Bihon, W., Burgess, T., Slippers, B., Wingfield, M.J. and Wingfield, B.D. (2011) Distribution of Diplodia pinea and its genotypic diversity within asymptomatic Pinus patula trees. Australasian Plant Pathology, 40 (5). pp. 540-548.

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

Wubetu Bihon[†], Treena Burgess[†*], Bernard Slippers[†], Michael J. Wingfield[†] and Brenda D. Wingfield[†]

[†]Department of Genetics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Lunnon Road, Hillcrest, Pretoria 0002, South Africa;

[*]School of Biological Sciences and Biotechnology, Murdoch University, Perth 6159, Australia.

Corresponding author: Brenda.wingfield@fabi.up.ac.za

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.

Key Words: Microsatellite markers, population diversity, vegetative compatibility groups, persistence

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*. 2004). 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*. (2004) 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 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 disturbed 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 (VCG's) and microsatellite markers are frequently used techniques to characterize and study genotypic diversity of fungi species. VCG's are 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 used showed high allelic diversity in populations of *D. scrobiculata* (Burgess *et al*. 2004a) the allelic diversity in populations of *D. pinea* was relatively low with most markers having a single dominant allele (Burgess *et al*. 2004b).

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 with 12 to 15 years of age at Balgown, in the KwaZulu-Natal midlands and two trees 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. (2004) 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 mobility of SSR products were compared to that 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. AABDCGDD). 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. 2001) (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 polymorphic loci within a tree ranged between 46 % and 69 %. Of the 36 alleles, nine were found in all five trees and 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 VCG's (Table 1). The 13 isolates obtained from Tree 1 were represented by eight VCG's. 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 VCG's were from within the same tree and from branches, but there were also VCG's shared between isolates from stem and branch or branch and cone. VCG's were shared only

between Tree 2 and 3 which were harvested within 50 m of each other in Mpumalanga.

Overall, there were fewer VCG's than microsat 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. The two isolates representing VCG 6 and 7 had the same microsatellite haplotypes. However the third isolate with the same microsatellite haplotype belonged to a different VCG.

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 (un published 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*. 2001b) 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 obtained from a single needle (Muller *et al*., 2001). Maximum infection and diversity reached when trees age and have increased foliage (McCutcheon and Carroll 1993; Gamboa and Bayman 2001). Moreover, mutations and natural recombination of *D. pinea* genotypes due to cryptic sexuality which have not been reported earlier in this fungus 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 breading for resistance and 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 endophtic infections when pine trees are subjected to physical or physiological stress.

Acknowledgements

We acknowledge the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB), Tree Pathology Co-operative Program (TPCP), National Research Foundation (NRF) and the International Foundation for Science, Stockholm, Sweden for financial support. We also thank Hardus Hatting, Brett Hurley and Jolanda Roux for assistance with the collection of samples and Mesfin Bogale is also acknowledged for his assistance in SSR primer design and validation.

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 (2004b) 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 (2004a) Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. *Mycological Research* **108**, 1399–1406.

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. Proceeding of National Academics 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. *Proceeding of the National Academy of Sciences of the United States of America* **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 (1991a) 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

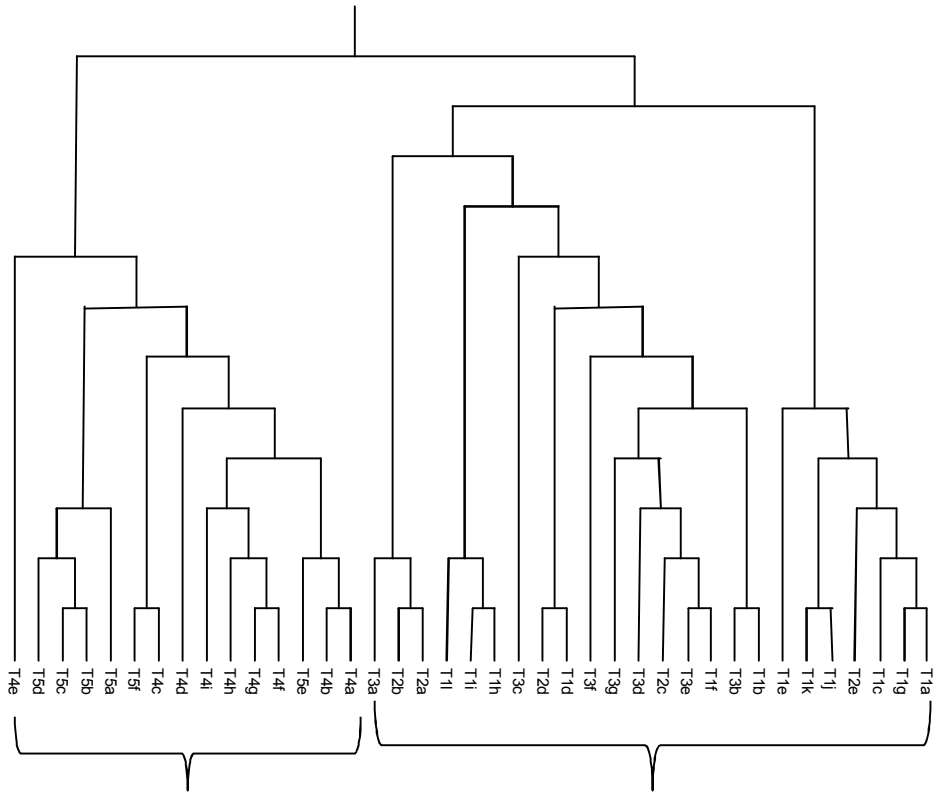
The forward primers were labelled with a phosphoramidite fluorescent dye indicated as FAM, NED, PET and VIC. ( ) Parenthesis under the core repeat motifs column indicate repeated nucleotides, subscript numbers indicates the number of repeats.

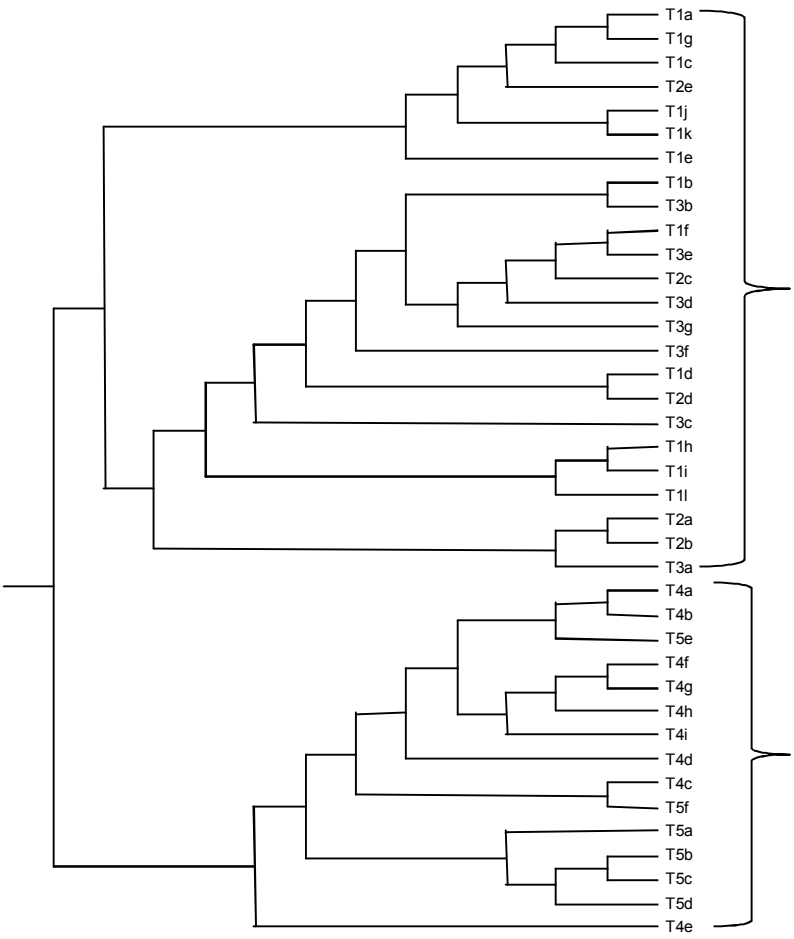
Table 3. Allele size (bp) and frequency at 13 SSR loci for *D. pinea* isolates from five *Pinus patula* trees used in this study

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.

Fig. 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.




T1a
T1g
T1c
T2e
T1j
T1k
T1e
T1b
T3b
T1f
T3e
T2c
T3d
T3g
T3f
T1d
T2d
T3c
T1h
T1i
T1l
T2a
T2b
T3a
T4a
T4b
T5e
T4f
T4g
T4h
T4i
T4d
T4c
T5f
T5a
T5b
T5c
T5d
T4e
Isolates from tree one to three
Isolates from four to five