

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

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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 °C and isolations were undertaken within two days.

Four pieces of wood (approximately 2.5 mm²) 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 °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 H₂O. 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)₅G, 5'HVH(GTG)₅, 5'NDB(CA)₇C,

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 °C and 60 °C. 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 Biosystems).

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 $H = 1 - \sum x_k^2$, 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 breeding 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 endophytic infections when pine trees are subjected to physical or physiological stress.

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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	CACAAAACBCAB	10	
	CMW29149	Branch	CAAAAABADABCB	11	
	CMW29150	Branch	BACAAAACABAB [*]	1	
	CMW29151	Branch	BACAAAACABAB [*]	1	
	CMW29152	Branch	BACAAAACBBCB	1	
	CMW29153	Branch	BACAAAACBAAB	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	BACAABBBBCBAAB	2	
	CMW29172	Cone	BACAAABABABCC	4	
Tree 3	CMW29168	Stem	BACAAABACABCB	14	
	CMW29169	Stem	BACAAAACABCB	4	
	CMW29170	Stem	BACAAABADABCB	15	
	CMW29157	Branch	BACAAABACABCB	3	
	CMW29158	Branch	BACAAAACAAAB	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	BACAABBBBCABCD	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 motifs	Fragment Length (bp)
WB1-a	SS12	PET -ACC ACC ACC ACC GTC AAG	62	(ACC) ₉ AC	107
WB1-b		GAA CGC CAT CGT CGT CAC			
WB2-a	SS13	FAM -GGC GTG TGT GAT GAG ATG AG	55	(CGAGA) ₄ CGAGC	180
WB2-b		GTC CTT TGT GTG TTG GGT TG			
WB4-a	SS14	NED -CAC CAC CAC CAA CAC CTT G	58	(CTT) ₅ (CCT) ₉	149
WB4-b		CGT GTT GGA AGC GAC GAC			
WB7-a	SS15	NED -GAA TCA CTG GCC GGT TTG	55	(GGA) ₅ AGA (GGA) ₄	99
WB7-b		GAG TCC AGC CTT TCC TCC TC			
WB8-a	SS16	VIC -GGG GAA AAG ACG TGT TGT TGT	55	(GA) ₁₁	99
WB8-b		CAG CAT CGT CGT CCC ATT AG			

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	<i>H</i>
SS1	377	0.273	0.286	0.279
	408	0.615	1.000	1.000	0.091	...	
	409	0.384	0.636	0.714	
SS2	193	1.000	1.000	0.857	1.000	1.000	0.049
	195	0.143	
SS5	499	0.154	0.500	0.143	0.368
	500	0.091	...	
	501	0.846	0.500	0.857	0.727	0.286	
	502	0.182	0.714	
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	0.132
	258	0.077	1.000	1.000	
	260	...	0.167	0.143	
SS10	279	0.615	0.167	0.143	0.402
	313	0.308	0.833	0.857	0.273	0.571	
	315	0.077	0.727	0.429	
SS11	171	0.769	0.667	1.000	0.192
	172	0.231	0.333	...	0.909	1.000	
	190	0.091	...	
SS12	98	0.077	0.261
	111	0.143	...	0.714	
	112	0.846	1.000	0.286	1.000	0.143	
	115	0.077	...	0.143	...	0.143	
SS13	156	0.615	0.833	1.000	1.000	1.000	0.150
	172	0.385	0.167	
SS14	159	0.462	0.167	0.286	0.818	0.857	0.359
	160	0.462	0.833	0.714	0.182	0.143	
	170	0.077	
SS15	62	0.539	0.833	0.361
	68	...	0.167	0.143	0.182	0.571	
	70	0.462	...	0.857	0.818	0.429	
SS16	98	0.143	0.089
	100	1.000	1.000	0.714	
	101	0.143	
	107	1.000	1.000	
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	
<i>H</i>		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.



