symptomatic trees after drought which was the same scenario for isolates from other countries. Those from Ethiopia (N = 57) were collected in the Shashmene state forest and the Wondogenet area. Isolation of the fungus was done using methods described previously (Bihon et al. 2010a).

SSR-PCR and allele size determination

DNA was extracted as described by (Bihon et al. 2010a) and PCR amplification was conducted using twelve fluorescently labelled microsatellite primers (SS1 to SS13) developed previously (Burgess et al. 2001b; Bihon et al. 2010a). All amplifications were performed in 25 µl reaction volumes containing 1x PCR buffer (Roche Diagnostics) without $MgCl_2$, 2.5 mM $MgCl_2$, 0.25 mM each dNTPs (Fermantas, Nunningen, Switzerland), 0.2 mM of each labelled forward (FAM, NED, PET, VIC) and reverse primers, 0.5 U of Taq polymerase (FABI). Amplifications were assessed using agarose gel-electrophorosis and DNA was visualised with GelRed™ nucleic acid gel stain under UV light (Biotium, California). PCR products were diluted and multiplexed before separation on a ABI Prism 3100 genetic analyzer (Applied Biosystems) as described previously (Bihon et al. 2010a).

Gene and genotype diversity

The frequency of alleles at each locus and gene diversity (h*) was calculated using the program POPGENE (Yeh et al., 1999). Genotypic diversity (G) was estimated using the equation $G = 1/\sum pi^2$ where *Pi* is the observed frequency of the ith phenotype (Stoddart and Taylor 1988).

In order to avoid bias arising from sample size, the genotypic diversity (G) was divided by the value of the number of isolates to give maximum percentage genotype diversity (G^*). Genotypic diversity was calculated for each population as $G^* = G/N*100$, where N = number of isolates. Contingency chi-square (χ^2) tests for differences in allele frequencies were calculated for each locus across clone corrected populations. Gene diversities across loci were recorded as significantly different to one another when the calculated χ^2 values were higher than the value on the chi-square table at $P < 0.05$ at each corresponding degrees of freedom (*d.f*).

Divergence of populations

Population divergence or differentiation theta "θ" was analyzed between pairs of populations using Multilocus program (Agapow and Burt 2000) with an estimate of Wright's Fst for haploids as θ = Q-q/1-q, where Q is the probability that two alleles from the same population are the same and q is the probability that two alleles from different populations are the same (Weir 1997). The alternative hypothesis that there is significance population divergence between populations was accepted when the calculated "θ" values were significantly different from the 1000 times randomized data sets at $P < 0.05$.

Linkage disequilibrium

Random mating or sexual recombination was evaluated indirectly through analysis of Index of Association (I_A) using the programme Multilocus 1.3 (Agapow and Burt 2000). In order to consider the effect of dependency of I_A on number of loci, rbarD ($\bar{r}_D$) was also analyzed using the same program. The observed I_A and $\bar{r}_D$ were compared with the output of 1000 times simulated random association of alleles. The null hypothesis (H_o) that there is random association of loci was accepted when the observed values for I_A were within the random data sets and probability (P) values showed no significance difference at $P < 0.05$.

Measure of genetic structure

To understand the genetic structure and relationships of individual genotypes, data were analysed using Parsimony in PAUP 4.0 (Swofford 2002). Alleles for each locus were numerically coded. Using these values an unweighted pair-group method with arithmetic mean (UPGMA) dendrogram was constructed in PAUP using the mean character differences to visualise the relationship between populations and that for isolates within populations.

Population structure was inferred and assigned in STRUCTURE 2.2 (Pritchard et al. 2000) using 100,000 replicates of Monte Carlo Markov Chain (MCMC) analysis after an initial burn-in of 20,000 for *K* ranging from 1 to 15 at 20 iterations. A Cluster identity of > 75 % was used to assign clusters. In addition, analysis of molecular variance (AMOVA) in GenAlEx version 6.1 (Peakall and Smose 2006) was carried out to differentiate percentage variations among and within populations.

Results

SSR amplification

Using the 12 SSR markers, 35 alleles were amplified for the 173 isolates representing the four populations of *D. pinea*. Of these, one unique allele was observed in each of the Australian, Argentinean and Ethiopian populations. Four unique alleles were found in the South African population. The number of alleles observed for the populations ranged from 18 (Australia) to 24 (South Africa) (Table 1). For the 12 SSR markers used, 10 loci were polymorphic in the South African isolates while only seven loci were polymorphic in the Australia isolates. In the Argentina and Ethiopia populations the same eight loci were polymorphic (Table 1).

Genotype and gene diversity

The highest level of genotype diversity was observed in the South African (43 %) and Argentinean (40 %) isolates. Only, moderate diversity was found in the Ethiopian isolates (23 %) and the Australian isolates exhibited lower (13 %) diversity (Table 1). Chi-square tests for the clone corrected populations showed significant differences ($P < 0.05$) in gene diversities at each of the loci. This was influenced by the monomorphic loci and unique alleles. Mean gene diversity across all loci ranges from 0.105 in Australia to 0.293 in Argentina populations.

Linkage disequilibrium

The I_A and $\bar{r}D$ values for all the populations showed that, there were no significant differences between the observed and randomized data-sets for the populations from South Africa, Ethiopia and Argentina. In these populations the observed I_A and $\bar{r}D$ values fell inside the distribution for randomized data sets. In contrast, significant differences were found between the observed and

randomized data sets for the Australian population (Table 1) and when the combined data set was analysed (I_A = 0.818, P < 0.001). Therefore, the null hypothesis that loci are randomly associated in the populations from South Africa, Argentina and Ethiopia was accepted. The hypothesis of random association could, however, not be accepted for the Australian population.

Genetic structure and variation

Analysis of molecular variance confirmed that there was a significant percent variation among and within populations responsible for the total variation. Forty two and fifty eight percent of the total molecular variance was accounted for among and within populations respectively. Pair-wise population differentiation "θ" was also significantly different between each population (Table 3). AMOVA analyses showed that percentage of genetic variation among individuals within populations was 58 % and that the variation among populations was 42 %.

A UPGMA phylogram constructed using the matrix of clone corrected multilocus haplotypes showed a clustering of populations based on their geographical origins (Fig. 1). South African and Ethiopian populations were, however, more closely related to each other than to the Argentinean and Australian populations. The Argentinean population was the most distant from the other populations considered. Analysis using STRUCTURE also showed that genotypes grouped based on geographic origin of the isolates and that there was a high level of admixture in the South African and Ethiopian populations (Fig. 2).

Discussion

In this study microsatellite analysis of four introduced *D. pinea* populations from South Africa, Ethiopia, Argentina and Australia showed that endophytic diversity was moderate to high in

populations from the first three of these countries. Strong evidence also emerged to suggest that recombination has occured in this fungus in those three countries. These data support the findings in previous studies on the pathogen in South Africa where diversity was high (Smith et al. 2000; Burgess et al. 2001a; Burgess et al. 2004). In the present study two additional introduced populations of *D. pinea* and the South African isolates were especially collected from a single area to prevent confusion arising from possible multiple introductions of the fungus into different areas. Diversity was low in the Australian population, which has previously been shown to have benefited from strong quarantine measures (Burgess et al. 2001a; Burgess et al. 2004) and there was no evidence of recombination in that population.

High levels of genotypic diversity in South African *D. pinea* were consistent with findings in previous studies where the introduced population in South Africa was more diverse than that seen in native pine forests of USA, Europe and Indonesia (Smith et al. 2000; Burgess et al. 2001a; Burgess et al. 2004). In those studies, the data were refelecting introductions of multiple genotypes over time (Fig. 3A). What was different in the present study is that all the isolates were collected from a single area, which would dispel the possibility of different introductions into different areas. An alternative explanation for the high level of genotypic diversity in the South African, Ethiopian and Argentinean populations in this study is that genotypic diversity increases due to out-crossing over time (Fig. 3B). This is the most plausible explanation for the results and it suggests that there is a cryptic sexual state in *D. pinea* in these countries. Although mutation can increase genetic diversity, this likely plays a negligible role as very few private alleles were found in the different populations (Halliburton 2004).

Diplodia pinea has always been thought to reproduce only asexually (Sutton 1980; Burgess et al. 2004), but a recent study confirmed that recombination of alleles occurs in the

South African populations of this fungus (Chapter 3). The current study provides evidence that recombination is a widespread phenomenon in *D. pinea* populations. Linkage disequilibrium analysis showed that *D. pinea* populations from South Africa, Ethiopia and Argentina were not significantly different from the randomized hypothetical data sets, thus providing evidence for recombination. The application of I_A values to infer the possibility of random association of genotypes due to cryptic sex as done in this study is not unusual and is an approach that has ben used for many other studies on fungi (Geiser et al. 1994; Kohli and Kohn 1998; Morgan et al. 2007; Groenewald et al. 2008).

Recombination in fungi for which there is no known sexual state is well recognised. For example *Aspergillus nidulans* (Geiser et al. 1994), *Fusarium oxysporum* and *Alternaria alternata* (Arie et al. 2000) and *Cercospora beticola*, (Groenewald et al. 2008) are asexual fungi in which cryptic recombination has been noted. This is thought to emerge from a sexual state that has not yet been discovered or from a parasexual cycle (Taylor et al. 1999). Population genetic studies can provide evidence for occurrence of cryptic sex in filamentous fungi in which sexual reproduction has not been previously reported (Kuck and Poggeler 2009) as has emerged in this study. Sexual structures typically occur in nature and can be very difficult to find. Although some effort has been made to find these for *D. pinea* (Wingfield, unpublished), it is entirely possible that they are present.

An interesting aspect of the results of this study is that there was significant geographic structure to the populations. This is based on distribution and frequency of different alleles. Yet, most alleles are still shared between the populations, with very few private alleles occurring in each population, which could have originated from mutation (Zhan and McDonald 2004). This implies that introductions into countries such as South Africa, Australia and Ethiopia might have

originated from a common source or sources, and that there has been limited exchange between the regions other than in the case of the Argentinian population that was distantly related to other populations. Mutation probably at a limited level and recombination has most likely further added to the differentiation of the populations since they were first introduced into the respective regions. A recent study showed that substructure in *D. pinea* population occurs even over relatively small geographic distances (Chapter 3). It has also been shown that the potential of *D. pinea* to be distributed via seed is very low (Bihon et al. 2010b) and the fungus is not insect vectored. Limited long distance dispersal, geographical barriers and reproductive isolation between populations should therefore lead to isolated populations that are differentiated from each other. Given the geographical distance between the populations considered in this study, natural spread would thus not be expected to influence gene flow.

The genetic distance between isolates from Argentina and the other three populations was high while the South Africa, Ethiopia and Australia populations were relatively closely related. There was evidence of genetic admixture, especially among South African and Ethiopian populations although this was not supported by the analysis of population differentiation. These admixtures could be due to the fact that pine germplasm has been shared between South Africa and Australia as suggested by Burgess and Wingfield (2002). It is not known whether plant material has been exchanged between South Africa and Ethiopia, but given the very long history of forestry and the fact that *Pinus patula* has been developed in as a plantation species in South Africa, this is very likely. This would also be consistent with the fact that South Africa has provided a source for forestry planting stock for many African countries and there is evidence that pathogens have also moved with this material (Hunter et al. 2008).

Movement of planting stock between countries is clearly contributing to pathogen introductions and every effort must be made to reduce this trend (Wingfield et al. 2001, 2008). The results of this study have also shown that cryptic recombination is a widespread phenomenon in most introduced *D. pinea* populations. Natural recombination can occur between different genotypes of well-known pathogens such as *D. pinea* and it is necessary to also recognise the dangers of not only of introducing new pathogens, but also genotypes of pathogens already present in countries.

References

Agapow PM, Burt A (2000) 'Multilocus 1.2' (Department of Biology, Imperial College: Ascot, UK).

Arie T, Kaneko I, Yoshida T, Noguchi M, Nomura Y, Yamaguchi I (2000) Mating-type genes from asexual phytopathogenic ascomycets *Fusarium oxysprum* and *Alternaria alternata*. Molecular Plant-Microbe Interactions **13**: 1330 – 1339.

Bihon W, Burgess T, Bernard S, MJ Wingfield, BD Wingfield (2010a) Distribution of *D. pinea* and its genotypic diversity within asymptomatic *P. patula* trees. Australasian Plant Pathology (*in press*).

Bihon W, Slippers B, Burgess T, Wingfield MJ, Wingfield BD (2010b) Sources of *Diplodia pinea* endophytic infections in *Pinus patula* and *P. radiata* seedlings in South Africa. Forest Pathology (*in press*).

Burgess T, Wingfield BD, Wingfield MJ (2001a) Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*. Mycological Research **105**: 1331 – 1339.

Burgess T, Wingfield MJ, Wingfield BD (2001b) Simple sequence repeat markers distinguished among morphotypes of *Sphaeropsis sapinea*. Applied Environmental Microbiology **67**: 354 – 362.

Burgess TI, Wingfield MJ (2002) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the southern hemisphere. International Forestry Review **4**: 56 – 65.

Burgess T, Wingfield MJ, Wingfield BD (2004) Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australasian Plant Pathology **33**: 513 – 519.

Feci E, Smith D, Stanosz DR (2003) Association of *Sphaeropsis sapinea* with insect-damaged red pine shoots and cones. Forest Pathology **33**: 7 – 13.

Geiser DM, Arnold ML, Timberlake WE (1994) Sexual origins of British *Aspergillus nidulans* isolates. Proceedings of the National Academy of Sciences, USA **91**: 2349 – 2352.

Goss EM, Larsen M, Chastagner GA, Givens DR, Grunwald NJ (2009) Population genetic analysis infers migration pathways of *Phytophthora ramorum* in US nurseries. PLoS Pathogens 5: e1000583, doi:10.1371/journal.ppat.1000583.

Groenewald M, Linde CC, Groenewald JZ and Crous PW (2008) Indirect evidence for sexual reproduction in *Cercospora beticola* populations from sugar beet. Plant Pathology **57**: 25 – 32.

Halliburton R (2004) Introduction to population genetics. Pearson Education, Inc., USA.

Hunter GC, van der Merwe NA, Burgess TI, Carnegie AJ, Wingfield BD, Crous PW, Wingfield MJ (2008) Global movement and population biology of *Mycosphaerella nubilosa*

infecting leaves of cold-tolerant *Eucalyptus globules* and *E. Nitens.* Plant Pathology **57**: 235 – 242.

Kohli Y and Kohn LM (1998) Random Association among Alleles in Clonal Populations of *Sclerotinia sclerotiorum.* Fungal Genetics and Biology **23**: 139 – 149.

Kuck U, Poggeler S (2009) Cryptic sex in fungi. Fungal Biology Reviews **23**: 86 – 90.

Linde CC (2010) Population genetic analyses of plant pathogens: new challenges and opportunities. Australasian Plant Pathology **39**: 23 – 28.

McDonald BA (1997) The Population Genetics of Fungi: Tools and Techniques. Phytopathology **87**: 448 – 453.

McDonald BA, Linde C (2002) Pathogen population genetics, evolutionary potential, and durable resistance. Annual Review of Phytopathology **40**: 349 – 379.

McDonald BA, McDermott JM (1993) Population Genetics of Plant Pathogenic Fungi: Electrophoretic markers give unprecedented precision to analyses of genetic structure of populations. BioScience **43**: 311 – 319.

Milgroom MG, Fry WE (1997) Contributions of population genetics to plant disease epidemiology and management. Advances in Botanical Research **24**: 1 – 30.

Milgroom MG, Sotirovski K, Spica D, Davis JE, Brewer MT, Milev M, Cortesi P (2008) Clonal population structure of the chestnut blight fungus in expanding ranges in south eastern *Europe.* Molecular Ecology **17**: 4446–4458.

Morgan JAT, Vredenburg VT, Rachowicz LJ, Knapp RA, Stice MJ, Tunstall REB, Parker JM, Longcore JE, Moitz C, Briggs CJ, Taylor JW (2007) Population genetics of the from-killing fungus *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Sciences, USA **104**: 13845 – 13850.

Nevo E (1978) Genetic Variation in Natural Populations: Patterns and Theory. Theoretical Population Biology **13**: 121 – 177.

Palmer MA, Stewart EL, Wingfield MJ (1987) Variation among isolates of *Sphaeropsis sapinea* in the north central United States. Phytopathology **77**: 944 – 948.

Peakall R, Smouse PE (2006) GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes **6**: 288 – 295.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics **155**: 945 – 959.

Smith H, Wingfield MJ, de Wet J, Coutinho TA (2000) Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. Plant Disease **84**: 139 – 142.

Stanosz GR, Swart WJ, Smith DR (1999) RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. Mycological Research **103**: 1193 – 1202.

Stoddart JA, Taylor JF (1988) Genotype diversity: Estimation and prediction in samples. Genetics **118**: 705 – 711.

Sutton BC (1980) The Coelomycetes. Commonwealth Mycology Institute, Kew, Surrey, England.

Swart WJ, KInox-Davis PS, Wingfield MJ (1985) *Sphaeiopsis sapinea,* with special reference to its occurrence on *Pinus* spp. in South Africa. South Africa Forestry Journal **35**: 1 – 8.

Swart WJ, Wingfield MJ (1991) Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. Plant Disease **75**: 761 – 766.

Swofford, D.L., 2002: PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Taylor JW, Jacobson DJ, Fisher MC (1999) The evolution of asexual fungi: reproduction, speciation and classification. Annual Review of Phytopathology **37**: 197 – 246.

Weir BS (1997) Genetic data analysis II. Sinauer Associates Inc.: Sunderland, MA.

Wingfield MJ, Slippers B, Roux J, Wingfield BD (2001) Worldwide Movement of Exotic Forest Fungi, Especially in the Tropics and the Southern Hemisphere. BioScience **51**: 134 – 140.

Wingfield MJ, Slippers B, Hurley BP, Coutinho TA, Wingfield BD, Roux J (2008) Eucalypt pests and diseases: growing threats to plantation productivity. Southern Forests **70**: 139 – 144.

Yeh FC, Yang RC, Boyle T (1999) POPEGENE version 1.31 (Microsoft windows based freeware for population genetic analysis. Alberta).

Zhan J, McDonald BA (2004) The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola*. Fungal Genetics and Biology **41**: 590 – 599.

Table 1. Comparison of *D. pinea* genotype diversity among six populations

Country	N	No of haplotypes	Polymorphic loci	Alleles	Unique alleles	G^*	I_A	$\bar{r}D$
Australia	28	10	7	18	1	13.861	0.939^*	0.237^*
Argentina	32	20	8	21	1	40.000	0.091^{NS}	0.013^{NS}
Ethiopia	57	24	8	21	1	24.464	0.009^{NS}	0.001^{NS}
RSA	56	33	10	24	4	43.75	-0.047^{NS}	-0.005^{NS}

N = total number of isolates, G^* = maximum percentage genotype diversity, RSA = Republic of South Africa, * P value < 0.05 indicating significant linkage disequilibrium and NS = non significance differences.

Table 2. Gene diversity and chi-square (χ^2) tests for differences in allele frequencies for 12 SSR loci across clone corrected populations of *D. pinea*

Locus	Australia	Argentina	Ethiopia	RSA	χ^2	df
SS1	0.000	0.498	0.289	0.057	97.62***	6
SS2	0.133	0.498	0.000	0.291	19.64***	3
SS7	0.000	0.000	0.000	0.359	16.34***	3
SS8	0.245	0.375	0.388	0.208	14.70*	6
SS9	0.337	0.482	0.035	0.327	51.91***	6
SS10	0.000	0.506	0.499	0.535	76.82***	9
SS11	0.294	0.000	0.495	0.057	92.39***	6
SS12	0.131	0.305	0.352	0.057	38.35***	6
SS13	0.000	0.000	0.216	0.000	53.54***	8
SS14	0.000	0.375	0.100	0.113	58.79***	6
SS15	0.131	0.471	0.000	0.291	15.46**	4
SS16	0.000	0.000	0.000	0.251	16.17*	6
Mean	0.105	0.293	0.198	0.212		

df = degree of freedom (number of populations – 1) (number of alleles – 1).
*, **, *** indicates significance differences at $P < 0.05$, 0.01 and 0.001 respectively.

Table 3. Pair wise comparisons of population differentiations (Θ) among *D. pinea* populations from different countries in the world

	Argentina	Ethiopia	RSA
Australia	0.465***	0.683***	0.515***
Argentina	-	0.616***	0.369***
Ethiopia		-	0.609***

*** Population are significant different from each other at $P < 0.001$.

Figure 1. UPGMA dendrogram for multilocus haplotypes of all populations

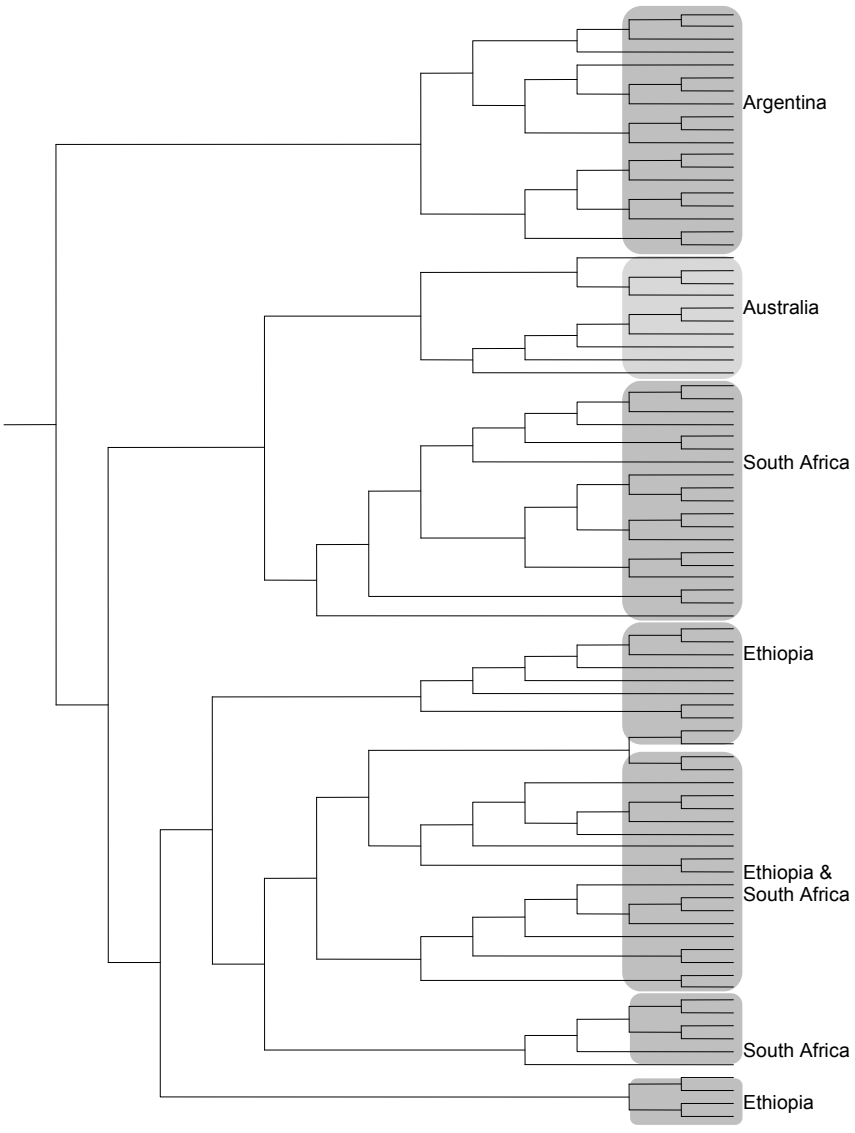


Figure 2. Genotype by STRUCTURE 2.2 revealed four underlying groups of probability of affiliation on the bases of the geographical sources of isolates. Differences in colour within a vertical bar indicate a multi-population affiliation of an individual genotype. The height of each colour within an individual is the measure of proportional affiliation.

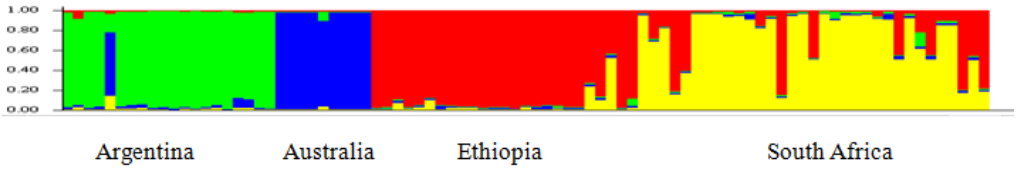
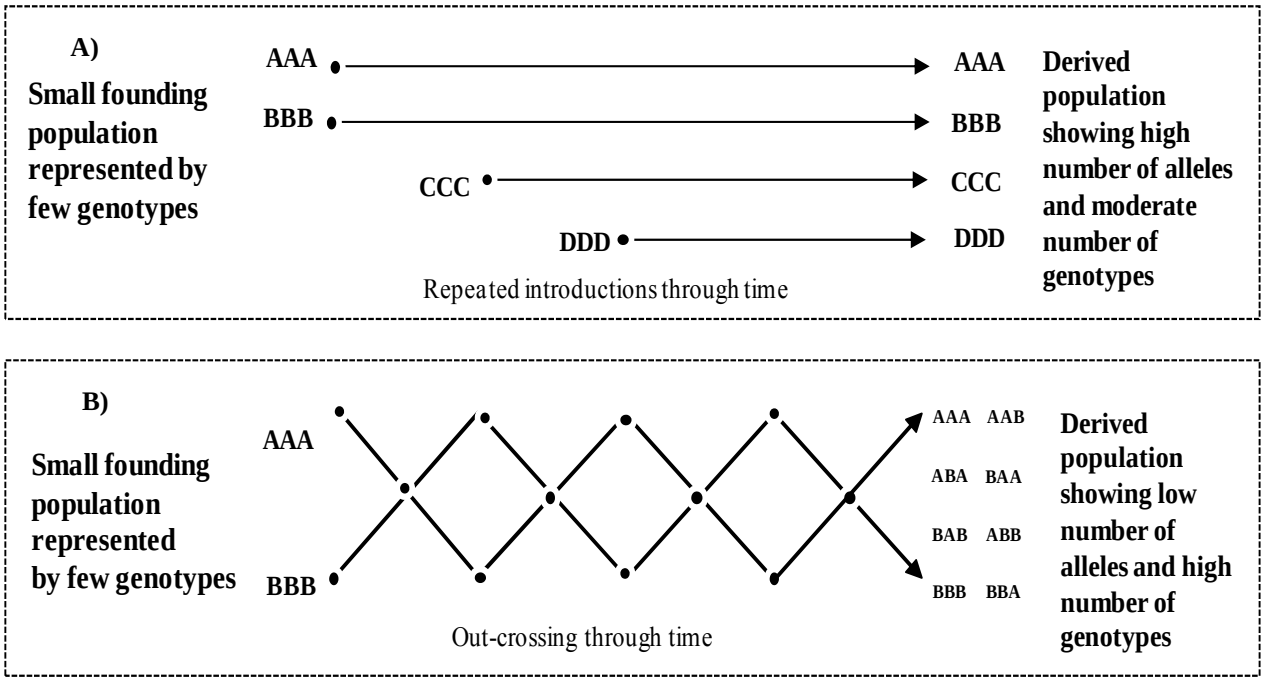


Figure 3. Two possible models explaining the high number of genotypes in introduced *D. pinea* populations. A) Constant number of genotypes acquired from a number of founding populations at different times B) High number of genotypes derived from a small number of founding genotypes but with out-crossing.



6

Sources of *Diplodia pinea* endophytic infections in *Pinus patula* and *P. radiata* seedlings in South Africa

In Press as:
Bihon W, Slippers B, Burgess T, Wingfield MJ, Wingfield BD (2010) Sources of *Diplodia pinea* endophytic infections in *Pinus patula* and *P. radiata* seedlings in South Africa. Forest Pathology

Abstract

Diplodia pinea, an opportunistic and latent pathogen, can significantly affect *Pinus* productivity world-wide. Despite being studied in South Africa for almost 100 years, the source of *D. pinea* inoculum responsible for seedling infection is unknown. The aim of this study was to determine the role of seed in vertical transmission of *D. pinea* and to investigate sources of inoculum leading to horizontal transmission to pine seedlings. Surface disinfected seeds were inoculated with spore and mycelium suspensions of *D. pinea* to determine its effect on germination. In addition, isolation of the fungus was done from surface disinfected seeds, asymptomatic seedlings collected from nurseries, plantations where pines naturally regenerate and recently established fields, to assess transmission and incidence of endophytic *D. pinea* infections. Inoculation of seeds with *D. pinea* spore suspensions affected speed and rate of germination. The fungus was isolated from surface disinfected seeds in only a few instances (2-3 %), and was not found in healthy seedlings collected from greenhouses and nurseries, suggesting that vertical transmission of the fungus does not occur or is rare. In contrast, *D. pinea* was isolated from 40 % of seedlings obtained from the understory of mature *P. patula* trees showing that horizontal transmission from mature to young trees sustains the *D. pinea* inoculum in South African pine plantations.

Introduction

Diplodia pinea (=*Sphaeropsis sapinea*) is an endophyte that exists as a latent pathogen without visible symptoms on pines in many parts of the world. It can be isolated from healthy tissue of pine trees at all stages from seedling to maturity, mostly in branches, twigs and reproductive organs. The fungus can also be isolated from asymptomatic wood of mature trees (Bihon et al. 2010).

Diplodia pinea results in disease when trees are exposed to physiological stress (Blodgett et al. 1997; Stanosz et al. 2001; Swart and Wingfield 1991). Infection results in a variety of symptoms, including damping-off, stem cankers, tip dieback, blue stain and where severe, large scale tree death (Palmer et al. 1987; Stanosz et al. 2005; Swart and Wingfield 1991). In South Africa, damage to *P. radiata* due to *D. pinea* was estimated to be up to 28 % loss of volume and 55 % potential loss of production after hail damage (Zwolinski et al. 1990). For this reason, *P. radiata* is not widely planted in South Africa in regions that receive summer rainfall (Swart and Wingfield 1991). However, *D. pinea* still remains one of the most important pathogens of *Pinus* spp. elsewhere in the country and particularly on *P. patula* after hail damage (Swart and Wingfield 1991).

Transmission of endophytic fungi can be horizontal through spores or mycelium, vertical (systemic) via seed infection or through a combination of these mechanisms (Carroll 1988). Many grass endophytes are seed-borne and persist within plants for the duration of the host life cycle (Caroll 1988; Schardl 2001). In this situation, seeds harbour viable propagules of the fungi that colonize the developing germlings, guaranteeing vertical transmission to the next generation (Schardl 2001; Ernst et al. 2003). Unlike grasses, most tree endophytes are thought to be transmitted horizontally (Arnold et al. 2003, 2007; Slippers and Wingfield 2007).

Diplodia pinea is commonly isolated from seedlings in nurseries (Palmer and Nicholls 1985; Stanosz et al. 2005; Stanosz et al. 2007) and from different parts of pine species including, needles, cones, branches, seed scales, seeds and piths of cones and mature wood (Flowers et al. 2003; Flowers et al. 2001; Smith et al. 2002; Vujanovic et al. 2000). The sources of infection in USA nurseries were mainly infected pine trees used as wind-breaks and leftover pruned branches (Palmer and Nicholls 1985; Stanosz et al. 2007) since the fungus can develop on dead pine tissue and litters of other hosts (Swart et al. 1987). Conidia of *D. pinea* are released from pycnidia in the presence of moisture and disseminated by wind and rain splash. Dispersal of conidia was positively correlated with moist weather in South African pine plantations (Swart et al. 1987). The fact that *D. pinea* was isolated from seeds and also found throughout the living tissue of seedlings, trees and cones, has led to the assumption that the "fungus can be transmitted via seed" and seeds were considered as the major sources of the inoculum in South Africa (Burgess and Wingfield 2002). This assumption has not been tested experimentally.

The objectives of this study were to investigate the transmission, presence and prevalence of *D. pinea* in seeds, seedlings from nurseries and the field, and to determine how seedlings become infected with the fungus. Pine seedlings were collected from different sources and isolation of the fungus conducted in the laboratory. Surface disinfected seeds of *P. radiata* and *P. patula* were also inoculated with spore and mycelium suspensions to see its effect on germination and persistence in seedlings in the greenhouse.

Materials and Methods

Isolation from seeds

Seeds of *P. radiata* and *P. patula* were obtained from Karatara and Ngodwana nurseries, South Africa, respectively. Seed were surface disinfested in 70 % EtOH for one minute and 3.5 % NaOCl for two minutes followed by 70 % EtOH for one minute. These treated seeds were then repeatedly washed using distilled water until clean water flowed out of the sieve. The seeds were then blotted on autoclaved filter paper and dried under room temperature. 100 seeds from each species were randomly taken and plated in five Petri dishes (i.e. 20 seeds per Petri dish) containing 1 % water agar (WA) m/v and incubated at 25 ^{0}C in the dark. This was repeated for an additional 200 *P. radiata* and *P. patula* seeds at two different times to have representative information.

Growth of *D. pinea* was monitored daily, starting two days after incubation. Colonies appearing to represent *D. pinea* were transferred to fresh malt extract agar. Their identities were confirmed based on their cultural characteristics and ITS rDNA sequences comparison (De Wet et al. 2003).

Seed inoculation and germination

Pinus radiata seeds were inoculated with mycelium and spore suspensions of *D. pinea*, strain CMW29144, to assess its effect on germination. Surface disinfested seeds were dipped into a mycelial suspension made from a 10 day-old culture on 2 % liquid malt extract (2 % m/v Biolab malt extract) and 100 seeds were immediately plated on 1 % water agar (WA) (1 % m/v Biolab agar). Another set of *P. radiata* seeds was dipped in spore suspensions collected by vortexing pycnidia in Eppendorf tubes using sterile distilled water. One hundred of these seeds were plated

on 1 % WA the day after treatment and 100 seeds were plated after 20 days of storage at room temperature. Equal numbers of surface disinfested non-inoculated seeds were used as a control treatment. Analysis of variance was conducted and treatment effects (control, inoculation with mycelium suspension, inoculation with spore suspension and planted one day after treatment and after 20 days of storage) were tested at the 5 % probability level. Mean separation was done using Student's t-test (P = 0.05) in JMP-5 (SAS Institute Inc., Carry, North Carolina).

Treated seeds were allowed to germinate and germination success was recorded from day 5. A seed was considered germinated when the radical protruded through the seed coat. Germlings were observed on the radical and plumule of the seedlings for defects due to the fungus. Germinating *P. radiata* seedlings, 10 spores treated and 10 non-treated controls were transferred to pots containing peat moss soil and placed in a greenhouse. These plants were allowed to establish for two months before they were harvested for *D. pinea* isolation.

Isolation of Diplodia pinea from seedlings

Isolations of *D. pinea* were done from seedlings raised from 1) commercial nurseries 2) naturally regenerated seedlings within a plantation 3) seeds and seedlings grown in the greenhouse and 4) planted seedlings in an open field.

One hundred asymptomatic *P. patula* seedlings were collected from each of three commercial nurseries in the Sabie area. In addition, approximately 65 naturally regenerated two-year-old seedlings were collected alongside plantations of mature *P. patula.* A second set of approximately one-year-old transplanted seedlings were collected from a cleared pine stand, but where there were no mature trees in the close proximity, close to Ngodwana, Mpumalanga. Plants were collected in separate plastic bags and taken to the laboratory for isolation.

In order to assess the presence of *D. pinea* in the seedlings raised under a controlled environment in the greenhouse, surface sterilized *P. radiata* seeds were sown in a tray containing peat moss soil. When germinated, seedlings were transferred into 11 cm diameter polyethylene bags. One year after transplanting, 100 seedlings were cut at the soil surface and isolation of the fungus was undertaken on 2.0 % MEA (2 % m/v Biolab malt extract, 1.5 %m/v Biolab Agar).

Persistence of Diplodia pinea in seedlings

Persistence of *D. pinea* was examined by inoculating the terminal shoots of one-year-old *P. radiata* seedlings with a 5 µl spore suspension at a concentration of 5.0 x 10^5 spores of strain CMW29144 per millilitre. The suspension was applied using micropipette without damaging the shoots. The same amount of distilled water was applied on five equal sized seedlings for the control. The inoculated seedlings were placed in a greenhouse, covered by polyethylene sheets for the first two days to maintain humidity required for spore germination, after which plants were watered regularly. Five months after inoculation, isolations were made from all inoculated and non-inoculated seedlings. Isolates recovered were characterized using microsatellite markers as described by Bihon et al. (2010) to confirm their identity with the strain used for inoculations.

Results

Isolation of D. pinea from seeds

Of a total of 300 *P. radiata* seeds tested at three different times (100 seeds at a time), *D. pinea* was isolated from seven seeds (2.3 %) as confirmed from their culture morphology and ITS sequences. No *D. pinea* was isolated from the 300 *P. patula* seeds.

Seed inoculation

Of the 100 surface treated *P. radiata* seeds that were inoculated with a mycelial suspension of *D. pinea*, 87 % germinated and 91 % of the non-treated control seeds were germinated. There was no significance difference between total germination percentage of mycelium suspension treated and control seeds at $P = 0.05$ (Fig. 3). There was some indication that seeds inoculated with a mycelial suspension germinated more slowly than the control seeds (Fig. 1). Thus, on the 7th day after plating on water agar, 43 % of control seeds had germinated compared to only 19 % of the treated seeds.

Treatment of seeds in the spore suspensions of *D. pinea* significantly reduced germination percentage (Fig. 2). In this experiment, 76 % of 100 seeds that were inoculated and immediately plated had germinated, whereas only 53 % of 100 seeds that had been inoculated and stored for 20 days germinated. The rest of these seeds never germinated. There were significant differences of total percentage germination between treatments at $P = 0.05$ indicated in fig. 3.

Lesions were observed on the radicals of some of the seedlings with both mycelium and spore treatments, while the non-treated controls showed no symptoms. No *D. pinea* could be isolated from ten seedlings germinated from the seed treated with a spore suspension after 2 months of growth in the greenhouse, which was also the case with seedlings from non-treated seeds.

Isolation from nursery and field-collected seedlings

No isolates of *Diplodia pinea* were isolated from any of the 300 *P. patula* seedlings from three nurseries (100 seedlings each) randomly sampled in the Sabie area. *Diplodia pinea* could also

not be isolated from the 100 one-year-old seedlings grown from seeds in a greenhouse and transplanted seedlings in the open fields. In contrast the fungus was isolated from 26 out of 65 (40 %) naturally regenerated approximately 1 to 2 years old seedlings collected alongside mature trees.

Persistence in inoculated plants

Diplodia pinea was recovered from 17 out of 25 seedlings (68 %) that were inoculated with a suspension of spores five months prior to termination of this experiment. The fungus was not isolated from control seedlings treated with distilled water. Microsatellite markers confirmed that the genotype of the recovered isolates was the same as that of the strain used in the inoculation (CMW29144).

Discussions

Results of this study confirmed that *D. pinea* is transmitted horizontally from mature trees, while no evidence could be found for any form of vertical transmission through seeds, as was thought previously. The fungus was isolated from surface treated *P. radiata* seeds very infrequently, but not from *P. patula* seeds. It was not present in seedlings collected from nurseries or seedlings grown in the greenhouse, but was recovered from seedlings growing under mature trees. These results suggest that mature trees may be a major source of inoculum of *D. pinea* in South Africa. An inoculation study also showed that *D. pinea* persists in inoculated *P. radiata* seedlings for at least five months after infection.

Diplodia pinea does not appear to frequently infect *Pinus* seeds. Previous studies reported isolating this fungus at a frequency of 4.6 % from seeds of 12 *Pinus* species (Vujanovic

et al. 2000). In another study, *D. pinea* was not obtained from healthy non-contaminated seeds, but was isolated from seeds collected from fallen cones on debris beneath trees (Fraedrich et al. 1994). The low rates of isolation in these previous studies and in the current study (2.3 % on *P. radiata* and 0 % on *P. patula*) suggest that seed transmission only plays a small part in the spread of *D. pinea*, but when large amounts of seed are transported from infested sources the cumulative threat might be significant.

The presence of the fungus in the seed is not necessarily an indication of transmission directly from the tree, as the seed could have acquired the fungus in the process of seed collection. High diversity of *D. pinea* genotypes in South Africa were previously reported (Burgess et al. 2004; Bihon et al. 2010) which could be due to multiple introductions, the presence of cryptic sex or mutation (Bihon et al. 2010). It was previously thought that imported seeds might account for the large diversity of *D. pinea* in South Africa (Burgess and Wingfield 2002). However, infected needles and cone tissues in seed batches could be the alternative sources of inoculum. It is also likely that seedlings or cuttings containing the fungus were imported into the country over the last three centuries and they could have contributed to introductions of the fungus.

Inoculation of seeds using a mycelial suspension of *D. pinea* affected germination speed, but the maximum percentage germination was not affected. Inoculation with a conidial suspension of *D. pinea*, not only reduced the speed of germination, but also significantly reduced percentage germination. These results are in agreement with previous studies. For example, *Diplodia pinea* has been reported to cause mortality and reduce total germination of seeds of *P. oocarpa*, *P. caribaea* and *P. pseudostrobus* after inoculation with conidia (Rees and Webber 1988). Mortality of seeds due to inoculation with *D. pinea* can be due to the fact that the fungus

could physically enter the seed coat via cracks or when the seed case opens prior to germination (Fraedrich et al. 1994; Rees and Webber 1988). Alternatively the fungus may penetrate the testa physically or using enzymes (Rees and Webber 1988). Once the fungus is inside the seed, it can affect the germination process of the seed and ultimately kill the embryo. On the other hand, the effect of mycelial suspension on speed of germination could be due to physical interference of the mycelium itself on the plate through competition for resources, rather than pathogenicity of the fungus (Gure et al. 2005).

In this study we show that *D. pinea* does not directly infect germinating seedlings. No infection could be detected in seedlings, even from seeds that were inoculated with conidia. Possible reasons for this could be that the seedlings had been raised from non-infected seeds or that *D. pinea* is present as small localized infections within a seedling which makes it difficult to isolate (Flowers et al. 2006). More likely, however, is that spread and infestation of *D. pinea* is exclusively horizontal, similar to other tree endophytes (Arnold et al. 2003 & 2007, Ganley and Newcombe 2006). This was also shown in previous studies where only one type of fungal endophyte was isolated from 2 % of *Pinus monticola* seeds, but fungal endophytes were isolated from 57 % of needles tested (Ganley and Newcombe 2006). In other seed-borne fungi (e.g. *Cladosporium cladosporides*, *Epicoccum purpurascens*), no seedlings raised from inoculated seeds of white pine (*P. strobes*) and white spruce (*P. glauca*) were infested after they were transferred to sterilized soil medium (Mittal and Wang 1993). The absence of the fungi in the seedlings raised from seeds therefore strengthen the previous reports that *D. pinea* infects seedlings when they are planted in the field, especially in close proximity to mature trees (Ganley et al. 2003).

Seedlings grown from various nurseries and open fields in South Africa were found to be free of *D. pinea*, in contrast to naturally regenerated seedlings collected alongside a mature pine plantation that had fairly high levels of infection. This was in contrast to the situation in USA where high seedling mortality was recorded in nurseries (Palmer and Nicholls 1985; Stanosz and Carlson 1996; Stanosz et al. 2007). In South Africa seedlings are raised in trays on metallic beds that are closed with screens, whereas in USA seedlings are often raised bare rooted under open conditions and surrounded by mature Pine tree windbreaks (Stanosz and Carlson 1996; Stanosz et al. 2005; Stanosz et al. 2007). Various authors have also shown that pruned branches from trees are sometimes left inside or around USA nurseries that contribute to inoculum levels in the nurseries (Stanosz and Carlson 1996; Stanosz et al. 2005; Stanosz et al. 2007).

Persistence of *D. pinea* as an endophyte in pine seedlings was confirmed by inoculating seedlings with an isolate of the fungus and recovering the same genotype five months later from healthy tissue. *Diplodia pinea* has previously been isolated from asymptomatic pine seedlings and mature trees, but the persistence of these infections have never been considered (Flowers et al. 2006; Flowers et al. 2001; Smith et al. 2002; Stanosz et al. 2005). In a recent study *D. pinea* has also been shown to be present in well-structured, healthy wood of *P. patula* trees (Bihon et al. 2010) and is most likely as the result of infections at different growth stages of the tree. *Diplodia pinea,* therefore, appears to be able to persist for very long periods of time in young and older tissues of a pine tree without causing disease.

In conclusion, it would appear that the South African nursery management conditions protect seedlings from infection by *D. pinea* and so reduce the transmission of the disease. Transmission of the pathogen is mainly horizontal through conidia that originate from mature pine trees. Planting disease free seedlings in close proximity to older pines, or where there is

debris from previous plantations, could contribute to their eventual infection. Removal of the diseased trees, twigs and debris from previous harvests is thus essential to inhibit conidial spread. Despite the fact that the fungus is present at a low level in seeds, it remains a potential route of introduction of new genotypes and thus movement of seed should be carefully monitored.

References

Arnold AE, Mejia LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences, USA **100**: 15649 – 15654.

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biology Reviews **21**: 51 – 66.

Bihon W, Burgess T, Bernard S, MJ Wingfield, BD Wingfield (2010) Distribution of *D. pinea* and its genotypic diversity within asymptomatic *P. patula* trees. Australasian Plant Pathology (*in press*).

Blodgett, JT, Kruger EL, Stanosz GR (1997) *Sphaeropsis sapinea* and water stress in a red pine plantation in central Wisconsin. Phytopathology **87**: 429 – 434.

Burgess T, Wingfield MJ (2002) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the southern hemisphere. International Forestry Review **4**: 56 – 65.

Burgess T, Wingfield MJ, Wingfield BD (2004) Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australasian Plant Pathology **33**: 513 – 519.

Carroll G (1988) Fungal Endophytes in Stems and Leaves: From Latent Pathogen to Mutualistic Symbiotic. Ecology **69**: 2 – 9.

De Wet J, Burges T, Slippers T, Preisig O, Wingfield BD, Wingfield MJ (2003) Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. Mycological Research **107**: 557 – 566.

Ernst M, Mendgen KW, Wirsel SGR (2003) Endophytic Fungal Mutualists: Seed-borne *Stagonospora* Spp. enhance reed biomass production in Axenic Microcosms. Molecular Plant-microbe Interaction **16**: 580 – 587.

Flowers J, Nuckles E, Hartman J, Vaillancourt L (2001) Latent infection of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. Plant Disease **85**: 1107–1112.

Flowers J, Hartman J, Vaillancourt L (2003) Detection of latent *Sphaeropsis sapinea* infections in Austrian pine tissues using nested polymerase chain reaction. Phytopathology **93**: 1471 – 1477.

Flowers J, Hartman J, Vaillancourt L (2006) Histology of *Diplodia pinea* in diseased and latently infected *Pinus nigra* shoots. Forest Pathology **36**: 447 – 459.

Fraedrich SW, Miller T, Zarnoch SJ (1994) Factors affecting the incidence of black seed rot in Slash pine. Canadian Journal of Forest Research **24**: 1717 – 1725.

Ganly RJ, Brunsfeld SJ, Newcombe G (2003) Species diversity of fungal endophytes in *Pinus monticola*. In Proceedings of the 8th International Congress of Plant Pathology. Offered paper, P. 152.

Ganly RJ, Newcombe G (2006) Fungal endophytes in seeds and needles of *Pinus monticola*. Mycological Research **110**: 318 – 327.

Gure A, Slippers B, Stenlid J (2005) Seed-borne *Botryosphaeria* spp. from native Prunus and Podocarpus trees in Ethiopia, with a description of the anamorph *Diplodia rosulata* sp. nov. Mycological Research **109**: 1005 – 1014.

Mittal RK, Wang BSP (1993) Effects of some seed-borne fungi on *Picea glauca* and *Pinus strobus* seeds. European Journal of Forest Pathology **23**: 138 – 146.

Palmer MA, Nicholls TH (1985) Shoot blight and collar rot of *Pinus resinosa* caused by *Sphaeropsis sapinea* in forest tree nurseries. Plant Disease **69**: 739 – 740.

Palmer MA, Stewart EL, Wingfield MJ (1987) Variation among isolates of *Sphaeropsis sapinea* in the North Central United States. Phytopathology **77**: 944 – 948.

Rees AA, Webber JF (1988) Pathogenicity of *Sphaeropsis sapinea* to seed, seedlings and saplings of some central American pines. Transactions of British Mycological Society **91**: 273 – 277.

Schardl CL (2001) *Epichloë festucae* and related mutualistic symbionts of grasses. Fungal Genetics and Biology **33**: 69 – 82.

Slippers B, Wingfield MJ (2007) Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. Fungal Biology reviews **21**: 90 – 106.

Smith H, Wingfield MJ, Coutinho TA (2002) The role of latent *Sphaeropsis sapinea* infections in post-hail associated die-back of *Pinus patula*. Forest Ecology and Management **164**: 177 – 184.

Stanosz GR, Carlson JC (1996) Association of mortality of recently planted seedlings and established saplings in red pine plantations with *Sphaeropsis* collar rot. Plant Disease **80**: 750 – 753.

Stanosz GR, Blodgett JT, Smith DR, Kruger EL (2001) Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. New Phytologist **149**: 531 – 538.

Stanosz GR, Smith DR, Albers JS (2005) Surveys for asymptomatic persistence of *Sphaeropsis sapinea* on or in stems of red pine seedlings from seven Great Lakes region nurseries. Forest Pathology **35**: 233 – 244.

Stanosz GR, Smith DR, Leisso R (2007) *Diplodia* shoot blight and asymptomatic persistence of *Diplodia pinea* on or in stems of jack pine nursery seedlings. Forest Pathology **37**: 145 – 154.

Swart WJ, Wingfield MJ (1987) Conidial dispersal of *Sphaeropsis sapinea* in three climatic regions of South Africa. Plant Disease **71**: 1038 – 1040.

Swart WJ, MJ Wingfield (1991) Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. Plant Disease **75**: 761 – 766.

Vujanovic YV, St-Arnaud M, Neumann PJ (2000) Susceptibility of cones and seeds to fungal infection in a pine (*Pinus* spp.) collection. Forest Pathology **30**: 305 – 320.

Zwolinski JB, Swart WJ, Wingfield MJ (1990) Economic impact of a post-hail outbreak of dieback induced by *Sphaeropsis sapinea*. European Journal of Forest Pathology **20**: 405 – 411.

Figure 1. Cumulative percentage germination of non-treated (solid line) and treated (broken line) *P. radiata* seeds treated in *D. pinea* mycelial suspensions.

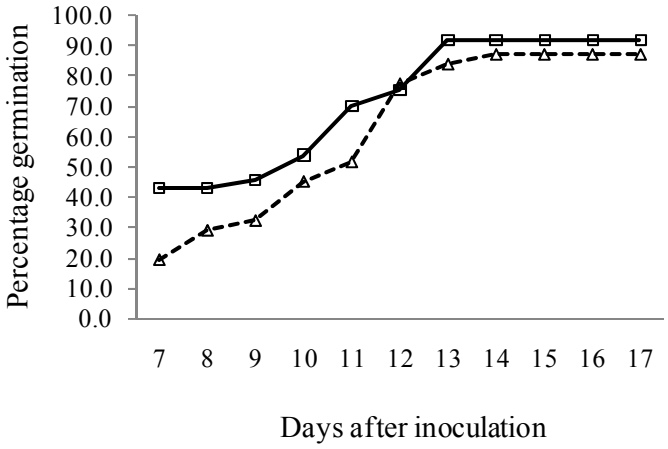


Figure 2. Cumulative percentage germination of *P. radiata* seeds that are not treated (-♦-and solid line), and treated in *D. pinea* spore suspensions, and immediately plated (-■-), or plated after 20 days of storage (-●-).

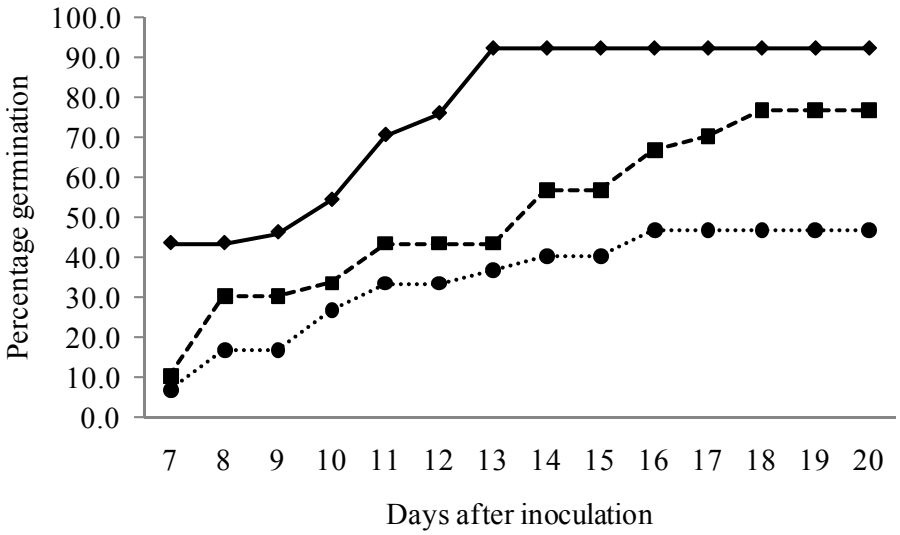
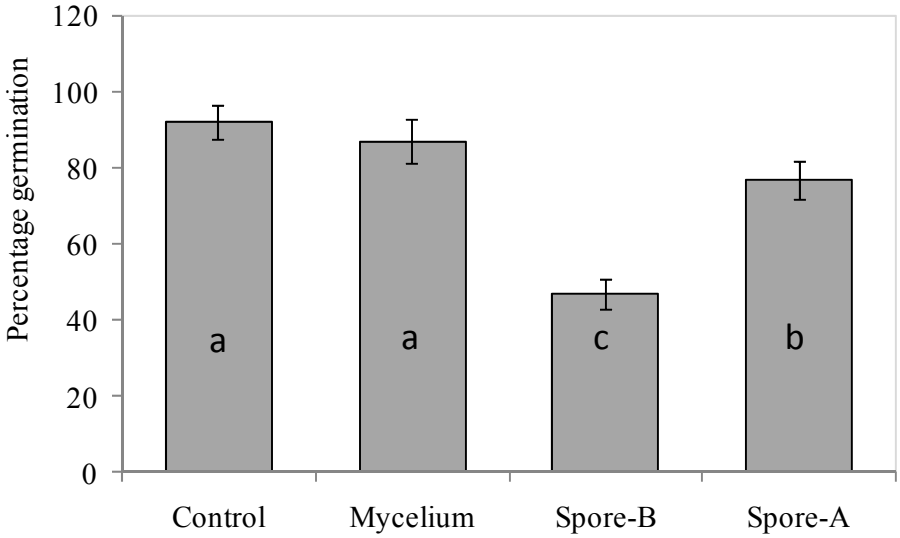


Figure 3. Comparison of four treatments on the total percentage germination of *P. radiata* seeds



Treatments with different letters in the bar are significantly different at P = 0.05. Spore-A = Seeds treated with spore suspension and plated the following day, Spore-B = Seeds treated with spore suspension and plated after 20 days of storage.

Summary

This study has significantly broadened and deepened the understanding of ecological aspects related to the spread and reproduction of *Diplodia pinea* as an endophyte, latent pathogen and causal agent of serious disease problems in plantations of *Pinus* spp. Analyses of genetic diversity in populations using microsatellite data has revealed very high levels of genetic diversity of populations of the pathogen at different spatial levels, ranging from within a single asymptomatic tree to within and between plantations over large geographic areas and in different countries where the fungus has been introduced. Analysis of the structure of the distribution of genotypes and the association of alleles within populations, suggest that sexual recombination is occurring in most environments in the Southern Hemisphere *D. pinea* populations. This indicates the presence of a cryptic sexual state in this fungus. The genetic diversity was structured and differentiated for regions separated by as little as 65 km to a country and continental scale. The diversity and likely sexual reproduction of *D. pinea* must complicate control strategies such as selection and breeding for resistance. It is thus essential to strengthen quarantine services aimed at minimizing the risk of introducing additional genotypes of *D. pinea*. In this regard, understanding the infection and spread between regions is essential. Results of this study demonstrate that this fungus infects seeds, but only at low levels, and is not transmitted vertically via seeds to seedlings. *Diplodia pinea* was also not isolated from seedlings in three commercial nurseries and open fields in South Africa. These results provide strong evidence that neither seeds nor seedlings are the primary sources of inoculum, but that the pathogen is mainly transmitted horizontally from mature trees and debris left in plantations. Finally extensive sampling conducted as part of this study led to the discovery of the sibling species, *D. scrobiculata* in South Africa and outside the Northern Hemisphere for the first time.