High genetic diversity of *Fusarium circinatum* associated with the first outbreak of pitch canker on *Pinus patula* in South Africa

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Abstract

The disease known as pitch canker results from infection of *Pinus* species by the fungus *Fusarium circinatum*. This fungus also causes a serious root disease of *Pinus* seedlings and cuttings in forestry nurseries. *Pinus radiata* and *P. patula* are especially susceptible to the pathogen, but there are no records of pitch canker on *P. patula* in established plantations. To date, only planting material of this tree species in nurseries or in plantations at the time of establishment have been infected by *F. circinatum*. Symptoms of pitch canker have recently emerged in an established *P. patula* plantation in South Africa and this study sought to determine whether the symptoms were caused by *F. circinatum*. Isolates from cankers were identified as *F. circinatum* using morphology and DNA-based diagnostic markers. Microsatellite markers were then used to determine the genetic diversity of a collection of 52 isolates. The entire population included 17 genotypes representing 30 alleles, with a greater number of genotypes collected from younger (three to six-year-old) than older (12 to 19-year-old) trees. Both mating types of *F. circinatum* were present, but no evidence of sexual recombination was inferred from population genetic analyses. This is the first record globally, of pitch canker on *P. patula* trees in managed plantations. It is of significant concern to South Africa, where *P. patula* is the most important *Pinus* species utilized for plantation forestry.

Keywords: microsatellite markers, plantation forestry, population genetics, tree disease

Introduction

Plantation-grown tree species are increasingly threatened by fungal pathogens (Wingfield *et al*. 2015). The ascomycete fungus *Fusarium circinatum*, for example, poses a significant threat to *Pinus* species in almost all of the regions globally where these trees are used to establish commercial plantations, as well as to native *Pinus* species in Europe and the United States of America (USA) (Gordon *et al*. 2001, Wingfield *et al*. 2008, Bezos *et al*. 2017). On established plantation trees, *F. circinatum* causes a disease known as pitch canker, which is characterized by resinous stem and branch cankers (McCain *et al*. 1987, Gordon *et al*. 2001). In production nurseries, *F. circinatum* causes a disease of *Pinus* seedlings, which is characterized by root and collar rot and high levels of mortality (Viljoen *et al*. 1994, Wingfield *et al*. 2008). Root and collar rot also develop in the field when nursery-infected, but asymptomatic plants are used for the establishment of plantations, resulting in significant post-planting mortality (Crous 2005, Mitchell *et al*. 2011, Morris *et al*. 2014).

Pinus patula (commonly known as Mexican weeping pine) is one of many *Pinus* species that have been reported to be susceptible to *F. circinatum* (Santos and Tovar 1991, Hodge and Dvorak 2000, Mitchell *et al*. 2012). This species is native to Central America and Mexico and it is widely planted as an exotic in tropical and temperate areas at altitudes between 1500 m and 3100 m (Dvorak and Donahue 1992). These include various regions in the Indian sub-continent, South America, Australia and Africa (Gillespie 1992). However, nearly half of the global area planted to *P. patula* occurs in eastern and southern Africa, where it represents the most important softwood plantation species (Dvorak 1997).

The association between *F. circinatum* and *P. patula* was first reported in the early 1990's (Viljoen *et al.* 1994). Initially, the pathogen was shown to cause a devastating root disease of *P. patula* seedlings in a single nursery in the Mpumalanga Province of South Africa (Viljoen *et al.* 1994). It subsequently spread to all planting stock production nurseries in the country (Britz *et al.* 2005). Subsequently, *F. circinatum* began to cause significant mortality at plantation establishment due to the planting of infected, but often asymptomatic seedlings in the field (Mitchell *et al.* 2011). Such problems with *F. circinatum*-associated root disease of planting material in production nurseries and in the field during plantation establishment have also been reported from Colombia where *P. patula* is used for plantation forestry (Steenkamp *et al.* 2012). Although detailed economic impact studies are lacking, research in South Africa suggests that *F. circinatum* represents the most important fungal pathogen of *P. patula* in the nursery environment and it is a significant impediment to the establishment of *P. patula* plantations (Wingfield *et al.* 2008, Mitchell *et al.* 2011, Mitchell *et al.* 2012).

Despite the occurrence of *F. circinatum*-associated root disease in nurseries and on young plants used to establish plantations, the symptoms of pitch canker have never been observed on mature *P. patula* trees in plantations. The manifestation of *F. circinatum* infection in the field has been reported only in Mexico from native stands of *P. patula* in Hidalgo (Britz *et al.* 2001). At that location, the pathogen was isolated from resinous lesions on branches of pitch canker-affected trees, as well as from the cones of trees that were apparently healthy (Britz *et al.* 2001). The possibility of mature or established *P. patula* being infected by *F. circinatum* has significant disease management implications in areas where this tree is planted. Moreover, the occurrence of pitch canker in established *P. patula* plantations in such regions is likely

to be associated with serious losses due to reduced growth, as has been shown for other *Pinus* species (Mitchell *et al*. 2011).

In this study, we considered a recent outbreak of pitch canker-like symptoms on established *P. patula* trees in plantations in the Limpopo Province of South Africa. The first objective was to confirm the presence of *F. circinatum* on symptomatic trees using morphology and DNA-based diagnostic procedures. A second objective was to evaluate the overall management risks associated with this disease outbreak by considering the population biology of the pathogen in this region. For this purpose, we inferred its mode of reproduction and estimated the overall genetic diversity of its population(s) in the region.

Materials and methods

Sample collection

Samples were collected from trees with cankers that ooze resin at four different compartments (designated PAT3, PAT12, PAT19 and PAT46) in established *P. patula* plantations in the Louis Trichardt region of the Limpopo Province of South Africa (Table I; Figure I). The distance between these compartments ranged from 300 to 1500 meters, while their sizes ranged from 2.8 to 8.3 ha. Compartments for sample collection were identified by the local foresters. At each compartment, as many symptomatic trees as could be found were selected and felled to allow collection of diseased plant material. This consisted of cutting sections of branches and stems with resin soaked lesions from trees using a machete or chainsaw. Samples were collected from trees ranging from three to 19-year-old (Table I). The PAT46 samples were collected from four and six-year-old trees that were planted in adjacent compartments (Figure I). In all cases, samples were collected only from trees

displaying pitch canker-like symptoms that were characterized by the presence of dead and dying branches and tree-tops, and large cankers on the main stems and branches from which copious amounts of resin exuded (Figure II a-d).

Table I: Sampling information of the *Fusarium circinatum* isolates obtained from established *P. patula* plantation trees in the Louis Trichardt region of the Limpopo Province.

Collection compartments[a]	Compartment size (ha)	No. of trees sampled	Age (year)	Number of isolates	Date sampled
PAT46*	6	16	4 and 6	24	May 2014
PAT19	8.3	3	19	4	May 2015
PAT3	4.82	9	3	10	July 2015
PAT12	2.796	10	12	14	April 2015

PAT46* represent a combined sample collection from PAT4 (4-year-old trees) and PAT6 (6-year-old trees)

[a]Collection compartments as represented in Figure I

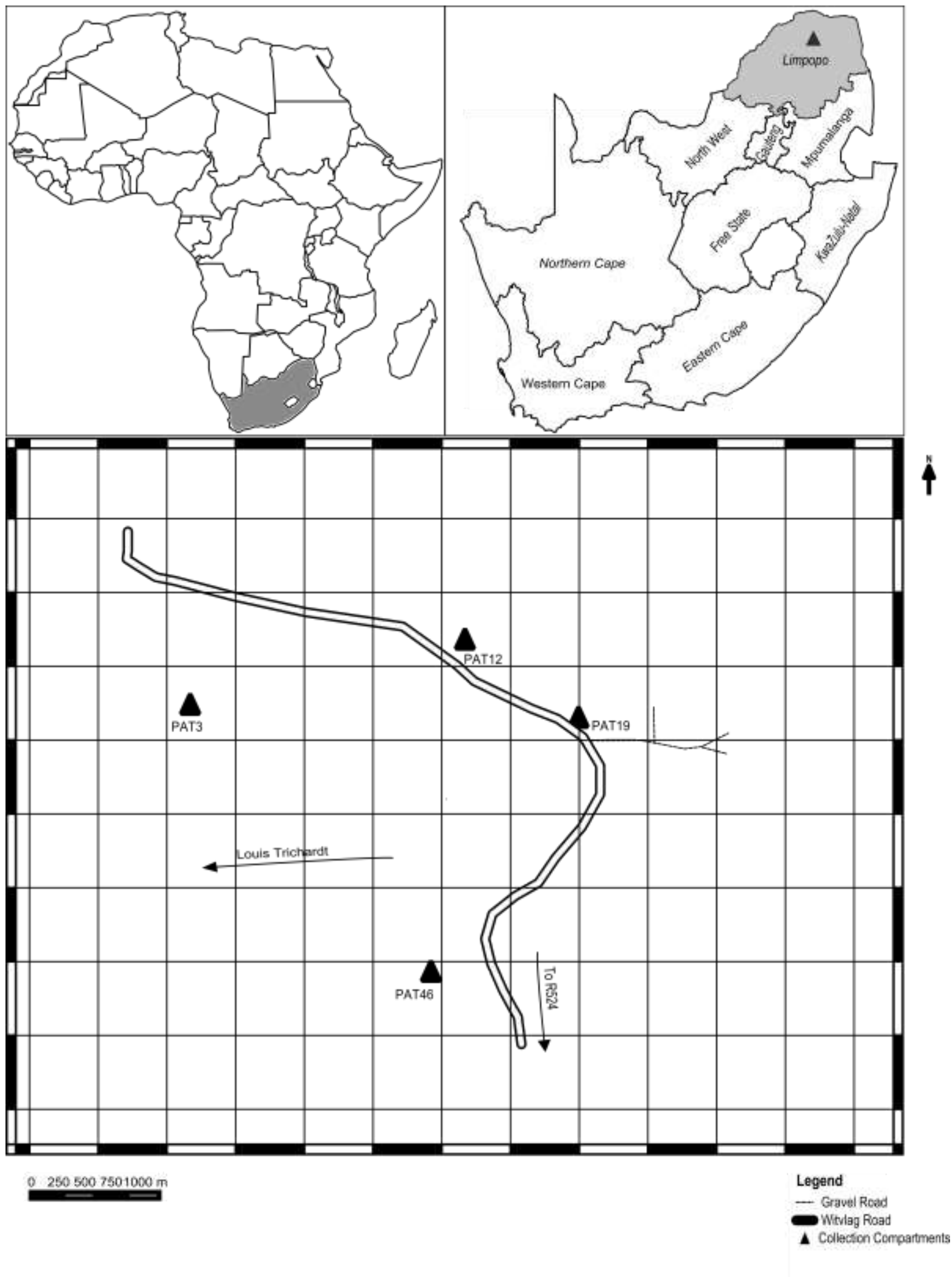

Figure I: Collection compartment location of *Fusarium circinatum* isolates from *P. patula* trees in the Louis Trichardt region of the Limpopo Province, South Africa.

Figure II: Symptoms associated with pitch canker disease of *P. patula* in Louis Trichardt, Limpopo, South Africa. (a) Flagging of branches (b) tip death (c) resin pockets in the cambium and xylem of affected trees (d) resin pockets and pitch soaking of xylem in a cross-section of deformed stem.

Fungal isolation and identification

For fungal isolation, individual plant samples were surface disinfested with 70% ethanol. The epidermis and outer bark layers covering lesions were removed to expose the leading edges of the cankers and at least four tissue pieces (*ca*. 5 mm^2) per lesion were plated onto *Fusarium* selective medium (Leslie and Summerell 2006) and incubated at 25°C. Cultures resembling *Fusarium* were then examined under a light microscope at 100 x magnification for the presence of poly-phialides and conidia on false-heads (Leslie and Summerell 2006). Single hyphal tips were transferred onto 0.5% potassium chloride (KCL) medium to induce sporulation in order to clearly distinguish conidia in chains from false-heads (Nirenberg and O'Donnell 1998). Isolates were then purified by transferring single hyphal tips to potato dextrose agar (20 g dextrose [Biolab], 5 g agar made up to 1 L with distilled water) and incubated for seven days at room temperature. All of the isolates included in this study are maintained in the *Fusarium* culture collection (CMWF) of the Tree Protection Cooperative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Genomic DNA was extracted and quantified from all of the isolates used in this study, as previously described by Fru *et al*. (2017). These DNA samples were then used to identify the isolates using two approaches. The first method involved a *F. circinatum*-diagnostic PCR (Schweigkofler *et al*. 2004, Steenkamp *et al*. 2014) to identify isolates likely representing the pitch canker fungus. Agarose gel electrophoresis was then used to vizualize the diagnostic amplicons.

The second approach involved sequence analysis of the diagnostic portion of the translation elongation factor 1α (TEF) gene (O'Donnell *et al*. 1998). Here, a TEF

fragment was amplifed and sequenced using primers EF1 and EF2 (O'Donnell *et al.* 1998) as previously described by Fru *et al.* (2017). The TEF sequences were then compared, using nucleotide BLAST searches (Altschul *et al.* 1990), to those in the Fusarium-ID database (http://isolate.fusariumb.org/index.php) (Geiser *et al.* 2004) and GenBank (Benson *et al.* 2013). These TEF sequences and representative sequences obtained from GenBank were also used for phylogenetic analysis based on maximum likelihood. As described in Fru *et al.* (2017), the latter involved sequence alignment with MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/), model testing with jModelTest 2.2 (Posada 2008), phylogenetic inference with PhyML 3.0 (Guindon *et al.* 2010; http://www.atgc-monpellier.fr/phyml/), and tree visualization with FigTree (Morariu *et al.* 2008).

Microsatellite and genetic analyses

All of the *F. circinatum* isolates obtained in this study were subjected to microsatellite analysis using the 10 primer sets (FCM2, FCM3, FCM4, FCM6, FCM7, FCM16, FCM20, FCM23, FCM24, FCM25) previously described by Santana *et al.* (2009). For this purpose, the allele sizes for each of the 10 microsatellite loci were determined using the ABI PRISM® GeneMapper v3 (Applied Biosystems) software (Fru *et al.* 2017). The allele data for each isolate were used to infer its corresponding multilocus genotype (MLG). For example, in an isolate with the MLG ASDFGHJKLQ, A was the allele for the first locus, S was the allele for the second locus etc.

Basic population genetic parameters were determined using POPGENE version 1.31 (Yeh *et al.* 1999). These included the number and percentage of polymorphic loci, as well as Nei's (1973) gene diversity (*h*) and Stoddart and Taylor's (1988) genotypic diversity (G). G was esitimated with the equation $G = 1/\sum 1/p^2_i$ where, *pi* is the

observed frequency of the *i*th genotype in the population (Stoddart and Taylor 1988). To account for any possible sample size bias, the maximum genotypic diversity (G*) was estimated using the equation (G/N) x 100, where N is the number of isolates (Chen *et al.* 1994). For the calculation of *h*, the equation $h = 1 - \sum x^2_k$ was used, where x is the frequency of the *k*th allele.

Analyses of allele distribution and differences in allele frequencies within the compartments, based on chi-square (X^2) tests, were done in GenAlEx version 6.3 (Peakall and Smouse 2012). Using POPPR in the R programme (Team 2013), population diversity based on the proportional abundance of MLGs, was also inferred using the Shannon diversity index (SI). The algorithm, $SI = -\sum p_i \ln p_i$ was applied, where p_i is the frequency of *i*th genotype in the population (Sheldon 1969). The SI value was then normalised for population comparison using the equation $H_s = SI/\ln N$, where *N* is the number of individuals in a population. This programme was also used to evaluate the arrangment of MLGs within the populations by applying the Evenness index (E_5) where $E_5 = (G-1)/(e^{Hs} - 1)$ (Grünward *et al.* 2003).

The population differentiation parameter theta (θ), which is an estimate of Wright's Fixation Index (F_{ST}) (Wright 1978), was estimated using Multilocus version 1.2 (Agapow and Burt 2000). The equation $\theta = (Q - q) / (1-q)$, was used, where Q is the probability that two alleles in a single population are the same, q the probability that two alleles from different populations are the same. The programme Network 5.0.0.0 (Fluxus Technology Ltd) was used to generate a minimum spanning network to infer the relationship among MLGs. Genetic diversity versus the number of loci for the entire population was tested with Multilocus to determine if a sufficient number of isolates and loci were used to infer statistically significant conclusions from the study.

Mode of reproduction

The mode of reproduction for the population of *F. circinatum* isolates from Limpopo was inferred using analyses of linkage disequilibrium as implemented in Multilocus (Agapow and Burt 2000). For these analyses, the association between alleles was tested by comparing observed and simulated values (based on 10 000 randomized datasets) for the index of association (I_A) and *rbarD* (r_D) (Smith *et al.* 1993). In these simulations, the null hypothesis (*H*o) of random association in the population was accepted when the observed I_A and r_D values were within the output of the simulated random allele data. To complement these analyses, the mating type of individual isolates was also determined following the mating type diagnostic PCR method with the primers GFmat1a, GFmat1b and GFmat2c, GFmat2d for Mat-1 and Mat-2, respectively, as previously described by Steenkamp *et al.* (2000). Chi-square tests were used to determine whether the observed mating type ratios differed significantly from the expected for random mating when mating type would segregate in a 1:1 Mendelian fashion. Significant deviation from this ratio (P = 0.05) would thus indicate rejection of the null hypothesis of random mating (Milgroom 1996).

Results

Sample collection

Symptoms typical of the pitch canker disease were observed only in one region of the Louis Trichardt area. This was in four compartments ranging in age from three to 19-year-old.

Fungal identification

Fifty-two isolates from 38 diseased *P. patula* trees were tentatively identified as *F. circinatum* based on morphological characteristics. The collection included multiple isolates originating from 11 trees (Table I). The number of isolates per collection compartment ranged from four (PAT19) to 24 in the case of PAT46 (Table I). All isolates produced single-celled, hyaline microconidia aggregated in false-heads on aerial poly-phialides and branched conidiophores (Nirenberg and O'Donnell 1998, Leslie and Summerell 2006). Isolates also produced sterile-coiled hyphae and macroconidia when grown on KCL (Leslie and Summerell 2006). On PDA, these isolates had white to purple aerial mycelium and the undersides of the plates were salmon colored, turning to violet with age (Leslie and Summerell 2006).

Application of the *F. circinatum*-diagnostic PCR (Steenkamp *et al*. 2014) generated the expected 360-bp amplicon from all 52 of the isolates, confirming that they were *F. circinatum*. Analysis of their TEF sequences also indicated that they shared 98% similarity with those of known *F. circinatum* isolates included in the *Fusarium*-ID database (Geiser *et al*. 2004) and GenBank. Phylogenetic analysis of the TEF sequences also showed that a set of representative isolates from *P. patula* cankers in Limpopo resided in a clade including known *F. circinatum* isolates (Figure III).

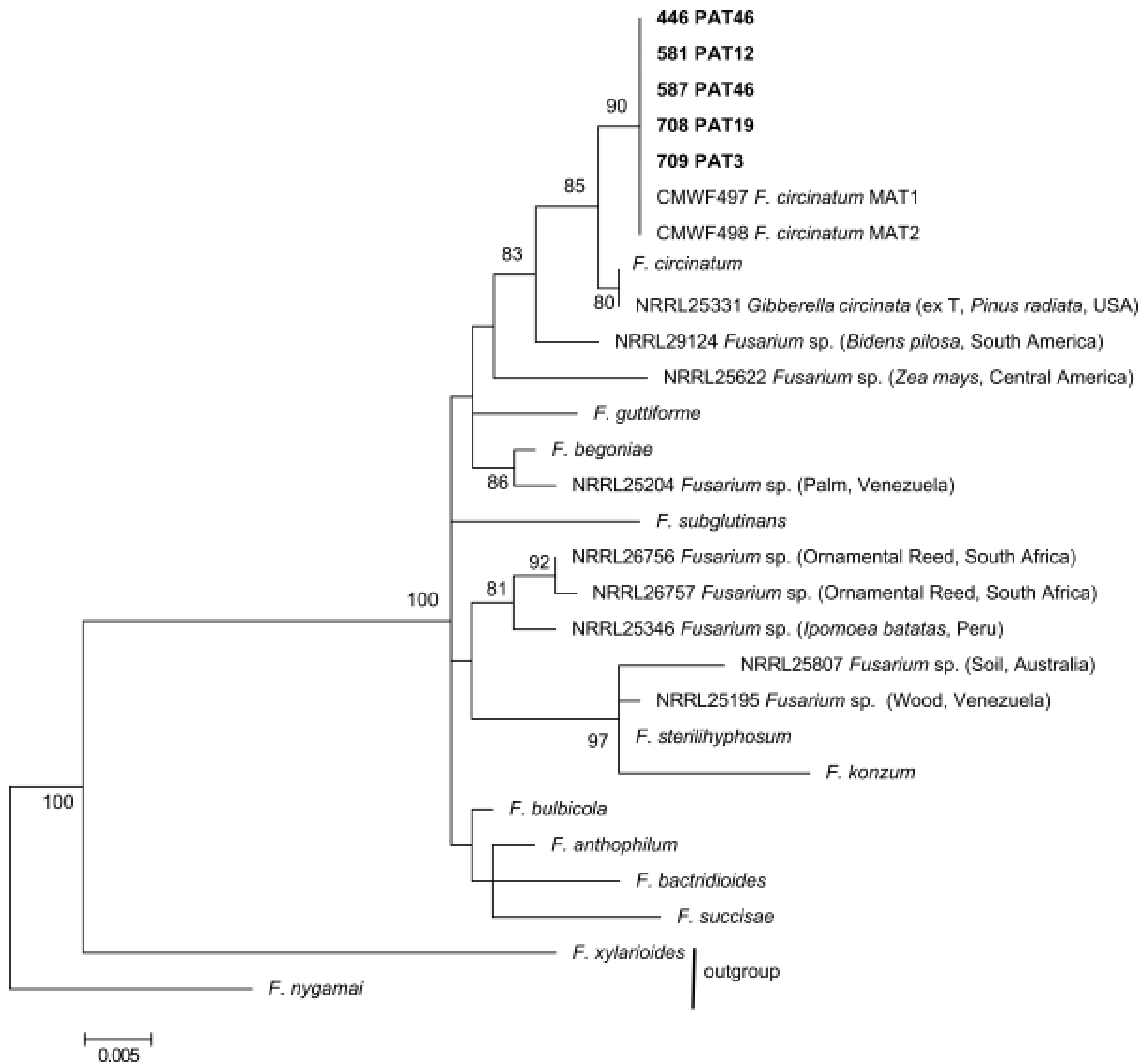

Figure III: Maximum likelihood (ML) tree constructed from TEF sequences including *F. circinatum* mating type tester strain (CMWF497 and CMWF498) and representatives of American clade of the *Fusarium fujikuroi* species complex. *Fusarium circinatum* isolates from *P. patula* appear in bold. Bootstrap support values ≥75% are indicated at the branch nodes and a scale bar represent substitutions per compartment.

Microsatellite and genetic analyses

All 10 of the microsatellite primer sets utilized in this study allowed for the amplification of a corresponding allele in each of the 52 isolates examined. Locus FCM7 was the most polymorphic, with seven alleles, while FCM6 had a single allele (Table II). For the 10 loci, a total of 30 alleles were identified in the set of 52 individuals (Table III).

Fourteen of these were shared among the collection of isolates obtained from all four compartments, while nine alleles were shared only between the isolate collections from the younger plantations (i.e., compartments PAT 3 and PAT46) (Table II). Neither of the collections obtained from the two older compartments (i.e., those from compartments PAT12 and PAT19) included unique alleles, which is in contrast to both of the collections from the younger plantations that contained unique alleles (1 in PAT3 and 6 in PAT46) (Table II). Overall, the allele frequencies across the four sampling compartments ranged from 0.04 to 1 (Table II). Based on chi-square (X^2) tests the allele frequencies between collection compartments were not significantly different at nine loci ($P > 0.05$), except for FCM24 (Table II). Mean gene diversity (h) for the entire collection of isolates was 0.31, but this ranged from 0.12 to 0.4 for the individual collections from compartments PAT3, PAT46, PAT12 and PAT19 (Table II).

Among the 52 *F. circinatum* isolates examined, a total of 17 MLGs (designated LM1-LM17), were identified (Table III). One MLG (LM4) was shared among all four isolate collections (Table IV). Two MLGs were uniquely shared between the isolate collections from the younger plantations (i.e., LM1 and LM17), while one MLG (LM13) was uniquely shared between the collections from the older plantations. Eleven MLGs were identified only in the PAT3 (i.e., LM5, LM6, LM14) or PAT46 (i.e., LM2, LM7, LM8, LM9, LM11, LM12, LM15, LM16) isolate collections. Two MLGs (LM1, LM17) occurred only in the PAT3 and PAT46 collections. The remaining two MLGs (LM3 and LM10) occurred in the collection of isolates from the PAT12 compartment, as well as one or both of the collections from the younger plantations (i.e., LM10 was present in PAT12, PAT3 and PAT46, while LM3 was present only in PAT12 and PAT46).

Table II: Allele frequencies, measure of gene diversity and the results for contingency X^2 tests for differences in allele frequency for the 10 microsatellite loci across the four *F. circinatum* collection compartments.

		Collection Compartments						
Locus	**Allele**	**PAT12**	**PAT19**	**PAT3**	**PAT46**	***h***	**DF**	X^2
FCM3	141	0.71	0.75	0.70	0.38	0.57	6	7.8
	147	0.29	0.25	0.20	0.42			
	160	0.00	0.00	0.10	0.21			
FCM20	182	1.00	1.00	0.90	0.92	0.11	3	1.7
	187	0.00	0.00	0.10	0.08			
FCM23	206	1.00	1.00	0.90	0.85	0.14	6	2.9
	221	0.00	0.00	0.00	0.04			
	257	0.00	0.00	0.10	0.08			
FCM24	105	0.00	0.00	0.10	0.38	0.31	3	9.9*[a]
	111	1.00	1.00	0.90	0.63			
FCM25	167	0.93	0.75	0.80	0.67	0.36	3	3.5
	172	0.07	0.25	0.20	0.33			
FCM7	179	0.00	0.00	0.10	0.29	0.67	15	16.6
	221	0.50	0.75	0.10	0.13			
	227	0.50	0.25	0.60	0.33			
	233	0.00	0.00	0.00	0.04			
	239	0.00	0.00	0.10	0.00			
	257	0.00	0.00	0.10	0.21			
FCM2	155	1.00	1.00	0.89	0.83	0.18	6	4.3
	163	0.00	0.00	0.11	0.08			
	167	0.00	0.00	0.00	0.08			
FCM4	135	0.93	0.75	0.80	0.58	0.4	12	11.9
	167	0.07	0.25	0.10	0.04			
	172	0.00	0.00	0.00	0.08			

	177	0.00	0.00	0.10	0.25			
	182	0.00	0.00	0.00	0.04			
FCM6	226	1.00	1.00	1.00	1.00			
FCM16	140	1.00	1.00	0.90	0.63	0.33	6	10.5
	188	0.00	0.00	0.10	0.21			
	254	0.00	0.00	0.00	0.17			
No. of alleles		14	14	24	29			
No. of unique alleles		0	0	1	6			
No. of isolates		10	4	14	24			
h		0.26(0.17)	0.15(0.19)	0.12(0.19)	0.4(0.24)	0.31(0.21)		
No. and percentage of polymorphic loci		9 (90%)	4 (40%)	4 (40%)	9 (90%)			

h = Nei's (1973) gene diversity. Values in parenthesis for this index are standard deviation.
X^2 = chi-square values, DF = degrees of freedom
[a]Asterisk indicate significant ($P < 0.05$) X^2

Based on genotype frequencies, LM1, LM4 and LM10 were the most dominant MLGs (Table IV, Figure IV). The frequency of LM4 was 31% across all the compartments sampled, followed by LM10 and LM1 with frequencies of 17% and 9% respectively across three of the compartments (PAT3, PAT46 and PAT12). Overall, the isolate collections from the two older plantation compartments (PAT12 and PAT19) contained fewer MLGs (but mostly the higher-frequency MLGs) than those from the younger plantations (Table IV). Common genotypes were thus mostly distributed across isolate collections. This even distribution of genotypes was also evident from the high E_5 values (e.g., ranging from 0.75 for PAT3 to 0.82 for PAT12) (Table III).

Table III: Summary statistics of the genetic diversity based on 10 microsatellite loci for the different collections of *Fusarium circinatum* isolates from mature *P. patula* trees examined in this study.

Collection compartments	Number of isolates	Number of alleles	Number of unique alleles	*H*[a]	Number of MLGs[b]	G[c]	G*[d]	E_5[e]	SI[f]	H_s[g]
PAT12	14	14	0	0.26	4	2.88	12.01	0.817	1.20	1.04
PAT19	4	14	0	0.15	2	1.6	40	0.795	0.56	0.93
PAT3	10	24	1	0.12	7	4.55	45.45	0.747	1.75	1.75
PAT46	24	29	6	0.4	13	8.73	36.36	0.807	2.36	1.71
Total	52	30	N/A	0.31	17	6.6	12.7	0.621	2.30	1.34

[a]*h*: gene diversity (Nei 1973) $h = 1 - \sum x^2_k$, where x is the frequency of the *k*th allele.

[b]MLG: multilocus genotype

[c]*G*: genotypic diversity (Stoddart and Taylor 1988) of the *F. circinatum* population collections in Limpopo, South Africa. *G* was esitimated with the equation $G = 1/\sum 1/p^2_i$ where, *pi* is the observed frequency of the *i*th genotype in the population

[d]G*: maximum genotypic diversity (G* = G/n x 100) (Chen *et al*. 1994) in the population expressed as a percentage (%), where G is the genotypic diversity of the population .

[e]E_5: Evenness index (Grünward *et al*. 2003); $E_5 = (G-1)/(e^{Hs} - 1)$

[f]SI: Shannon diversity index (Sheldon 1969); $SI = -\sum p_i \ln p_i$ was applied, where p_i is the frequency of *i*th genotype in the population

[g]H_s: normalised Shannon diversity index; $H_s = SI/\ln N$, where *N* is the number of individuals in a population

Table IV: Frequency and distribution of *Fusarium circinatum* genotypes in the four collection compartments at Louis Trichardt, Limpopo, South Africa.

Genotype	Genotype Frequency	Collection compartments
LM1	9.62	PAT46, PAT3
LM2	1.92	PAT46
LM3	7.69	PAT46, PAT12
LM4	30.80	PAT12, PAT46, PAT19, PAT3
LM5	1.92	PAT3
LM6	1.92	PAT3
LM7	1.92	PAT46
LM8	1.92	PAT46
LM9	5.77	PAT46
LM10	17.31	PAT12, PAT46, PAT3
LM11	1.92	PAT46
LM12	1.92	PAT46
LM13	3.85	PAT12, PAT19
LM14	1.92	PAT3
LM15	1.92	PAT46
LM16	1.92	PAT46
LM17	5.77	PAT46, PAT3

Figure IV: Minimum-spanning network of *F. circinatum* genotypes (LM1 - LM17) sampled from four *P. patula* plantation compartments in Louis Trichardt, Limpopo Province, South Africa. The compartments are represented by the four different colours in the circles. Sizes of the circles are proportional to the frequency of genotypes.

The graph of genotypic diversity versus loci reached a plateau, indicating that the numbers of loci were adequate to discriminate individuals in the Limpopo population (data not shown). Genotype diversity of isolates from older *P. patula* plantation

compartments was 2.88 (PAT12) and 1.6 (PAT19), while the isolate collections from the younger plantations were characterized by G values (Table III) of 4.55 (PAT3) and 8.73 (PAT46). Overall, all of the isolates in the collection likely represent members of the same population because little to no differentiation was detected among the various isolate collections from the four compartments (Table V). The only comparison for which a statistically significant θ value was estimated was for the PAT46 and PAT12 collections that were moderately differentiated (Weir and Cockerham 1984) from one another.

Table V: Comparison of genetic differentiation estimated with θ[a] (above diagonal) and gene flow expressed as *M*[b] (below diagonal) between pairs of *Fusarium circinatum* collections sampled from four compartments in *P. patula* plantations.

	PAT19	PAT12	PAT46	PAT3
PAT19	-	0	0.001	0
PAT12	-7.168	-	0.118*	0.023
PAT46	525.82	3.75	-	0
PAT3	-4.057	21.09	26.67	-

[a] θ is Wright's F_{ST} estimate (Weir 1996) with θ = (Q-q) / (1-q) where Q is the probability that two alleles in a single population are the same and q is the probability that two alleles from different populations are the same (Weir 1996).

[b] M is the level of gene flow calculated with the formula $M = [(1/\theta) - 1] / 2$ (Cockerham and Weir 1993). Negative values for θ were treated as zero meaning no population differentiation between isolate collections. Values with asterick (*) indicate the null hypothesis of no differentiation is rejected at $P < 0.05$ for θ values.

Mode of reproduction

For the microsatellite data from the entire set of isolates, I_A and r_D, values of 1.19 and 0.26 (P < 0.0001) were obtained (Table VI). These values fall well outside what would be expected for a normal distribution (as calculated from 10 000 randomized

datasets). The null hypothesis of random allele association was consequently rejected, which suggested the absence of recombination in the collection of isolates from Limpopo. Similar results were also obtained when the four isolate collections were examined individually (Table VI).

Table VI: Observed index of association (I_A) and *rD* values and the mating type ratios for each of the isolate collections from *P. patula* plantation trees.

Collection compartments	I_A[a]	*rD*[b]	*P*[c]	Mat 1 : Mat 2[d]	X^{2}[e]
PAT19	n/a	n/a	n/a	4 : 0	n/a
PAT12	0.78	0.67	0.002	11 : 3	4.57*
PAT46	1.76	0.22	0.001	10 : 0	10*
PAT3	2.01	0.25	0.003	17 : 7	4.17*
Combined	1.19	0.26	<0.0001	42 : 10	19.70*

[a] I_A is a measure of multilocus linkage disequilibrium (Smith *et al.* 1993). The sample size for compartment PAT19 was too small for I_A, *rD, P* and X^2.

[b] *rD* is a measure of multilocus linkage disequilibrium independent of sample size (Smith *et al.* 1993).

[c] *P* value for the null hypothesis that the alleles are randomly associated in the population.

[d] Ratio of Mat-1 and Mat-2 mating type individuals in each of the collection compartments.

[e] Chi-square (X^2) test to determine the significant difference between the expected frequencies and the observed frequencies in the Mat 1 and Mat 2 mating type individuals in the collections

Only ten of the 52 isolates were of the Mat-2 mating type suggesting a significant bias towards Mat-1 (Table VI). Both Mat-1 and Mat-2 individuals were recovered from thirteen percent of the diseased trees. No Mat-2 isolates were detected in the PAT46 and PAT19 collections. The mating type ratios for isolates from all of the compartments deviated significantly from the expected 1:1 ratio. This was also true for

the combined data set, where the ratio was 42:10 (X^2 = 19.90, P = 0.05) for Mat-1 and Mat-2, respectively (Table VI).

Discussion

Results of this study provide the first evidence of pitch canker on established *P. patula* trees in plantations. In South Africa, pitch canker is now known on at least three commercially deployed *Pinus* species. These include *P. radiata* and *P. greggii* in the Western, Eastern Cape and KwaZulu-Natal Provinces (Coutinho *et al*. 2007, Mitchell *et al*. 2011, Fru *et al*. 2017) and now *P. patula* in the Limpopo Province of the country. The discovery of pitch canker on *P. patula* emerging from this study is of concern, because this is the most commonly planted softwood species in South Africa (Mitchell *et al*. 2012, DAFF 2015) and Colombia (Rodas *et al*. 2016).

It is unclear what combination of events might have led to the pitch canker outbreak on *P. patula* in Limpopo. For example, asexual reproduction giving rise to suitable inoculum loads as described by Correll *et al.* (1991), the presence of a susceptible host and favorable abiotic factors (high relative temperature and regular mist events in the affected area) could have been contributing factors. Also, the host trees could have been pre-disposed to infection due to a hailstorm that occurred in December 2013, which would have provided the pathogen with wounds as entry points. *Fusarium circinatum*, which is a wound-based pathogen (Sakamoto and Gordon 2006, Wingfield *et al*. 2008), could therefore, have benefited from the hail induced-wounds on a susceptible host.

The genetic diversity of *F. circinatum* in the *P. patula* plantation in the Louis Trichardt region was high (G* = 12.70). This is higher than in other reports for populations of the pitch canker fungus in the Eastern (G* = 11.27) and Western (G* = 1.93) Cape Provinces, as well as in the Demagtenberg (G* = 2.72) area of KZN (Santana *et al.* 2016, Fru *et al.* 2017). This could have emerged from multiple introductions of *F. circinatum* genotypes spreading across the different plantation compartments in Limpopo. It is consistent with the fact that only 46% of the alleles in Limpopo were shared across all the plantation compartments examined. Examples of such multiple introductions have been reported in previous population studies of pitch canker in South African *Pinus* plantations (Steenkamp *et al.* 2014, Santana *et al.* 2016, Fru *et al.* 2017).

Sexual recombination could have influenced the high level of genetic diversity in the Limpopo *F. circinatum* population. However, the results of the present study suggested that the population was largely asexual, despite the presence of both Mat-1 and Mat-2 individuals in the Limpopo population. This is similar to the case for other *F. circinatum* populations associated with pitch canker outbreaks elsewhere in South Africa, that are largely clonal (Santana *et al.* 2016) or dominated by isolates of a single mating type (Fru *et al.* 2017).

It has been suggested that increases in allele frequency can result from fit alleles being retained during clonal reproduction (McDonald and Linde 2002). Such a genetic structure has also been shown in populations of *F. circinatum* from plantations in Spain (Berbegal *et al.* 2013). In that case, high diversity of the pathogen was suggested to be due to multiple independent genotype introductions that underwent clonal divergence and admixture (Berbegal *et al.* 2013). This premise would probably

also hold true for the population of *F. circinatum* associated with pitch canker in Limpopo and other regions in South Africa.

Comparisons of *F. circinatum* populations from the younger (3 to 6-year-old) and older (12 to 19-year-old) plantations in the Louis Trichardt region, showed a higher diversity among the isolates from younger trees. This suggests that genotypes of *F. circinatum* were introduced into the area with planting material in the case of the younger plantation compartments. Plants that leave nurseries with latent infections can develop active disease when planted in the field, and lead to establishment of the pathogen in the field (Gordon *et al.* 2015). Wind-dispersed spores from a build-up of inoculum could then have led to infection of older pre-disposed trees in the established plantations. It is possible that the lower diversity of the isolates from older tree compartments, reflect genotypes that have a higher level of fitness and thus capable of surviving in the plantation setting.

The high level of genetic diversity of *F. circinatum* and the presence of both mating types in the population from *P. patula* in Limpopo could pose significant management challenges. This is because both these factors potentially increase the evolutionary potential of the pathogen in the region (McDonald and Linde 2002). Evolutionary potential increases with the population size of a pathogen (i.e., the population sizes of more diverse populations are higher). Sexual recombination can also increase the evolutionary potential of a pathogen. Although it has never been recorded in the field, *F. circinatum* readily produces sexual offspring under laboratory conditions (Britz *et al.* 1998). Given suitable conditions, the Mat-1 and Mat-2 individuals present in the Limpopo population could thus mate to produce recombinant offspring, which would further increase the risk of *F. circinatum* overcoming host defenses.

The identification of pitch canker on established *P. patula* trees is of concern to the forestry industry in South Africa. Despite the fact that considerable effort has been made to select and breed *F. circinatum*-tolerant hybrids of *Pinus* species, a significant proportion of South African plantations are planted to *P. patula*. Greater effort should also be placed into nursery hygiene to reduce *F. circinatum* infections, as well as regional quarantine to reduce the movement of infected planting material into the field.

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