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Unique clones of the pitch canker fungus, *Fusarium circinatum*, associated with a new disease outbreak in South Africa

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Abstract Pitch canker of pines is caused by the fungus *Fusarium circinatum*. In South Africa, this pathogen has mostly been a nursery problem. From 2005, however, outbreaks of pitch canker have been reported from established *Pinus radiata* and *P. greggii* in the Western and Eastern Cape Provinces. Most recently, pitch canker-like symptoms were observed on 10-year-old *P. greggii* trees in a plantation in the midlands of the KwaZulu-Natal (KZN) Province. The aim of this study was to: (i) identify the causal agent of the observed symptoms, (ii) determine the genetic diversity, and (iii) the mode of reproduction of this fungal population. Furthermore, the aggressiveness of isolates from these trees was compared with that of isolates obtained previously from *P. patula* in South Africa. Isolates from the *P. greggii* trees in KZN were confirmed as *F. circinatum* based on both morphology and DNA sequence analyses. Microsatellite marker analyses revealed the presence of five genotypes of *F. circinatum*, not previously reported from other plantations in South Africa, with one of these genotypes being dominant. These genotypes were all pathogenic to *P. patula* and *P. elliottii*. No evidence of sexual reproduction was detected in the KZN population of the fungus. This was consistent with the fact that isolates from *P. greggii* were all of the MAT-2 mating type, in contrast to previously collected isolates from across South Africa that included both mating types. The results suggest that the outbreak of pitch canker on *P. greggii* in KZN represents a separate introduction of *F. circinatum* into the region with important implications for managing the disease.

Keywords Mating type · Microsatellite analyses · *Pinus greggii* · Population diversity

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Introduction

Fusarium circinatum Nirenberg & O'Donnell, the causal agent of pine pitch canker disease, has been known in South Africa since the late nineteen-eighties (Viljoen et al. 1994). It was first detected in the country from a single nursery, resulting in root disease of seedlings (Viljoen et al. 1994). For many years, the fungus was known only as a nursery pathogen and the cause of field establishment problems of especially *P. patula* (Mitchell et al. 2011). However, in 2005, true pine pitch canker disease as manifested by cankers on the stems and branches of established trees was reported on *P. radiata*

in the Western Cape Province of the country (Coutinho et al. 2007). Since then, it has become a common problem in the coastal and mountain regions of the Western and Eastern Cape Provinces of South Africa (Mitchell et al. 2011). The pitch canker fungus remains one of the most important constraints to the sustainable planting of *Pinus* species in South Africa and globally (Wingfield et al. 2008; Mitchell et al. 2011).

Fusarium circinatum is an introduced pathogen in South Africa (Viljoen et al. 1997; Wikler and Gordon 2000; Britz et al. 2005; Berbegal et al. 2013). This fact has emerged from population genetic data showing a low genotypic diversity of *F. circinatum* in South Africa relative to Mexico, which is in the region thought to be its center of origin (Wikler and Gordon 2000). Outbreaks of pitch canker on *P. radiata* and *P. greggii* in the Western and Eastern Cape Provinces, respectively, are further thought to be the result of multiple distinct introductions of the pathogen into the various plantations (Steenkamp et al. 2014; Santana et al. 2016).

Genetic diversity in *F. circinatum* populations is influenced by many factors including reproduction and recombination (Correll et al. 1992). Recombination resulting from sexual reproduction was once suggested as the source of genetic diversity in South African commercial pine nurseries. This was based only on indirect evidence (i.e. high numbers of vegetative compatibility groups [VCG] and the distribution of mating types) (Viljoen et al. 1997; Britz et al. 1998, 2005), because sexual structures have never been found in the field. However, for plantation collections of *F. circinatum* in South Africa, inferences using microsatellite data showed no evidence of sexual reproduction (Santana et al. 2016). This apparent asexuality is also supported by the dominance of a single mating type of the fungus in plantation populations (Steenkamp et al. 2014; Santana et al. 2016).

Pitch canker outbreaks are commonly associated with one or a small number of clones of *F. circinatum*. For example, Correll et al. (1992) used VCG assays to show that isolates affecting native and landscape pine trees in California were clonal. Expansion of the disease range in California was further confirmed to be largely caused by clones from the original infection (Gordon et al. 1996). In Spain, pitch canker on *P. radiata* is also thought to have been caused by a clonal population of *F. circinatum* introduced into the Basque Country (Iturritxa et al. 2011). A later study using a larger sample size showed that the pathogen is represented by two populations in Spain, suggesting two independent introductions that have undergone clonal divergence and admixture (Berbegal et al. 2013). This is similar to the distribution patterns of the fungus in South Africa, where plantation infections are caused by a limited number of *F. circinatum* clones, which apparently originate from nursery populations of the pathogen (Steenkamp et al. 2014; Santana et al. 2016).

Recently, pine pitch canker-like symptoms were observed on 10-year-old *P. greggii* trees in a trial plantation in the Midlands of the KwaZulu-Natal Province (KZN) of South Africa. This plantation falls within the summer rainfall region of South Africa and in the second largest plantation forestry region of the country (DAFF, 2009). It is situated at about 1570 m above sea level and lies within the warm temperate climatic zone of the country (Engelbrecht and Engelbrecht 2016). Overall, the climatic conditions associated with this plantation are broadly similar to those associated with the pitch canker-affected pine plantations in the Western and Eastern Cape Provinces of the country (Schulze and Maharaj 1997; Santana et al. 2016).

Assuming that the symptoms observed in KZN were caused by *F. circinatum*, it would be reasonable to hypothesize that the population responsible for the outbreak would be of a relatively small size and reproducing mainly asexually. It was previously speculated that such population genetic dynamics might be driven by host associated factors, the exploitation of which could provide avenues for developing improved planting stock (Steenkamp et al. 2014; Santana et al. 2016). Our overall goal was, therefore, to determine the population biology of the pathogen associated with *P. greggii* in this plantation and to evaluate the aggressiveness of isolates relative to those routinely used to assay resistance in commercial planting stock. To achieve these goals, our first aim was to identify the causal agent of the symptoms observed on *P. greggii* in KZN. We then determined the genetic diversity and mode of reproduction of the pathogen responsible for the disease outbreak. Finally, we subjected representatives of the KZN collection of isolates to pathogenicity tests using seedlings of relatively susceptible (i.e., *P. patula*) and tolerant (i.e., *P. elliottii*) pine hosts.

Materials and methods

Disease and fungal isolates

Sampling took place in a diseased *P. greggii* plantation in the Demagtenberg plantation in the Karkloof region,

near Howick (KZN). Sampling was randomly conducted by collecting material from multiple tree lesions and different sections of the plantation, at four time points, spanning from Summer to Spring. Plant samples were placed, separately for each tree, in plastic bags and taken to the laboratory. Isolations were made from symptomatic tissues on stems, branches, branch tips and cones.

Plant samples were surface disinfested with 70 % ethanol and cut open to expose the leading edges of the cankers. Small pieces of a symptomatic tissue (ca. 75 mm2) were cut from the edges of the lesions and plated onto *Fusarium* selective (FS) medium (Nelson et al. 1983). These plates where then incubated for 3–5 days at 23 °C under florescent light.

Cultures resembling those of the genus *Fusarium* were transferred to 0.25 % potassium chloride (KCl) agar plates to induce sporulation. Inoculated plates were incubated for five days at 25 °C under fluorescent light and inspected under a light microscope at 100× magnification for characteristic structures of *Fusarium* species, as described by Nirenberg and O'Donnell (1998). Fungal isolates with these structures were transferred onto fresh potato dextrose agar (PDA) plates for another round of incubation to evaluate colony characteristics. Cultures on PDA were, thereafter, used to prepare pure cultures for DNA extraction. All cultures were deposited 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.

Identification of the pathogen

Freeze-dried fungal mycelium from fresh cultures was ground to a fine powder, in Eppendorf tubes, with sterilized steel beads using a Retsch cell disrupter. This powder served as starting material for total genomic DNA (gDNA) isolation using a modification of the method described by Möller et al. (1992). DNA was quantified with a Thermo Scientific NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to a final working concentration of 50 ng/μl gDNA for Polymerase Chain Reaction (PCR) experiments. This DNA was then used in a PCR-based diagnostic method (Schweigkofler et al. 2004) to rapidly screen the isolates and determine whether they represented *F. circinatum*.

These presumptive identifications were confirmed by sequence analysis of a portion of the translation elongation factor 1-alpha (*tef1*) gene. For this purpose, a portion of the *tef1* gene was PCR amplified using Ef-1 (forward; 5′-ATG GGT AAG GAG GAC AAG AC-3′) and Ef-2 (reverse; 5′-GGA AGT ACC AGT GAT CAT GTT-3′), following similar conditions to those used by O'Donnell et al. (1998) and Swett et al. (2014). The resulting PCR products were purified with gel filtration columns using 6 % Sephadex G-50 (50–150 μm bead size) (Sigma). The clean amplicons were then sequenced in both directions using the original PCR primers, the ABI PRISM™ BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM™ 3500 × 1 Genetic Analyzer (Applied Biosystems, CA, USA).

Bidirectional reads were assembled into consensus sequences with the CLC Main Workbench 6.1 software package (CLC Bio, Aarhus, Denmark). These sequences were then compared to those in the FUSARIUM-ID v. 1.0 (http://isolate.fusariumdb.org) (Geiser et al. 2004) and NCBI (http://www.ncbi.nlm.nih.gov) nucleotide databases using BLAST searches. BLAST hits showing 97 % and higher similarity to the newly produced sequences were then downloaded, in addition to *F. oxysporum,* to use for out-group purposes. These downloaded *tef1* sequences, together with the new sequences were then aligned using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/), after which the alignment was checked with MUSCLE (Edgar 2004) as implemented in MEGA v. 5.1 (Tamura et al. 2011). The dataset was then subjected to Maximum Likelihood (ML) analysis using PhyML 3.0 (Guindon et al. 2010; http://www.atgc-monpellier.fr/phyml/) and the best-fit model parameters as indicated by jModelTest 2.2 (Posada 2008). Branch support was estimated using the same parameters based on 1000 bootstrap replicates. Trees were visualized using FigTree (Morariu et al. 2008).

Microsatellite-based genotyping

Isolates confirmed as those of *F. circinatum* were selected from 33 *P. greggii* trees in order to determine their population structure. Some trees yielded isolates from both stems and branches, while other isolates were collected only from a stem, branch or shoot tip of individual trees. A total of 72 isolates (March, 18 isolates; May, 26 isolates; July, six isolates; and September, 22 isolates) were used for genotyping. To allow for comparisons with previous work, we also included 35 isolates of *F. circinatum* to represent the genotypes identified by Santana et al. (2016).

Microsatellite alleles for each of the 107 isolates were determined using ten fluorescently labeled primer sets (FCM3, FCM20, FCM23, FCM24, FCM25, FCM7, FCM4, FCM6 and FCM16) as described by Santana et al. (2009). Microsatellite amplicons were diluted, pooled and run on an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). To allow for allele size determination, each sample included GeneScan™ LIZ-500 (Applied Biosystems) molecular size standard. Sizes of the amplicons were automatically assigned using Applied Biosystem's ABI PRISM® GeneMapper v3 software.

Analysis of genetic diversity

Alleles at different microsatellite loci were coded using letters for each allele. The genotype of individual isolates was inferred using its allele information from each respective locus (e.g. DFGHJJHGHK, where D was the allele for the first locus, F for the second etc.) in a sequential order. The frequency of alleles and gene diversity (h) for the KZN F. circinatum population was calculated using the programme POPGENE (Yeh et al. 1999). Genotypic diversity was estimated using the eq. $G = 1/\sum p_i^2$ where p_i is the observed frequency of the i^{th} phenotype (Stoddart and Taylor 1988). To eliminate bias arising from sample size, the genotype diversity (G) was divided by the value of the number of isolates to give maximum percentage genotypic diversity (G*). The maximum genotypic diversity was calculated as $G^* = (G/N) \times 100$, where N is the number of isolates. To test whether the number of loci used in scoring genotypic diversity was sufficient, the genotypic diversity was plotted against the number of loci (1000 random samplings) using MULTILOCUS version 1.3 (Agapow and Burt 2001).

Mode of reproduction

Mating types of all isolates from KZN were determined following the method described by Santana et al. (2016) and based on the diagnostic method initially introduced by Steenkamp et al. (2000). Mode of reproduction was also inferred from the microsatellite data by investigating multilocus linkage disequilibrium as Index of Association (I_A) in the program Multilocus 1.3 (Agapow and Burt 2001). Consideration for the effect of dependency of I_A on the number of loci was achieved by the analysis of rbarD ($\acute{r}D$) in the same program. A comparison of the observed I_A and $\acute{r}D$ was made with the output of 1000 times simulated random alleles. The null hypothesis (H_o) that there is random association of loci was accepted when the observed values for I_A were within the random data set and probability (P) values showed no significant difference at $P < 0.001$. This inference method was used with a clone and non-clone-corrected data set.

Pathogenicity tests

Inoculation experiments with 20 seedlings each of 9-month-old *P. patula* and *P. elliottii* were conducted using five isolates CMWF1830, CMWF1806, CMWF1844, CMWF1829, CMWF1867 representing different genotypes of *F. circinatum* from the KZN collection. These tests also included five *F. circinatum* isolates known to be pathogenic and that were collected in previous studies. The latter five isolates originated from routine screenings from diseased *P. patula* seedlings during 2002–2003 and included the virulent isolate (FCC3579), used in resistance screening trials (Porter et al. 2009; Mitchell et al. 2012). The pathogenicity tests, which involved removal of growing tips of seedlings and inoculation with a spore suspension, were conducted as described by Mitchell et al. (2012). Negative controls were treated with 15 % glycerol only.

Inoculated seedlings were maintained in the greenhouse under natural photoperiods at 25 ± 4 °C. Treatments were arranged in a completely randomized design with 20 replicates for each treatment. The entire experiment was repeated once. Plants were observed weekly and lesion lengths were measured at the end of the sixth week. Re-isolations from six trees for each treatment were made and the resulting fungi were identified to confirm that the inoculated strains were responsible for the lesions observed (Koch's postulates). The generated data set was tested for normal distribution based on D'Agostino and Pearson omnibus normality test. One-way ANOVA with Tukey's Multiple Comparison post-test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com).

Results

Disease and fungal isolates

Diseased *P. greggii* trees in the KZN plantation showed typical symptoms of pitch canker. These included branch

'flagging', needle yellowing, die-back of the growing tips, and resinous stem cankers (Fig. 1a, b, and c).

Observation of cultures on KCI showed sterile-coiled hyphae in the aerial mycelium with monophialides and polyphialides on proliferating conidiophores. They also produced conidia, aggregated in false heads, in the aerial mycelium, typical of F. circinatum. On PDA, mycelium was white or slightly pink, with a violet pigmentation at the centre of the cultures (Nirenberg and O'Donnell 1998).

All 72 *Fusarium* isolates examined, tested positive using the *F. circinatum*-specific diagnostic method (Schweigkofler et al. 2004). The identities of all isolates were also confirmed by analyses of the *tef1* gene sequences. They were 97 % similar to those of other *F. circinatum* isolates in the *Fusarium*-ID database (http://isolate.fusariumdb.org). Based on the *tef1* phylogeny, the KZN isolates also grouped closely with the type strain of *F. circinatum* and other isolates of this species collected previously in South Africa (Fig. 2).

Microsatellite-based genetic diversity

All 10 microsatellite markers designed for *F. circinatum* (Santana et al. 2009), gave amplification products for the *F. circinatum* isolates examined in this study (in other words, no null alleles were detected). The 72 *F. circinatum* isolates from KZN contained 22 alleles across the 10 microsatellite markers tested (Table 1). Of these, 77 % were also found in the representative set of isolates from other regions of South Africa (Santana et al. 2016). Allele frequencies per locus in the KZN collection ranged from 0.014 to 0.986. The percentage of polymorphic loci in this collection (KZN) was 90 % and the gene diversity (*h*), based on frequency of alleles in this population was 0.27.

A total of five genotypes or multilocus haplotypes (BBEBBDBBBA, CBBAACBBBB, CBBAADBBBB, DABCADAABD, DBBAADAABD) were identified among isolates collected from the diseased *P. greggii* trees in the KZN plantation. None of these genotypes were present in the collection of isolates previously

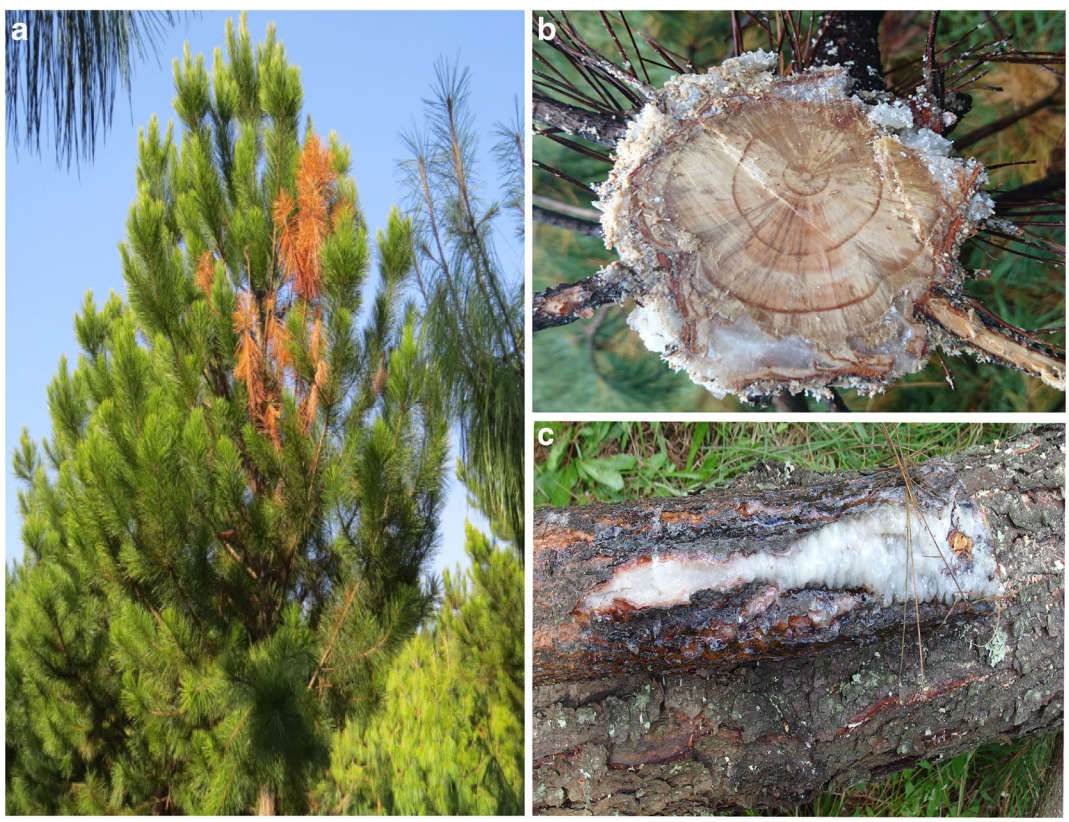

Fig. 1 Pitch canker symptoms on *P. greggii* trees in South Africa. **a** Flagging of an infected branch, **b** Pitch-soaked xylem and **c** Resinous stem canker on the main stem of an infected tree

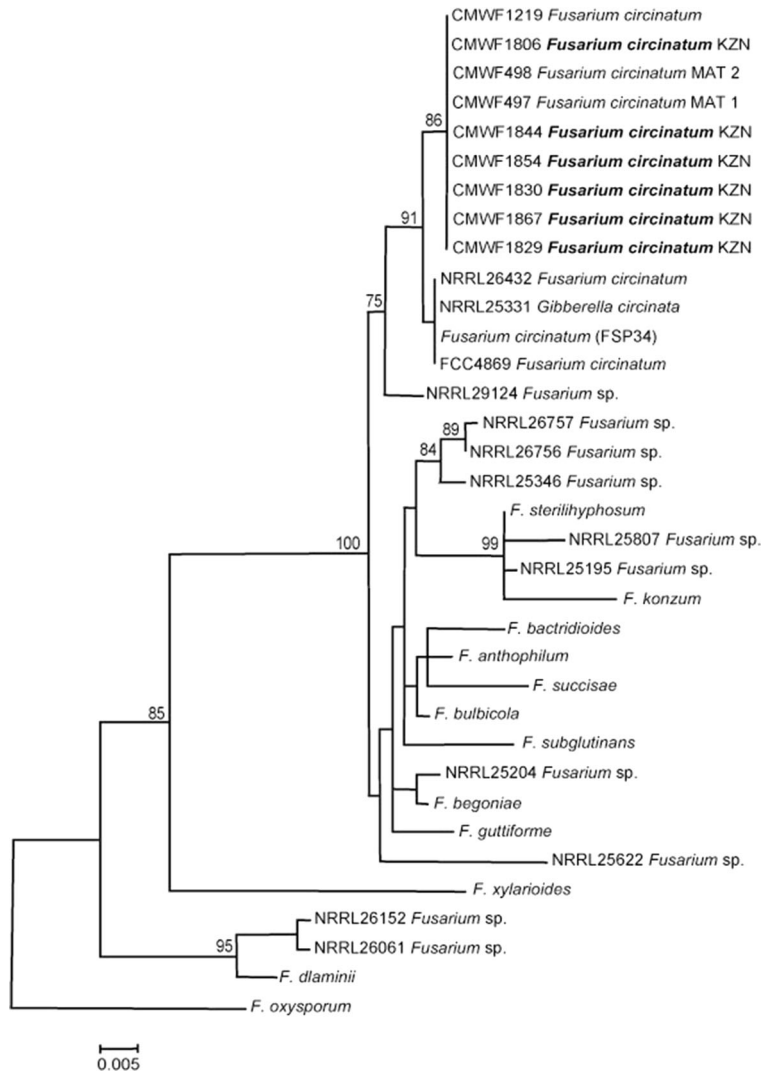

Fig. 2 A *tef1*-based maximum likelihood tree showing the relationship between *F. circinatum* and other members of the American clade of the *F. fujikuroi* complex (Geiser et al. 2013). Isolates identified from this study are indicated in bold. Other known strains, including the *F. circinatum* mating type tester strains (CMWF497 and CMWF498) are also included. *F. oxysporum* was used as out-group. Bootstrap values ≥75 % are indicated at nodes, and the scale bar represents substitutions per site

examined by Santana et al. (2016). Two genotypes (CBBAADBBBB, DABCADAABD) from KZN were dominant, representing 63.9 % and 31.9 % of the isolates respectively. The remaining genotypes represented 1.4 % of the population. The genotypic diversity (G) was 1.96 with a maximum genotypic diversity (G*) of 2.72 for the KZN population. A plateau was reached for the graph plotted for genotypic diversity versus number of loci (data not shown), indicating that the number of microsatellite markers used was adequate to estimate the genetic diversity in this population.

The two dominant genotypes (CBBAADBBBB, DABCADAABD) were identified from the different seasons of the year (March to September) and they were shared across the different plant tissue types on the 33 trees sampled in KZN. About 75 % of individual genotypes were from stem tissue, 16 % from branch tissue and the remaining isolates were from dying shoots or insect galleries.

Mode of reproduction

PCR-based diagnoses showed that all of the isolates from the KZN plantation harbored the MAT-2 idiomorph at the mating type locus. Analysis of linkage disequilibrium, as inferred by $\acute{r}D$ and I_A, was done to evaluate the potential of random mating within the KZN population. The $\acute{r}D$ values were 0.161020 and 0.683632, while the I_A values were 1.27919 and 4.59588 for the respective clone-corrected and clone-

Table 1 Allele frequencies and gene diversity (*h*) values for KZN and representative isolates (SA) of genotypes from other *F. circinatum* populations in South Africa

Locus	Allele Size	Allele Configuration	Allele frequencies KZN	SA
FCM3				
	127	A		0.03
	141	B	0.02	0.46
	147	C	0.65	0.46
	155	D	0.33	0.1
			h = 0.46	
FCM20				
	153	A	0.32*	
	182	B	0.68	0.8
	187	C		0.2
			h = 0.43	
FCM23				
	201	A		0.3
	206	B	0.99	0.3
	220	C		0.03
	240	D		0.2
	245	E	0.01*	
	255	F		0.2
	260	G		0.1
			h = 0.03	
FCM24				
	105	A	0.67	0.4
	111	B	0.01	0.6
	135	C	0.32*	
			h = 0.45	
FCM25				
	167	A	0.99	0.66
	172	B	0.01	0.34
			h = 0.03	
FCM7				
	172	A		0.06
	202	B		0.03
	209	C	0.01	0.1
	214	D	0.99	0.2
	220	E		0.2
	226	F		0.03
	231	G		0.03
	243	H		0.03
	255	I		0.2
	261	J		0.03
			h = 0.03	
FCM2				
	148	A	0.33*	
	155	B	0.67	0.46
	163	C		0.03
	167	D		0.3
	172	E		0.2
	175	F		0.05
			h = 0.44	
FCM4				
	105	A	0.33*	
	135	B	0.67	0.49
	145	C		0.2
	167	D		0.11
	173	E		0.03
	177	F		0.11
	183	G		0.03
	198	H		0.03
			h = 0.44	
FCM6				
	220	A		0.03
	226	B	1.00	0.97
			h = 0.00	
FCM16				
	140	A	0.02	0.69
	153	B	0.7	0.03
	165	C		0.03
	182	D	0.33*	
	188	E		0.2
	200	F		0.03
	252	G		0.03
	275	H		0.03
			h = 0.46	
Mean			*h* = 0.27	

h = Nei's (1973) gene diversity

*= are unique alleles found in KZN only

uncorrected data sets. The I_A for both data sets was significantly greater than that for randomized data set (clone-corrected, $P = 0.02$; clone-uncorrected $P < 0.0001$). These tests, therefore, allowed rejection of the null hypothesis that recombination was occurring in the KZN population of F. circinatum.

Pathogenicity tests

Shoot tip inoculations showed that all the *F. circinatum* isolates used in the study were pathogenic to *P. elliottii* and *P. patula*. The appearance of lesions on all *F. circinatum* inoculated plants was noticeable within two weeks post-inoculation. No symptoms developed on plants used as negative controls. *Fusarium circinatum* was re-isolated from lesions on the randomly selected 30 % of inoculated plants.

Lesion lengths generated from both *P. patula* and *P. elliottii* were combined, and justified by the fact that there was no statistical significance between the two experimental repeats. On *P. patula* seedlings, lesion lengths for isolates from KZN ranged from 46 to 65 mm (Fig. 3a). Two isolates (CMWF1829, CMWF1830), were the most aggressive genotypes on this host and showed a significant difference ($P < 0.05$) to known isolates, including FCC3579 used for pathogenicity trials in South Africa. Among the KZN isolates, CMWF1844 was the least aggressive, but the lesion lengths it induced were not significantly different from those caused by FCC3599 (Fig. 3a). The same isolates produced shorter lesion lengths on *P. elliottii* seedlings. Following the square root transformation (Bartlett 1936) of lesion lengths generated on *P. elliottii*, there was no significant difference between isolates from KZN and those of known aggressiveness (Fig. 3b).

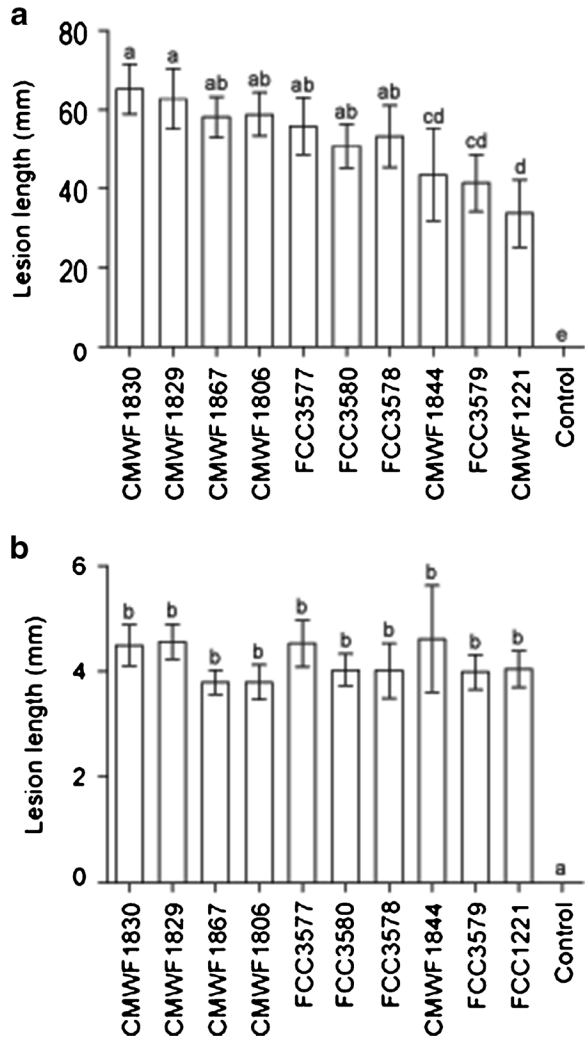

Fig. 3 Mean lesion lengths 60 d post-inoculation with five *F. circinatum* isolates representing genotypes from KZN (CMWF1806, CMWF1844, CMWF1854, CMWF1830, and CMWF1867) with FCC3579 as positive control and four other virulent isolates (FCC3580, FCC3577, FCC3578 and CMWF1221) on **a** *Pinus patula* and **b** *Pinus elliottii*. The bar graphs are based on experiments carried out on 40 replicates. Results are in means ± standard error at 95 % confidence interval. Means designated with the same letter/s are not signficantly different according to Tukey's HSD test at $P < 0.05$

Discussion

Results of this study showed that the disease observed in a trial planting of 10-year-old *P. greggii* in the KZN plantation was caused by the pitch canker pathogen, *F. circinatum*. The symptoms associated with this disease were similar to those known for pitch canker (Wingfield et al. 2008) and reported when the disease first emerged on established plantation trees of *P. radiata* in the Western Cape Province of South Africa (Coutinho et al. 2007). The current study represents the first confirmed report of pitch canker in the eastern part of South Africa. It is of significant concern because this area lies within the major commercial pine growing region of the country.

The genetic diversity of the KZN population of *F. circinatum* was low relative to that known for collections from diseased pine seedlings in nurseries in South Africa (Viljoen et al. 1997; Britz et al. 2005). The genetic diversity of the KZN population was also lower than that of the fungus population associated with pitch canker on *P. greggii* in the Eastern Cape Province (Santana et al. 2016). This is clearly illustrated by the fact that the 72 isolates examined in this study represented only five mutlilocus genotypes. None of the *F. circinatum* genotypes identified from the KZN site corresponded to those previously identified within the

nursery setting. This is also true for the collections of the fungus from the Western and Eastern Cape pitch canker-affected plantations. The distinct nature of the five KZN genotypes was, however, primarily due to unique combinations of known alleles for the respective microsatellite loci. Only six of the 22 alleles identified have not been found previously in South Africa populations of the pathogen. These data, therefore, suggest a separate, but likely South African, source of the inoculum responsible for KZN outbreak of pitch canker.

There was no evidence of sexual reproduction in the KZN population of *F. circinatum*. This is despite the fact that the genotypes identified in the present study consisted mostly of different combinations of microsatellite alleles previously identified in South Africa. Based on linkage disequilibrium analyses, the null hypothesis of random mating or assortment was rejected. Examination of the actual mating types of individual isolates also showed that there is very little potential for sexual recombination in the KZN population because all of the isolates were MAT-2. This is different from what has been seen in previous studies of nursery populations (Britz et al. 1998) and plantation populations (Steenkamp et al. 2014; Santana et al. 2016) where the MAT-1 mating type predominated. However, a similar situation has been reported in Spain with clonal populations, where MAT-1 and MAT-2 were common in different populations despite not being separated by any physical barriers (Iturritxa et al. 2011; Berbegal et al. 2013).

Inoculation tests with representative isolates of the multilocus genotypes recovered from the disease outbreak in KZN showed pathogenicity to *P. patula* and *P. elliottii*. These two species were chosen for inoculation because they are known, respectively, to be susceptible and relatively tolerant to *F. circinatum* (Mitchell et al. 2012) and could serve as an appropriate pathogenicity assay. Tested isolates produced longer lesions on *P. patula* relative to *P. elliottii*, which is consistent with the known biology of the pathogen (Mitchell et al. 2014). Symptom manifestation on the inoculated plants was similar to that shown in previous pathogenicity studies using South African isolates (Mitchell et al. 2012). However, some of the KZN isolates were significantly more aggressive on *P. patula* than previously tested isolates. This emphasizes the need for adjustments to be made in the selection of disease tolerant material and management procedures.

Results of this study support our hypothesis that new outbreaks of pitch canker in South Africa will most likely be caused by asexually reproducing populations of *F. circinatum* that include a small number of genotypes. Similar to other outbreaks in South African *Pinus* plantations (Steenkamp et al. 2014; Santana et al. 2016), the effective size (N_e) of the KZN population is probably very small. According to Wright's equation involving mating type differences (Wright 1931), N_e is zero or approaches zero for the *F. circinatum* population associated with the *P. greggii* pitch canker outbreak in KZN. This is because all of the isolates collected in the present study were of the same mating type. We have previously also shown that the predominance of asexual reproduction is linked to an overall lack of female fertile or hermaphrodite isolates in the plantation setting in the Western Cape Province (Steenkamp et al. 2014). If this was also the case for the KZN population, Leslie and Klein's (1996) derivation of N_e based on female fertility and sterility differences would also approach zero. Such small effective population sizes are characteristic of those that would have arisen relatively recently from founder populations and/or those of pathogens in their epidemic phase (McDonald and Linde 2002; Burnett 2003).

The population dynamics of *F. circinatum* in the pine plantations suggests a low evolutionary potential for the pathogen in these settings (McDonald and Linde 2002). This is because of its asexual reproductive mode and low N_e, as well as an overall low potential for natural gene/genotype flow. Other important *Fusarium* pathogens that also fall in this risk category include *F. oxysporum* and its various *formae speciales* (McDonald and Linde 2002). From a practical perspective, it should be possible to exploit the low evolutionary potential of *F. circinatum* in the South African plantation setting to ultimately improve planting stock. However, such improvements would remain at risk from anthropogenic activity. This is because accidental introduction of isolates into a new region will affect the gene/genotype flow potential of the fungus. Also, introduction of female fertile strains into a region could accentuate the sexual reproductive mode and accordingly also increase the pathogen's N_e (McDonald and Linde 2002). The combined effects of such lapses in silvicultural practice would be an increase in the evolutionary potential of the fungus and an increased risk of susceptibility in apparently improved planting stock (McDonald and Linde 2002).

The pitch canker outbreak described in this study confirms initial fears regarding the introduction, spread

and establishment of *F. circinatum* in South Africa (Wingfield et al. 2008). The unmitigated presence of the pitch canker fungus in plantations in recent years presents a serious threat to the South African forestry industry. Therefore, an understanding of the biology of F. circinatum in commercial plantations in South Africa is important for the management and control of potential disease outbreaks.

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