High diversity and clonality are hallmarks of *Fusarium circinatum* in South Africa

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ABSTRACT

Pitch canker on plantation-grown *Pinus* species, caused by the fungal pathogen *Fusarium circinatum,* first appeared in the western and southern Cape regions of South Africa. However, outbreaks have subsequently been reported from the major plantation growing regions of KwaZulu-Natal and Limpopo in the eastern, summer rainfall regions of the country. It is more than 10 years since the last detailed population genetics studies on *F. circinatum* in the region were conducted. To shed light on the population biology of *F. circinatum* in this region of South Africa, we used microsatellite markers and mating-type assays to study a collection of 296 isolates from different nurseries and plantation sites. Our results showed that populations in the region are highly diverse, but strongly interconnected, with various genotypes shared across nursery and plantation collection sites. In contrast to nursery populations, those associated with pitch canker outbreaks were characterized by the presence of relatively small numbers of dominant genotypes that were generally widespread across the region. Opposite mating-type individuals occurred in most of the isolate collections, but multilocus linkage disequilibrium analyses pointed towards clonality being the main reproductive mode of *F. circinatum* in the region. Most of the pathogen's genetic variation could probably have resulted from multiple different introductions into the country and more specifically, into the summer rainfall region. Because the spread and establishment of invasive pathogens are typically driven by aggressive clones, the results of this study provide important considerations for current and future *Pinus* disease management strategies.

Keywords: microsatellite markers, pine nurseries, pine plantations, population genetics

1 INTRODUCTION

Pitch canker is a disease of *Pinus* species caused by the fungus *Fusarium circinatum* (Wingfield et al., 2008). The pathogen typically requires wounds for infection, which on older trees lead to tip and branch dieback, and the formation of resinous branch and stem cankers (Coutinho et al., 2007; Wingfield et al., 2008). In seedlings, infections typically cause a severe root and root collar disease that ultimately lead to death (Wingfield et al., 2008). Seedlings may also

be infected with the fungus without displaying any apparent disease symptoms (Swett et al., 2018), and such symptomless infected planting stock can result in *F. circinatum*-associated root and root collar disease up to 6 years after plantation establishment (Mitchell et al., 2011). Consequently, *F. circinatum* poses a significant economic risk to plantation forestry wherever susceptible *Pinus* species are propagated (Drenkhan et al., 2020; Wingfield et al., 2008). For example, in South Africa high economic losses are incurred annually, with about 25% of seedlings, especially of *Pinus patula*, typically dying of *F. circinatum*-associated disease within 1 year of being established in the field (Mitchell et al., 2011).

F. circinatum is capable of both sexual and asexual reproduction. During asexual reproduction, conidiation and, to some extent, hyphal fragmentation give rise to genetically identical or clonal progeny. Sexual reproduction of the fungus is heterothallic, requiring isolates of opposite mating-type (i.e., *MAT1-1* and *MAT1-2*) to produce meiotic offspring (Martin et al., 2011). However, the sexual stage of the pitch canker fungus has never been observed in plantations (Drenkhan et al., 2020). This is also true for South Africa where genetic diversity of the pathogen in seedling nurseries was initially attributed only to sexual reproduction (Britz et al., 2005). These initial conclusions were supported by mating-type distribution patterns showing that both mating-types, often at similar frequencies, were usually present in the nursery environment (Britz et al., 2005; Steenkamp et al., 2014). By contrast, in the South African plantation environment, the fungus appears not to undergo sexual reproduction as one of the mating-types is typically absent or significantly under-represented (Fru et al., 2017, 2019; Santana et al., 2016; Steenkamp et al., 2014).

Although numerous population genetic studies have been conducted on *F. circinatum* during the course of the last three decades, comparisons of data obtained from these studies are either complex or not feasible. A major reason for this is the different methodologies used in the various studies. For example, recent studies from Europe and South Africa relied on microsatellite markers (e.g., Berbegal et al., 2013; Santana et al., 2016), while earlier studies on other populations from South Africa and the United States mainly employed vegetative compatibility group (VCG) assays (e.g., Correll et al., 1992; Viljoen et al., 1997). Both methods are useful to estimate genetic diversity, but VCG assays have limited value when seeking to infer population structure and dynamics (McDonald, 1997). Another issue that complicates comparisons among studies relates to the sampling intensity/strategy employed, which can affect the observed allelic frequencies, thereby influencing inferences of population genetic processes (McDonald & Linde, 2002; Milgroom, 1996). An apt example of such discrepancies potentially arising from the sampling strategy are those described by Berbegal et al. (2013) and Santana et al. (2016) for *F. circinatum* isolates from South Africa. Berbegal et al. (2013) used a small number of representative isolates from South Africa to infer evidence for sexual reproduction, while Santana et al. (2016) rejected the null hypothesis of random mating in their study of an extensive collection of randomly sampled isolates.

The objective of this study was to obtain a holistic view of the reproductive mode and population structure of *F. circinatum* in South Africa. All previous microsatellite-based studies conducted in the country have focused on populations of the fungus associated with pitch canker outbreaks in the plantation setting (Fru et al., 2017, 2019; Santana et al., 2016). None of these considered populations associated with disease outbreaks in nurseries or the link between nursery and plantation populations. To address these knowledge gaps, we

considered the population biology of *F. circinatum* in the summer rainfall region of South Africa where more than 70% of the country's commercial *Pinus*-based forestry activities occur (DAFF, 2019). The first studies in the region were restricted to the nursery setting and were conducted more than two decades ago, first using VCG assays (Viljoen et al., 1997) and later sequence-characterized markers with limited variability (Britz et al., 2005). Therefore, in the current study, we first obtained isolates from plants with symptoms in nurseries and plantations, as well as from plantations that are in the process of being established on the field. The microsatellite and mating-type data for these fungi were then combined with those from previous studies (Fru et al., 2017, 2019) to determine genetic diversity and infer the reproductive mode of the pathogen across the respective nurseries and plantations in the summer rainfall region of South Africa.

2 MATERIALS AND METHODS

2.1 Sample collection, fungal isolation and DNA extraction

The microsatellite and mating-type data for three sets of *F. circinatum* isolates were used in this study. One of these included isolates collected from pitch canker-affected plantation trees in the summer rainfall region, for which the relevant data had previously been published (Fru et al., 2017, 2019). Data for the two remaining sets were generated in the current study, and included isolates from the study conducted by Britz et al. (2005) and those originating from the current study. Isolates from all three sets are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

For the newly collected isolates, fresh samples from dying seedlings were obtained from nurseries in Tzaneen (Limpopo Province), Sabie and Ngodwana (Mpumalanga Province). Similarly, samples were obtained from tissue with symptoms on plantation trees in the Carolina region (Mpumalanga Province) and the Karkloof region (KwaZulu-Natal Province). Tree ages for field samples ranged from 3 to 18 years old (Table 1) and symptoms included root collar disease (Carolina), as well as stem and branch cankers (Karkloof).

TABLE 1. Geographical locations, age of trees and number of individuals examined for each of the 10 South African *Fusarium circinatum* collections analysed in this study

Collection code[a]	Province	Location	Species	Tissue type infected	Approx. age (years)	No. of isolates[b]
GRE10 (C)	KwaZulu-Natal (KZN)	Demagtenberg	*Pinus greggii*	Stem and branches	10	72
GRE12 (C)	KwaZulu-Natal (KZN)	Karkloof	*P. greggii*	Stem and branches	12	45
GRE5 (C)	KwaZulu-Natal (KZN)	Karkloof	*P. greggii*	Stem and branches	5	24
PAT12 + 18 (C)	Limpopo (L)	Soutpansberg	*Pinus patula*	Stem and branches	12, 18	18
PAT4 + 6 (C)	Limpopo (L)	Soutpansberg	*P. patula*	Stem and branches	4, 6	24
PAT3 (C)	Limpopo (L)	Soutpansberg	*P. patula*	Stem and branches	3	10
PATrc3 (E)	Mpumalanga (MP)	Carolina	*P. patula*	Root collar infections	3	13
PATni (S)	Limpopo (L)	Tzaneen	*P. patula*	Seedling roots	–	25
PATnii (S)	Mpumalanga (MP)	Ngodwana	*P. patula*	Seedling roots	–	29
PATniii (S)	Mpumalanga (MP)	Sabie	*P. patula*	Seedling roots	–	36

[a] The collection codes were designed to confer information regarding the *Pinus* host (GRE and PAT for either *P. greggii* or *P. patula*) and age of the plant (indicated in Arabic numerals). For clarification, 'rc' was added to the code for the samples from infected root collar tissue of establishment plants. An 'n' was added to the code for the collections from seedlings in the three nurseries targeted (indicated with i, ii and iii). Collections containing isolates from diseased establishment plants are indicated with (E), those with isolates from diseased seedling roots are indicted with (S), while those from plantation trees with pitch canker symptoms are indicated with (C).

[b] Of the isolates in collection PATnii, 10 came from the study by Britz et al. (2005). Collection GRE10 came from the study by Fru et al. (2017). The isolate collections PAT12 + 18, PAT4 + 6 and PAT3 are from the study by Fru et al. (2019).

For isolation of the fungi from seedlings, small pieces of approximately 4–6 mm of infected tissue (root, collar, stem or shoot tips) were removed with a sterile scalpel, surface disinfested with 70% ethanol and plated onto *Fusarium* selective medium (FSM) following a similar method to Fru et al. (2017). The isolation plates were incubated for 3–5 days at 23–25°C under fluorescent light. Isolations from plantation tree samples were done by surface disinfestation of small pieces of tissue with symptoms with 70% ethanol, after which the bark was removed with a sterile scalpel to expose lesions. Small sections were then taken from the leading edges of lesions (2–5 mm in length and 2–5 mm wide, with a depth of 2–4 mm) and plated onto FSM.

Pure cultures were obtained following a similar approach as Fru et al. (2017) where small pieces of hyphal tissue were transferred onto agar medium containing 0.25% potassium chloride. Inoculated plates were incubated for 5 days at 23°C under fluorescent light and then examined under a light microscope at 100× magnification for characteristic structures of *Fusarium* species. Single hyphal tips were then transferred to plates containing potato dextrose agar (PDA; 20 g/L potato dextrose agar powder, 5 g/L agar bacteriological powder; Merck). Following incubation at 23°C under fluorescent light, fungal growth was evaluated for

colony and microscopic characteristics as described by Leslie and Summerell (2006). The identities of all presumptive *F. circinatum* isolates were then confirmed with a standard diagnostic PCR following the method described by Fru et al. (2017).

2.2 Gene and genotypic diversity

A set of 10 microsatellite markers (FCM2, FCM3, FCM4, FCM6, FCM7, FCM16, FCM20, FCM23, FCM24 and FCM25) previously developed for *F. circinatum* (Santana et al., 2009) was used for genotyping. For this purpose, isolates were subjected to PCR, with reaction mixture and thermal cycling conditions for the 10 primer sets as described by Fru et al. (2017). Amplicons were then diluted, pooled and run on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems) for fragment analysis. To allow for allele sizing, each sample included 0.14 µl GeneScan-LIZ-500 (Applied Biosystems) molecular marker. Alleles were scored from the obtained fragment lengths using Applied Biosystem's GeneMapper v. 3 software.

Each isolate was represented by a multilocus genotype (MLG) generated from the identified alleles across the 10 microsatellite loci. Each MLG was arbitrarily assigned a unique number from one to the total number of observed MLGs. Assigned MLGs were plotted against the number of loci sampled with the function *genotype_curve* in POPPR package (Kamvar et al., 2014) in R (R Core Team, 2013) to ascertain if the number of loci was sufficient to discriminate individuals. Within-locus allelic diversity was determined by analysing data obtained from isolates across all collection sites using the function *locus_table* of POPPR. Number of alleles (Na), effective alleles (Ne), gene (or allele) diversity (*h*) and evenness were calculated in the same package. Percentage polymorphism (P) per site was then calculated using Popgene v. 1.31 (Yeh et al., 1999). The program HP-rare (Kalinowski, 2005) was used to calculate allelic richness (Rs) and private allelic richness (pRs). POPPR was also used to determine the number of observed versus expected MLGs (eMLG) adjusted for sample size, with rarefaction to the smallest sample of 10 isolates (from Soutpansberg, Limpopo). The same package was used to determine evenness (E_5) that expresses the ratio of abundant versus rare genotypes. Genotypic diversity (*G*) was calculated with the non-clone-corrected data as applied in Stoddart and Taylor's index of MLG diversity (Stoddart & Taylor, 1988) in POPPR. This is based on the formula $G = \frac{1}{\sum_{x=0}^{N}\left[f_x\left(\frac{x}{N}\right)^2\right]}$ where *N* is the sample size and is the number of genotypes observed

times in the sample. The maximum possible $\hat{G}$, which occurs when each individual in a sample has a different genotype, was calculated to control for collections with different sample sizes. This was achieved by dividing *G* from each collection by *N* to get the percentage of maximum possible diversity that was obtained for that collection.

2.3 Population structure and genetic differentiation

The program MULTILOCUS v. 1.3b (Agapow & Burt, 2001) was used to infer θ (a derivation of Wright's F_{ST} for estimating population differentiation) between different collection sites. Popgene was used to estimate the number of migrants (M) between isolate collections. STRUCTURE v. 2.3.4 was used to estimate the most probable number of genetic groups within collections (Falush et al., 2003; Pritchard et al., 2000). The program uses a Bayesian clustering

algorithm to assign individuals to a specific number of clusters (*K*), employing an ancestry model with admixture and correlated allele frequencies. To determine an appropriate *K*-value, 20 independent runs for each *K*-value were performed (*K* = 1 through to *K* = 10 were tested) with every run having a burn-in of 750,000 iterations, followed by 500,000 Markov chain Monte Carlo (MCMC) iterations. The best *K*-value was identified with the model of Evanno et al. (2005) as implemented in STRUCTURE HARVESTER (Earl & VonHoldt, 2012). The program CLUMPAK (Kopelman et al., 2015) was used to visualize the clustering pattern. Additionally, the entire MLG data set was also subjected to discriminant analysis of principal components (DAPC) using the R package *adegenet*. This approach infers genetic clustering of genetically related individuals and maximizes discrimination between groups in the absence of evolutionary assumptions (Jombart et al., 2010).

Analysis of molecular variance (AMOVA) was used to test for genetic variation between and within *F. circinatum* collections in POPPR of the R program. These estimates were tested by applying 999 permutations of genotypes among collections to determine levels of significance at $p \leq 0.001$ (Peakall & Smouse, 2006). MLG relatedness was examined using a minimum spanning network analysis following the method of Bruvo et al. (2004). Visualization of the network was then achieved in POPPR.

2.4 Mating-type and recombination tests

The likelihood of random mating was evaluated using mating-type frequencies and estimates of microsatellite-based linkage disequilibrium (Milgroom, 1996). For this purpose, the mating-type of isolates was determined following the PCR-based method employed by Santana et al. (2016). Likelihood of random mating as evidenced by Mendelian segregation of mating-type was then evaluated with chi-square (χ^2) tests. Microsatellite-based linkage disequilibrium was estimated in POPPR on the non-clone- and clone-corrected MLG data set. From this, the index of association (I_A) and $\overline{r}_d$ was calculated, where the latter is an alternative of I_A adjusted for the number of loci (Smith et al., 1993). The observed I_A and $\overline{r}_d$ estimates were then compared to those inferred from 1000 randomly reshuffled genotype combinations, and a significant departure from the simulated data set was suggestive of the absence of recombination.

3 RESULTS

3.1 Isolate collections

This study included a total of 296 *F. circinatum* isolates that originated from tissue of *P. patula* (nurseries and plantations) and *P. greggii* (plantations only) with symptoms (Table 1). Of these, 134 originated from previous studies. These included 10 isolates from the Ngodwana nursery that were studied by Britz et al. (2005) and 124 isolates from pitch canker symptoms on established trees in plantations. The latter included 72 isolates from the Demagtenberg plantation in KwaZulu-Natal and 52 from the Soutpansberg plantation in Limpopo (Fru et al., 2017, 2019). The remaining 162 isolates were obtained during the current study and included 69 from stem and branch tissues with symptoms collected from 5- and 12-year-old trees in the Karkloof plantations, as well as 13 isolates originating from root collar infections that developed on trees postplanting in the field in the Carolina area. In addition, a total of

80 isolates were obtained from nursery seedlings with root disease in Tzaneen (*n* = 25), Ngodwana (*n* = 19) and Sabie (*n* = 36).

Based on their origins in terms of geographical location, *Pinus* host, tissue type with symptoms and host plant age, the 296 isolates were represented by 10 isolate collections (Table 1). Those associated with pitch canker on different age classes of *P. greggii* and *P. patula* were included in three isolate collections each (i.e., collections GRE5, GRE10 and GRE12 and collections PAT3, PAT4 + 6 and PAT12 + 18, respectively). The isolates associated with nursery seedlings were included in three collections (PATni, PATnii and PATniii), while those from establishment plants with root collar disease also represented a separate collection (PATrc3).

3.2 Gene and genotypic diversity

A fragment was amplified for all 296 isolates examined using each of the 10 microsatellite markers designed for *F. circinatum* (Santana et al., 2009). Analysis of these fragments revealed a total of 64 alleles, ranging from two for locus FCM25 to 12 for locus FCM23, with a mean of 6.4 alleles across the 10 collection sites (Table 2). All the loci were polymorphic. The Simpson diversity index (1 − *D*) (Simpson, 1949), which is a measure of allele richness across the sites, ranged from 0.013 (FCM6) to 0.78 (FCM7), while allele evenness ranged between 0.33 (FCM6) and 0.9 (FCM25) for the 10 loci (Table 2). The full set of 10 microsatellite markers was sufficient to discriminate different genotypes (Figure S1).

TABLE 2. Summary statistics for the 10 microsatellite loci examined in this study

Locus	*n* [a]	1 – *D*[b]	H_{exp} [c]	Evenness[d]
FCM3	6	0.709	0.711	0.892
FCM20	3	0.439	0.440	0.682
FCM23	12	0.679	0.681	0.587
FCM24	3	0.561	0.563	0.869
FCM25	2	0.499	0.501	0.998
FCM7	11	0.777	0.780	0.736
FCM2	7	0.537	0.539	0.569
FCM4	9	0.673	0.675	0.688
FCM6	2	0.013	0.013	0.329
FCM16	9	0.668	0.670	0.587
Total (*N*)	64			
Mean	6.4	0.555	0.557	0.694

[a] Number of observed alleles (with the primers used, no null alleles were detected for any of these loci).

[b] Simpson index of diversity (Simpson, 1949). This was calculated with the formula $D = 1 - [\sum n(n-1)/N(N-1)]$, where *n* = number of observed alleles per locus and *N* represents the total number of alleles of all the loci.

[c] Nei's unbiased gene diversity, $H_{exp} = [n/(n-1)] \times [1 - \sum(p^2)]$, where *p* is the frequency at a given locus and *n* is the number of observed alleles (Nei, 1978).

[d] Evenness of alleles based Simpson's dominance index (Simpson, 1949).

TABLE 3. Parameters describing the genetic diversity, based on 10 microsatellite loci, within each of the *Fusarium circinatum* isolate collections originating from the summer rainfall regions in South Africa

Collection[a]	Location[b]	*N*[c]	P	Na	Ne	*h*	Rs	MLG	eMLG	*G*	$\hat{G}$	E_5	pRs
GRE10 (C)	Demagtenberg, KZN	72	90	2.2 (0.63)	1.50 (0.42)	0.28 (0.22)	1.70	5	2.40	1.96	2.72	0.74	0.61
GRE12 (C)	Karkloof, KZN	45	90	3.2 (1.90)	2.04 (0.81)	0.43 (0.22)	2.52	14	5.78	5.29	11.76	0.61	0.35
GRE5 (C)	Karkloof, KZN	24	70	2.4 (1.08)	1.62 (0.83)	0.28 (0.25)	1.97	8	5.30	4.57	19.04	0.75	0.02
PAT12 + 18 (C)	Soutpansberg, LP	18	40	1.4 (0.52)	1.21 (0.34)	0.13 (0.19)	1.38	4	3.68	2.66	14.78	0.75	0
PAT4 + 6 (C)	Soutpansberg, LP	24	90	2.9 (1.29)	1.98 (0.88)	0.41 (0.23)	2.46	13	7.35	8.73	36.38	0.81	0.12
PAT3 (C)	Soutpansberg, LP	10	90	2.4 (1.58)	1.47 (0.43)	0.28 (0.17)	2.30	7	7.00	4.55	45.50	0.75	0.02
PATni (S)	Tzaneen, LP	25	90	2.3 (0.82)	2.06 (0.69)	0.46 (0.20)	2.26	7	4.79	4.14	16.56	0.78	0.21
PATnii (S)	Ngodwana, MP	29	90	3.5 (1.96)	1.89 (0.57)	0.42 (0.20)	2.63	15	6.68	5.64	19.45	0.55	0.21
PATniii (S)	Sabie, MP	36	100	3.3 (1.83)	2.13 (0.93)	0.44 (0.25)	2.52	15	6.45	6.75	18.75	0.67	0.21
PATrc3 (E)	Carolina, MP	13	70	1.9 (0.74)	1.66 (0.55)	0.32 (0.25)	1.88	4	3.54	2.68	20.62	0.82	0
All nurseries		103	100	3.3 (1.83)	2.12 (0.92)	0.44 (0.25)	4.30	34	8.43	8.56	8.31	0.50	4.30
All plantations		193	90	5.1 (2.92)	2.59 (0.96)	0.55 (0.22)	4.70	38	7.03	9.75	5.05	0.75	4.70
All isolates		296	100	6.4 (3.80)	2.60 (0.98)	0.56 (0.21)		68	7.92	15.88	5.36	0.54	

[a] Collections containing isolates from diseased establishment plants are indicated with (E), those with isolates from diseased seedling roots are indicted with (S), while those from plantation trees with pitch canker symptoms are indicated with (C).

[b] See Table 1 for additional location and host information. MP, Mpumalanga; LP, Limpopo; KZN, KwaZulu-Natal.

[c] *N* = number of individuals examined, P = percentage polymorphism, Na = mean observed number of alleles, Ne = effective number of alleles (Kimura & Crow, 1964), *h* = Nei's (1973) gene diversity, MLG = number of multilocus genotypes observed, eMLG = expected number of MLG at the smallest sample size ≥10 based on rarefaction (Hurlbert, 1971), *G* = Stoddart and Taylor's index of MLG diversity (Stoddart & Taylor, 1988), $\hat{G}$ = The maximum value of G to control for sample size, E_5 = index of evenness for multilocus genotypes (Grünwald et

al., 2003), Rs = allelic richness, pRs = private allelic richness. For Na, Ne and *h*, the values in parentheses represent standard deviation.

No clear trends could be discerned in the diversity indices for the collections originating from the plantation environment versus those obtained from diseased seedlings (Table 3). However, all three seedling collections were mostly associated with higher values for gene and genotypic diversity (*h* and *G*), as well as for the observed and expected number of alleles (Na and Ne). Nevertheless, among the 10 isolate collections examined, PAT12 + 18 (i.e., isolates from stems and branches with symptoms of 12- and 18-year-old *P. patula* in the Soutpansberg region) were generally associated with the lowest values for the respective diversity estimates. This collection, together with the collection PATrc3 (i.e., isolates from root collar infections on establishment plants in the Carolina region), was also devoid of private alleles.

A total of 68 MLGs (Table S1) were identified across all the collection sites, which corresponded to an eMLG value of 7.92 adjusted for the smallest sample size of 10 (Table 3). Among the 193 isolates obtained from stem and branch cankers (collections GRE12, GRE10, PAT12 + 18, GRE5, PAT4 + 6, PAT3), 51 MLGs were identified. The 90 isolates from nursery collections (PATni, PATnii, PATniii) represented 37 MLGs. From the 13 isolates obtained from root collars of infected trees in the field (collection PATrc3), four MLGs (Table 3) were identified. When MLGs from the isolate collections were normalized for differences in sample size (Grünwald et al., 2003), the resulting eMLG values ranged from 2.4 to 7.35 for the collections originating from cankers, 4.79–6.68 for the nursery isolates, and was 3.54 for the PATrc3 collection (Table 3). Additionally, for the 10 isolate collections (irrespective of source), E_5 values ranged from 0.61 for GRE12 to 0.82 for PATrc3 (E_5 ranges between 0 and 1, where $E_5 = 0$ shows that one MLG dominates and $E_5 = 1$ indicates that MLGs are equally abundant). This was also evident when comparing the frequencies of MLGs in the various collections (Table S1).

The majority of genotypes (54 of 68 MLGs) were found only in a single isolate collection (Figure S2). For those genotypes occurring in multiple sampling locations, one genotype (MLG25) was found in six of the 10 isolate collections. These included all three nursery collections (PATnii, PATniii, PATni), the collection from establishment plants (PATrc3), as well as two collections (GRE10 and GRE5) from a plantation site in KwaZulu-Natal (Figure 1). Additionally, eight genotypes (MLG10, MLG25, MLG33, MLG35, MLG45, MLG46, MLG58 and MLG68) were shared among the collections obtained from plantation trees with pitch canker symptoms. Of these, three genotypes (MLG33, MLG66 and MLG68) occurred in both the *P. greggii* and *P. patula* collections. The two most abundant genotypes (MLG25, MLG68) were also shared among nursery collections, as well as those from the pitch canker-affected plantation trees. The collection of isolates from postestablishment trees (PATrc3) with root collar disease contained the abundant genotype MLG25, as well as MLG6 that also occurred in a collection from trees with pitch canker (i.e., GRE10) and MLG51 that was also found in one of the nursery collections (i.e., PATnii) (Figure 1).

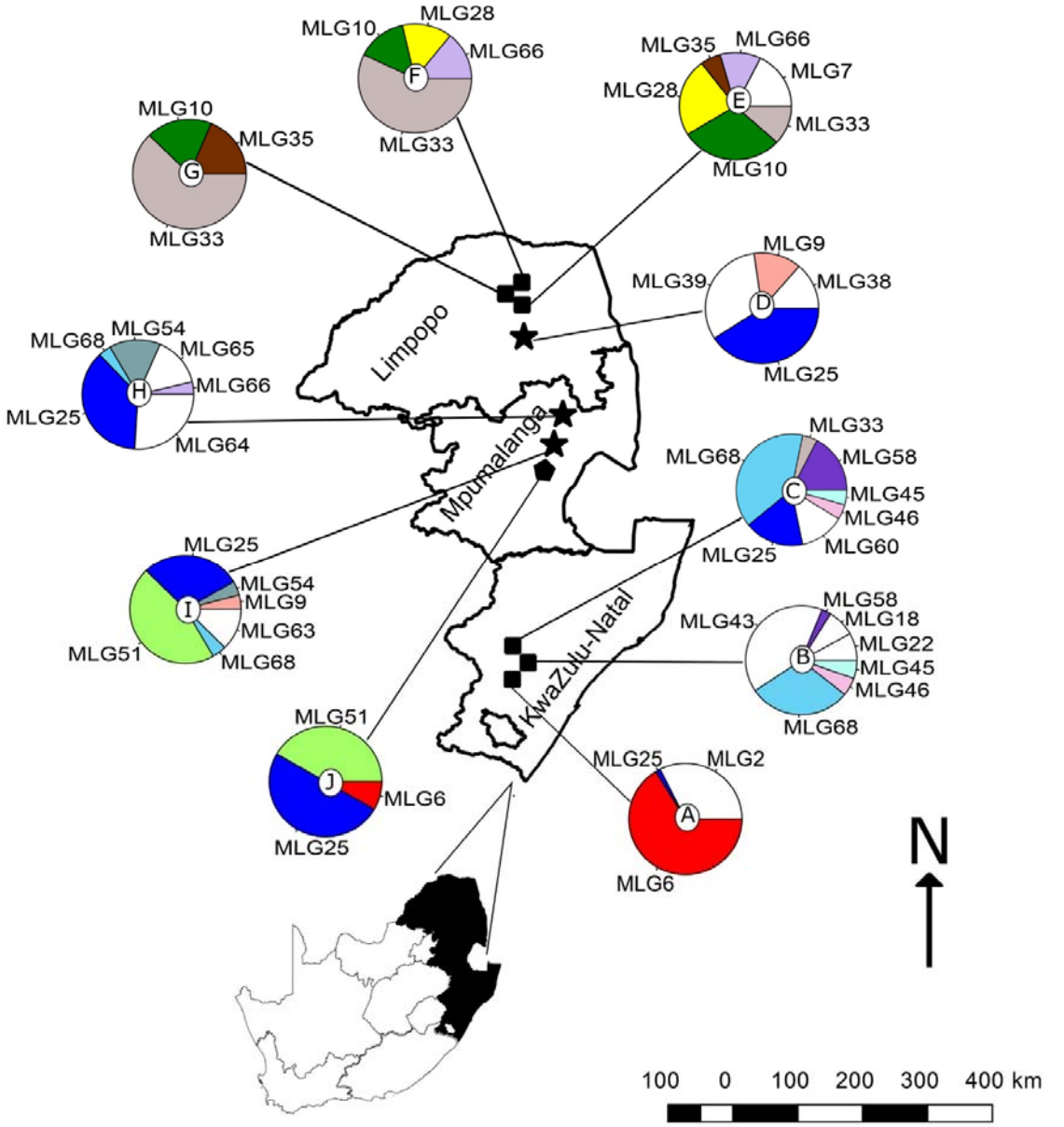

FIGURE 1. Map of distribution of *Fusarium circinatum* genotypes in the eastern, summer rainfall region of South Africa. In the pie charts, only multilocus genotypes (MLGs) with three or more isolates are represented, of which only those occurring in more than one location are indicated in colour. The black symbols indicate the type of collection site, with stars (★) for nursery sites, squares (■) for plantation sites and a pentagon (⬟) for the establishment site (see Table 1). Within the pie charts, capital letters in the central circles denote collection sites as follows: (a) Demagtenberg plantation (collection GRE10 from 10-year-old *Pinus greggii* trees), (b) Karkloof plantation (collection GRE12 from 12-year-old *P. greggii* trees), (c) Karkloof plantation (collection GRE5 from 5-year-old *P. greggii* trees), (d) Tzaneen nursery (collection PATnii from *Pinus patula* seedlings), (e) Soutpansberg plantation (collection PAT4 + 6 from 4- and 6-year-old *P. patula* trees), (f) Soutpansberg plantation (collection PAT3 from 3-year-old *P. patula*), (g) Soutpansberg plantation (collection PAT12 + 18 from 12 and 18-year-old *P. patula*), (h) Sabie nursery (collection PATniii from *P. patula* seedlings), (i) Ngodwana nursery (collection PATnii from *P. patula* seedlings), and (j) Carolina plantation establishment site (collection PATrc3 from 3-year-old *P. patula* trees displaying root collar disease).

3.3 Population structure and genetic differentiation

MULTILOCUS analysis of population subdivision indicated a general lack of genetic differentiation between most pairs of isolate collections (Table 4). The only exceptions were comparisons involving the PAT12 + 18 and PATnii collections. For most comparisons involving PAT12 + 18 from Soutpansberg, especially with those outside this region, significant θ values ($p \leq 0.05$), suggestive of moderate to strong differentiation, were observed. The same level of differentiation was also found for comparisons between the PATnii nursery collection from Ngodowana and those from the Tzaneen nursery (i.e., PATni), and plantations in the Karkloof region (i.e., GRE10) and Soutpansberg (i.e., PAT12 + 18 and PAT3) areas. In other words, except for the PAT12 + 18 and PATnii collections, little to no genetic differentiation was detected between the isolates from establishment plants (i.e., PATrc3) and other isolate collections, between isolates from *P. greggii* and the other collections, and between nursery collections (i.e., PATni and PATnii) and the other collections. Generally, where genetic differentiation of collection pairs was statistically significant for the non-clone-corrected data, this was also true for the clone-corrected data set (Table 4).

TABLE 4. Comparison of the estimates of genetic differentiation (θ) and gene flow (*M*) between pairs of *Fusarium circinatum* isolate collections from the summer rainfall region of South Africa

Collection[a]	GRE10 (C)	GRE12 (C)	PAT12 + 18 (C)	PAT4 + 6 (C)	PAT3 (C)	GRE5 (C)	PATrc3 (E)	PATnii (S)	PATniii (S)	PATni (S)
GRE10 (C)	–	0.54:0.43	**0.52**:0.45	0.37:0.87	0.47:0.58	0.65:0.27	0.46:0.58	**0.45**:0.61	0.51:0.49	0.44:0.65
GRE12 (C)	0.28:1.28	–	**0.49**:0.53	0.29:1.23	0.36:0.88	0.23:1.71	0.21:1.89	0.12:3.65	0.18:2.35	0.28:1.27
PAT12 + 18 (C)	**0.25**:1.50	**0.29**:1.25	–	**0.12**:3.52	0.01:34.34	**0.62**:0.31	**0.45**:0.61	**0.39**:0.78	**0.35**:0.95	**0.34**:0.98
PAT4 + 6 (C)	0.14:3.03	0.17:2.50	0.00:29.88	–	0.02:26.68	0.42:0.69	0.19:2.13	**0.16**:2.57	0.20:2.01	0.19:2.09
PAT3 (C)	0.17:2.53	0.19:2.18	0.00:6.36	0.00:−8.60	–	0.48:0.55	0.28:1.27	**0.25**:1.49	0.20:1.96	0.21:1.84
GRE5 (C)	0.38:0.81	0.06:8.57	**0.36**:0.90	0.22:1.82	0.21:1.84	–	0.41:0.72	0.33:0.99	0.13:3.23	0.36:0.89
PATrc3 (E)	0.04:13.24	0.08:5.43	**0.17**:2.49	0.02:29.5	0.05:9.05	0.22:1.78	–	0.05:9.34	0.21:1.94	0.19:2.08
PATnii (S)	**0.14**:3.00	0.05:9.19	**0.12**:3.81	**0.02**:23.5	**0.03**:15.09	0.13:3.32	0.00:−8.51	–	0.19:2.18	**0.23**:1.65
PATniii (S)	0.27:1.35	0.07:6.54	0.22:1.77	0.11:4.10	0.11:4.11	0.01:42.34	0.13:3.48	0.05:8.99	–	0.19:2.20
PATni (S)	0.13:3.49	0.16:2.56	0.14:3.09	0.08:6.08	0.08:5.62	0.20:2.05	0.06:7.56	0.07:6.80	0.13:3.34	–

Note: Values are ratios of θ:*M*. Here θ is an estimate of Wright's F_{ST} (Weir & Cockerham, 1996) and calculated using $\theta = (Q - q)/(1 - q)$, 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. M is the level of gene flow calculated using $M = [(1/\theta) - 1]/2$ (Cockerham & Weir, 1993). Values below the diagonal are calculated from clone-corrected data from each data collection. Bold values represent significance at $p \leq 0.05$ based on 1,000,000 randomization for each data set. Negative values for θ were considered as zero, implying no population differentiation between isolate collection.

[a] Collections containing isolates from diseased establishment plants are indicated with (E), those with isolates from diseased seedling roots are indicted with (S), while those from plantation trees with pitch canker symptoms

are indicated with (C). See Table 1 for additional location and host information. MP, Mpumalanga; LP, Limpopo; KZN, KwaZulu-Natal.

STRUCTURE analysis was done on the full microsatellite data set for the *F. circinatum* isolates. A plot of Δ*K* values from all 20 runs indicated *K* = 9, suggesting that each isolate had ancestry from one or more of nine genetically distinct sources (Figure S3). Three of the examined collections (GRE5, GRE10 and PAT12 + 18) were dominated by isolates with >90% membership probability to one particular inferred ancestral population (Figure 2). This pattern was similar to what was seen with DAPC (Figure S4). Nevertheless, such genotype clustering across all isolate collections revealed largely the same pattern as what was observed using the θ-values estimated with MULTILOCUS. For example, isolates from the *P. patula* Soutpansberg collections (PAT12 + 18, PAT4 + 6 and PAT3) shared substantial membership to one or more of the same ancestral populations. This high level of genetic relationship among isolate collections was also supported by the AMOVA results (Table 5). For all four of the comparisons (i.e., between and within individual isolate collections, individual nursery collections, plantation and nursery collections, individual plantation collections), most of the genetic variation was distributed within rather than between collections.

TABLE 5. Analysis of molecular variance (AMOVA) among and between different *Fusarium circinatum* collections obtained from the summer rainfall region of South Africa

Source of variation	***df***	**Sum of squares**	**Mean squares**	**Percentage variation**	**ϕ_{ST} (*p*-value)**
Between isolate collections	9	297.48	33.10	37	0.37 (0.001)
Within isolate collections	286	524.62	1.83	63	
Between the plantation and nursery collections	1	28.39	28.39	7	0.07 (0.001)
Within the plantation and nursery collections	294	793.71	2.7	93	
Between the plantation collections	1	48.14	48.14	19	0.19 (0.001)
Within the plantation collections	191	481.70	2.52	81	
Between the nursery collections	3	44.99	14.99	19	0.19 (0.001)
Within the nursery collections	99	218.87	2.21	81	

Note: AMOVA was performed with *poppr.amova* function in POPPR (R Core Team, 2013). The establishment isolates were included in the nursery grouping for AMOVA.

The minimum spanning network inferred from the microsatellite data allowed visualization of relationships among the *F. circinatum* MLGs (Figure 3). Although genetic distances among MLGs from particular geographical regions (e.g., MLG27, MLG28, MLG30, MLG32, MLG33 from the Soutpansberg collections) were often small, a number of closely related MLGs were shared across isolate collections (e.g., MLG64 and MLG66, and MLG24 and MLG25) in the collections from pitch canker-affected plantation trees of *P. patula*, as well as the PATniii and PATnii nursery collections. Notably, the PATrc3 isolates from establishment plants with root collar disease were represented by MLGs that are closely related to those from nurseries (e.g., MLG50, MLG51 and MLG54), or those from nurseries and pitch canker-affected plantation trees of *P. patula* (e.g., MLG24, MLG25, MLG26 and MLG37).

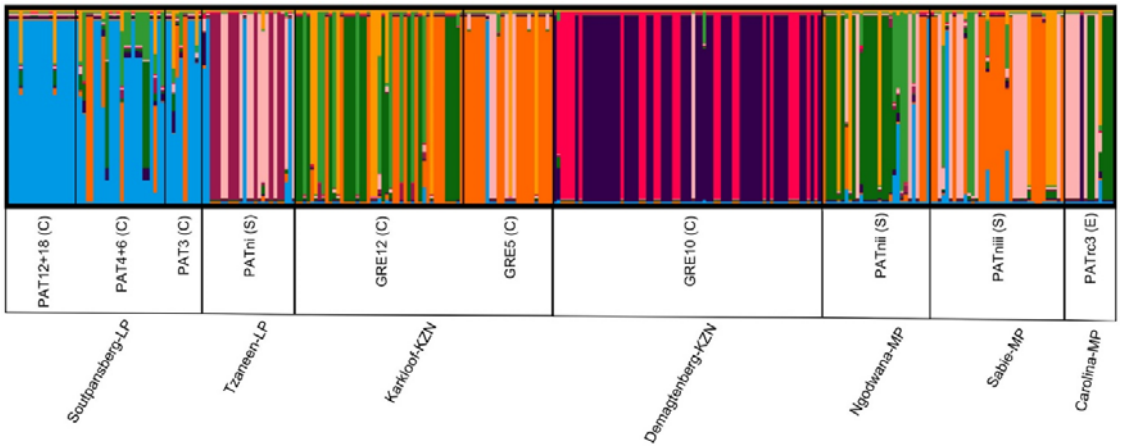

FIGURE 2. Estimated genetic structure of the pitch canker fungus, *Fusarium circinatum* from 10 sample collection sites (field and nursery) in the summer rainfall region of South Africa inferred using STRUCTURE v. 2.3.4 (Falush et al., 2003). Vertical bars represent individuals of different multilocus genotypes (MLGs) and each MLG shows the membership coefficient of individuals at *K* = 9.

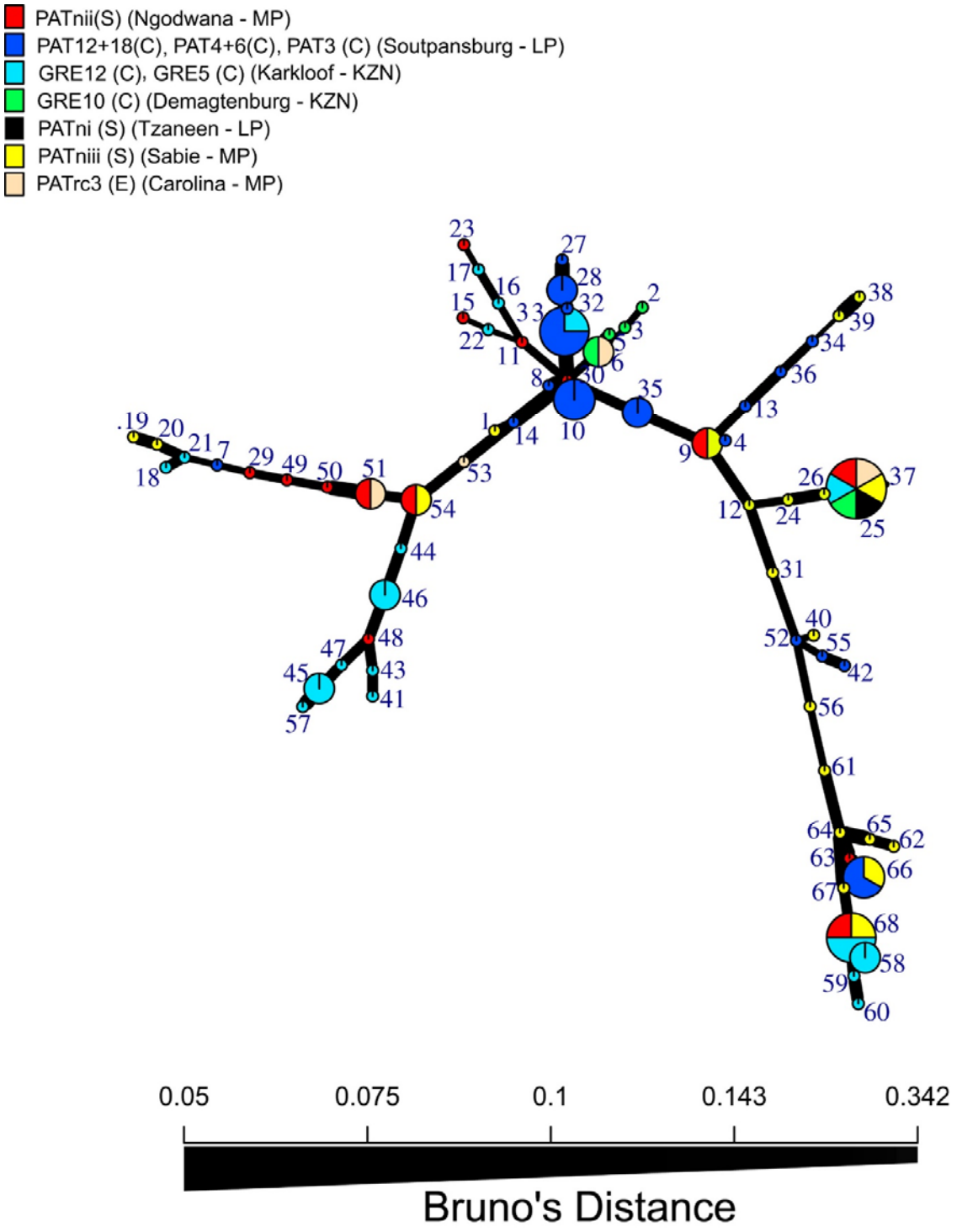

FIGURE 3. Minimum spanning network based on Bruvo's genetic distance (Bruvo et al., 2004) using 10 microsatellite markers for *Fusarium circinatum* populations. Nodes (circles) are individual multilocus genotypes. Genotypes represented at ≥3 locations/sites are labelled with their multilocus genotype (MLG) number designation. Node colours represent geographical site membership proportional to the pie size. Node sizes are relative to the number of samples in the nodes. Lines represent minimum genetic distance between individual genotypes determined by Prim's algorithm (Kamvar et al., 2014). Nodes that are more closely related have darker and thicker lines, whereas nodes more distantly related have lighter and thinner lines or no lines at all.

3.4 Mating-type and tests for recombination

Most collections included both *MAT-1* and *MAT-2* isolates (Table 6). The only exceptions were the collections GRE10 and PAT3, with the former collection containing only *MAT-2* isolates and the latter collection only *MAT-1* isolates (Table 6). Except for the PATnii and PATniii isolate collections, the observed mating-type ratios all deviated significantly ($p = 0.05$) from the expected 1:1 ratio following Mendelian genetics. Furthermore, single MLGs were mostly represented by isolates of the same mating-type. The only exceptions were MLG7, MLG25, MLG33, MLG35, MLG43 and MLG65, which included isolates of opposite mating-type (Table 7). Of these, MLG25, MLG43 and MLG65 were very closely related (i.e., they differed only at one or two microsatellite loci) to both an MLG containing *MAT-1* isolates and an MLG with *MAT-2* isolates. For example, MLG65 differed at locus FCM4 from MLG64 and from MLG62, containing *MAT-1* and *MAT-2* isolates, respectively. The closest relatives of MLG33 and MLG35 were all *MAT-1* containing MLGs, while MLG7 had no such close relatives as it differed from all other MLGs at >2 microsatellite loci.

TABLE 6. Mating-type ratios and multilocus tests for recombination in the collections of *Fusarium circinatum* examined in this study

Collection[a]	Mating-type ratio[b]		Recombination tests[c]		
	MAT-1*:*MAT-2	χ^2	I_A	$\bar{r}_d$	***p*-value**
GRE10 (C)	0:72	72*	4.60 (1.28)	0.68 (0.16)	0.001, *n* = 72 (0.015, *n* = 5)
GRE12 (C)	8:37	18.69*	2.80 (1.43)	0.35 (0.18)	0.001, *n* = 45 (0.001, *n* = 14)
GRE5 (C)	2:22	16.67*	3.29 (2.05)	0.57 (0.35)	0.001, *n* = 24 (0.001, *n* = 8)
PAT12 + 18 (C)	15:3	8*	1.15 (0.17)	0.39 (0.06)	0.001, *n* = 18 (0.618, *n* = 4)
PAT4 + 6 (C)	17:7	4.17*	1.76 (1.11)	0.22 (0.14)	0.001, *n* = 24 (0.001, *n* = 13)
PAT3 (C)	10:0	10*	2.01 (1.36)	0.25 (0.17)	0.003, *n* = 10 (0.01, *n* = 7)
PATrc3 (E)	1:12	9.31*	3.19 (−0.15)	0.54 (−0.03)	0.001, *n* = 13 (0.596, *n* = 4)
PATni (S)	4:21	11.56*	4.67 (2.97)	0.59 (0.38)	0.001, *n* = 25 (0.001, *n* = 7)
PATnii (S)	14:15	0.034 ns	2.05 (0.47)	0.26 (0.06)	0.001, *n* = 29 (0.006, *n* = 15)
PATniii (S)	18:18	0 ns	2.85 (2.01)	0.33 (0.23)	0.001, *n* = 36 (0.001, *n* = 15)
All plantations	52:141	41.04*	2.08 (1.28)	0.24 (0.16)	0.001, *n* = 193 (0.001, *n* = 38)
All nurseries	36:54	3.6*	1.97 (0.98)	0.23 (0.11)	0.001, *n* = 90 (0.001, *n* = 32)
All isolates	89:207	47.04*	1.72 (0.96)	0.21 (0.11)	0.001, *n* = 296 (0.001, *n* = 68)

[a] Collections containing isolates from diseased establishment plants are indicated with (E), those with isolates from diseased seedling roots are indicted with (S), while those from plantation trees with pitch canker symptoms are indicated with (C).

[b] Mating-type ratio of *MAT-1*:*MAT-2* is determined using *MAT-1* to *MAT-2* count of individuals present in a collection. The χ^2 values were calculated to assess a segration deviation from the expected 1:1 ratio. Values indicated with an asterisk (*) show mating-type ratios that depart from the null hypothesis of 1:1 at $\alpha = 0.05$. Those that are under the null hypothesis are indicated with 'ns' (not significant).

[c] These tests were performed with MULTILOCUS v. 1.3b (Agapow & Burt, 2001) using all individuals in each data set. The results of those performed on clone-corrected data sets are indicated in parentheses. I_A: index of association measure of multilocus linkage disequilibrium (Smith et al., 1993). $\bar{r}_d$: measure of multilocus linkage disequilibrium independent of sample size (Smith et al., 1993).

TABLE 7. Microsatellite-based multilocus genotypes (MLGs) that were represented by isolates of both mating-types, together with the mating-type of polymorphic loci of their most closely related MLGs

Genotypes with both mating-types	Mating-type and microsatellite loci at which closely related genotypes differ		
	Most closely related genotype[a]	Mating-type	Polymorphic locus
MLG7	MLG21	*MAT-2*	FCM3, FCM4, FCM23
	MLG29	*MAT-1*	FCM3, FCM4, FCM16, FCM25
MLG25	MLG26	*MAT-2*	FCM4
	MLG37	*MAT-1*	FCM7, FCM23
MLG33	MLG30	*MAT-1*	FCM7, FCM24
	MLG32	*MAT-1*	FCM7
MLG35	MLG9	*MAT-1*	FCM3, FCM4
	MLG10	*MAT-1*	FCM4
MLG43	MLG41	*MAT-2*	FCM23
	MLG48	*MAT-1*	FCM16, FCM24
MLG65	MLG62	*MAT-2*	FCM4
	MLG64	*MAT-1*	FCM4

[a] Most closely related MLGs were identified using results of the minimum spanning network analysis presented in Figure 3.

Based on gametic disequilibrium, there was no evidence of recombination in any of the isolate collections (Table 6). This was clearly evident with the non-clone-corrected data, where all I_A and $\overline{r}_d$ tests rejected the null hypothesis of random mating at $p \leq 0.003$. When only the clone-corrected data was considered, the I_A and $\overline{r}_d$ tests for most of the isolate collections ranged from −0.15 to 2.97 and −0.03 to 0.38, respectively, with many rejecting the null hypothesis of random mating at $p \leq 0.001$ (Table 6). The exceptions were for collections GRE10, PAT12 + 18 and PATrc3, where failure to reject the null hypothesis was probably due to the limited sample size ($n \leq 5$) for the clone-corrected data (Grünwald et al., 2017).

4 DISCUSSION

The results of this study showed that *F. circinatum* populations occurring in seedling production nurseries in the summer rainfall region of South Africa are genetically diverse. Despite employing a different genetic marker system, this is broadly in agreement with previous reports from nurseries in the region, where normalized allele (*h*) and genotypic diversity ($\hat{G}$) values of 0.42–0.46 and 12.9%–19.7%, respectively, were reported (Britz et al., 2005). Only Berbegal et al. (2013) have previously considered this issue outside South Africa, even though *F. circinatum* is often encountered in nurseries (reviewed by Drenkhan et al., 2020). They showed that populations of *F. circinatum* in Spanish seedling nurseries are also characterized by high allelic and genotypic diversity (Berbegal et al., 2013). The high levels of genetic diversity in nurseries could be ascribed to various factors or combinations of factors. These include multiple initial introductions via contaminated seed (Britz et al., 2005; Wikler & Gordon, 2000) with subsequent exchange of pathogen genotypes through trade/movement of infected plants (Britz et al., 2005; Drenkhan et al., 2020), or alternatively the various molecular processes driving emergence of new genotypes (Berbegal et al., 2013; Britz et al., 2005).

In the South African plantation environment, diversity patterns observed for *F. circinatum* associated with different pitch canker outbreaks varied considerably. For some of the collections from plantations in the summer rainfall region of South Africa, we recorded high diversity, which, in some cases, exceeded what was observed in the nursery collections (results of this study and Fru et al., 2019), while others generally lacked diversity (results of this study and Fru et al., 2017). As was the case in other well-sampled studies, such differences in genetic diversity among populations associated with pitch canker outbreaks was not surprising. In Spain, for example, populations from the Basque Country and Cantabria lacked any diversity, while those from western and northern Spain were more diverse (Berbegal et al., 2013). Comparable examples are also found for the winter rainfall region of South Africa. For example, the population from Tokai in the Western Cape Province, where the first outbreak of pitch canker occurred on established trees (Coutinho et al., 2007; Steenkamp et al., 2014), had low *h* and $\hat{G}$ values (i.e., 0.003 and 1.7%), while one from Maclear in the Eastern Cape Province had much higher values (i.e., 0.5 and 32.1%) (Santana et al., 2016).

Our microsatellite analyses revealed that *F. circinatum* populations from the summer rainfall region of South Africa are characterized by limited genetic differentiation. Statistically significant estimates of θ (Agapow & Burt, 2001) were mostly not recorded, irrespective of the isolate collections analysed. Even for the two collections that were significantly differentiated from all other collections, substantial levels of gene flow among all collections could be inferred, especially using the clone-corrected data. Lack of differentiation was highlighted by the sharing of MLGs, many across isolate collections, as well as the membership of isolates to various ancestral populations as shown with Bayesian clustering (Pritchard et al., 2000). This was different from what has been reported from South Africa's winter rainfall region, where geographic distance appeared to be correlated with moderate to strong genetic differentiation (Santana et al., 2016). However, similar to the situation in the winter rainfall region, we observed a strong genetic connection between populations of the fungus from nurseries and those collected from established trees in plantations. Ninety-three percent of the molecular variance in our microsatellite data was distributed within the combined nursery and plantation population sample, with only 7% of the diversity separating nursery from plantation populations. Overall, the population biology of *F. circinatum* in the summer rainfall region has thus been influenced by significant movement of genotypes within and between nursery and plantation populations.

Various ecological attributes most likely underpin the population biology of *F. circinatum* in the summer rainfall region of South Africa. These include factors affecting the dispersal of the pathogen by wind, water splash and insect vectors, as well as its capacity to survive for extended time periods in soil or host debris (Wingfield et al., 2008). Another notable factor is its ability to colonize *Pinus* seedlings without symptoms (Wingfield et al., 2008), as well as various non-*Pinus* plants (Hernandez-Escribano et al., 2018; Swett & Gordon, 2015). Any one of these factors or combinations of them could thus drive repeated outbreaks of root/root collar disease of seedlings in nurseries (Fourie et al., 2014; Gebeyehu & Wingfield, 2003) and of pitch canker in plantations (Coutinho et al., 2007; Gordon et al., 2001; Pérez-Sierra et al., 2007). For example, the re-use of inadequately cleaned seedling containers, combined with infected but symptomless planting stock (Morris et al., 2014), partly explains the link between inoculum sources for pitch canker outbreaks and genotypes that occur in nurseries

and compartments where new plantations are being established. Also, understorey grasses in plantations may serve as an inoculum source because these plants have been shown to host genotypes associated with pitch canker outbreaks in various South African plantations (Herron et al., 2020). Although more research is needed to determine if or how these potential inoculum sources contribute to pitch canker outbreaks in plantations, human activity undoubtedly enhances the inherent dispersal abilities of the pathogen, thereby significantly impacting its population biology in all commercial forestry settings (Drenkhan et al., 2020; Wingfield et al., 2008).

In contrast to the nursery environment, the likelihood of sexually compatible isolates of *F. circinatum* encountering one another in the plantation environment is small. This was reflected in mating-type frequencies in the plantation collections that deviated significantly from the expected 1:1 ratio of occurrence of *MAT-1* and *MAT-2* isolates following Mendelian segregation (Tibayrenc et al., 1991). There have been similar reports for pitch canker-associated populations from other parts of South Africa (Fru et al., 2017, 2019; Santana et al., 2016; Steenkamp et al., 2014). In Spain, pitch canker outbreaks in the País Vasco and Cantabria regions were also associated with populations dominated by one mating-type (Berbegal et al., 2013). Apart from not allowing sexual reproduction, such non-random mating-type frequencies probably also accelerate the loss of female fertility in the respective populations. Indeed, loss of female fertility has been implicated as a major contributor to the clonal or asexual mode of reproduction of *F. circinatum* in populations from the winter rainfall region of South Africa (Steenkamp et al., 2014).

The apparent absence of sexual reproduction in *F. circinatum* populations was supported by our microsatellite data. For all the collections analysed, including the nursery populations for which mating-type distribution suggested recent random mating, significant microsatellite-based linkage disequilibrium was observed. There was no evidence that alleles at the 10 microsatellite loci recombined freely to allow for the creation of new genotypes. Therefore, populations of *F. circinatum* in the summer rainfall region of South Africa display the hallmarks of a haploid eukaryote in which clonality is the prevailing reproductive mode (Tibayrenc et al., 1991). In other words, our isolate collections were devoid of recombinant MLGs and contained many widespread and over-represented MLGs, while evidence for nonrandom association among the microsatellite loci were absent (Smith et al., 1993; Tibayrenc et al., 1991). This is similar to previous reports for pitch canker-associated populations in South Africa's winter rainfall regions (Santana et al., 2016; Steenkamp et al., 2014) and for those in various regions of Spain (Berbegal et al., 2013). Within these settings, the pathogen would benefit from the advantages of clonal reproduction, particularly the ability to rapidly generate large numbers of fit propagules that can colonize the plant host and persist in the environment, thereby enabling it to spread and cause disease outbreaks (McDonald & Linde, 2002). In this sense, *F. circinatum* represents an apt example of a pathogen in which clonal reproduction has facilitated high levels of anthropogenic exchange (Drenth et al., 2019), resulting in its establishment in many parts of the world where *Pinus* species are cultivated or where susceptible *Pinus* species occur naturally (Drenkhan et al., 2020).

The lack of evidence for sexual reproduction in populations of *F. circinatum* might be the consequence of one or more life history traits that hinder our ability to infer or detect this

mode of reproduction (Smith et al., 1993; Taylor et al., 2015). The fact that the pathogen's sexual stage is readily inducible in the laboratory (e.g., Britz et al., 2002), combined with the occurrence of female fertile isolates in some populations (Britz et al., 2005; Wikler et al., 2000), suggests that *F. circinatum* populations must engage in some level of sexual reproduction or at least must have done so recently (Schurko et al., 2009). However, the exact mechanism involved in such recombination events might not conform to what is typically known for heterothallic species of *Fusarium* (Martin et al., 2011). For example, a recent study showed that some strains of *F. circinatum* apparently have the capacity to engage in a form of homothallism under certain conditions (Zhao et al., 2019). Another aspect prolly hindering the search for evidence of sexual reproduction in *F. circinatum* is our incomplete knowledge of its ecology. Most isolate collections with which population studies were conducted were made during disease outbreaks where sampling could be biased towards clones (Drenth et al., 2019; Smith et al., 1993). Also, most of these collections were made in regions where the fungus has been introduced, with only one small collection, so far, from Mexico/Central America where it is thought to be native (Britz et al., 2001; Wikler & Gordon, 2000). Furthermore, the paradigm that *F. circinatum* is specific to *Pinus* has changed during the last decade because the fungus has been isolated, often as an endophyte, from various other plants (reviewed by Drenkhan et al., 2020). What we know about the reproductive biology of *F. circinatum* is thus likely to be strongly biased by its ability to cause disease on susceptible *Pinus* species (Swett & Gordon, 2015).

Our findings have shown that mutation might represent an important source of the variation observed in *F. circinatum* populations. Many of the more dominant MLGs differed at only one or a few microsatellite loci from co-occurring single-isolate MLGs. Hypermutability at microsatellite loci is a common phenomenon and the use of such loci may introduce error into population studies (Bhargava & Fuentes, 2010). This is because clonal members of a group would differ at hypermutable microsatellite loci, essentially leading to an overestimation of genetic diversity. This property of microsatellites may also introduce error in the form of homoplasy, which would lead to an underestimation of genetic diversity (Bhargava & Fuentes, 2010; Putman & Carbone, 2014). It occurs where an allele at such a locus in a group of apparently clonal individuals is not derived from their common ancestor, but rather from a more recent mutation in one or more of the individuals. Either of these situations could also explain why some of the *F. circinatum* MLGs represented by both mating-types often differed at only one microsatellite locus. The notion that spontaneous mutation could cause changes in multilocus traits in *F. circinatum* was recently demonstrated using genomic information of the fungus (Gordon et al., 2021). Consequently, our future research will seek to use whole genome sequence data to determine the role of this phenomenon in the population biology of the pitch canker fungus.

Despite large gaps in our knowledge regarding the population biology of *F. circinatum*, the results of this study have significant practical value. Clonality reduces the evolutionary potential of the pathogen, which would safeguard resistance/tolerance to it in commercial planting stock (McDonald & Linde, 2002). Our results have also highlighted the importance of nursery and silvicultural practices to minimize anthropogenic exchange (sensu Drenth et al., 2019) in *Pinus*-based forestry (Mitchell et al., 2011).

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DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

REFERENCES

Agapow, P.M. & Burt, A. (2001) Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes*, 1, 101–102.

Berbegal, M., Perez-Sierra, A., Armengol, J. & Grünwald, N.J. (2013) Evidence for multiple introductions and clonality in Spanish populations of *Fusarium circinatum*. *Phytopathology*, 103, 851–861.

Bhargava, A. & Fuentes, F.F. (2010) Mutational dynamics of microsatellites. *Molecular Biotechnology*, 44, 250–266.

Britz, H., Coutinho, T.A., Gordon, T.R. & Wingfield, M.J. (2001) Characterisation of the pitch canker fungus, *Fusarium circinatum* from Mexico. *South African Journal of Botany*, 67, 609–614.

Britz, H., Coutinho, T.A., Wingfield, B.D., Marasas, W.F.O. & Wingfield, M.J. (2005) Diversity and differentiation in two populations of *Gibberella circinata* in South Africa. *Plant Pathology*, 54, 46–52.

Britz, H., Coutinho, T.A., Wingfield, M.J. & Marasas, W.F.O. (2002) Validation of the description of *Gibberella circinata* and morphological differentiation of the anamorph *Fusarium circinatum*. *Sydowia*, 54, 9–22.

Bruvo, R., Michiels, N.K., D'Souza, T.G. & Schulenburg, H. (2004) A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology*, 13, 2101–2106.

Cockerham, C.C. & Weir, B.S. (1993) Estimation of gene flow from *F*-statistics. *Evolution*, 47, 855–863.

Correll, J.C., Gordon, T.R. & McCain, A.H. (1992) Genetic diversity in California and Florida populations of the pitch canker fungus *Fusarium subglutinans* f. sp. *pini*. *Phytopathology*, 82, 415–420.

Coutinho, T.A., Steenkamp, E.T., Mongwaketsi, K., Wilmot, M. & Wingfield, M.J. (2007) First outbreak of pitch canker in a South African pine plantation. *Australasian Plant Pathology*, 36, 256–261.

Department of Agriculture, Forestry and Fisheries (DAFF). (2019) Report on commercial timber resources and primary roundwood processing in South Africa 2017/18. Pretoria: The Directorate, Forestry Technical and Information Services.

Drenkhan, R., Ganley, B., Martín-García, J., Vahalík, P., Adamson, K., Adamčíková, K. et al. (2020) Global geographic distribution and host range of *Fusarium circinatum*, the causal agent of pine pitch canker. *Forests*, 11, 724.

Drenth, A., McTaggart, A.R. & Wingfield, B.D. (2019) Fungal clones win the battle, but recombination wins the war. *IMA Fungus*, 10, 18.

Earl, D.A. & VonHoldt, B.M. (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, 4, 359–361.

Evanno, G., Regnaut, S. & Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, 14, 2611–2620.

Falush, D., Stephens, M. & Pritchard, J.K. (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics*, 164, 1567–1587.

Fourie, G., Wingfield, M.J., Wingfield, B.D., Jones, N.B., Morris, A.R. & Steenkamp, E.T. (2014) Culture-independent detection and quantification of *Fusarium circinatum* in a pine-producing seedling nursery. *Southern Forests*, 76, 137–143.

Fru, F.F., Steenkamp, E.T., Wingfield, M.J. & Roux, J. (2019) High genetic diversity detected for isolates of *Fusarium circinatum* associated with the first outbreak of pitch canker on *Pinus patula* in South Africa. *Southern Forests*, 81, 69–78.

Fru, F.F., Steenkamp, E.T., Wingfield, M.J., Santana, Q.C. & Roux, J. (2017) Unique clones of the pitch canker fungus, *Fusarium circinatum*, associated with a new disease outbreak in South Africa. *European Journal of Plant Pathology*, 148, 97–107.

Gebeyehu, S. & Wingfield, M.J. (2003) Pine weevil *Pissodes nemorensis*: threat to South African pine plantations and options for control. *South African Journal of Science*, 99, 531–536.

Gordon, T.R., Henry, P.M., Jenner, B.N. & Scott, J.C. (2021) Spontaneous changes in somatic compatibility in *Fusarium circinatum*. *Fungal Biology*, 125, 725–732.

Gordon, T.R., Storer, A.J. & Wood, D.L. (2001) The pitch canker epidemic in California. *Plant Disease*, 85, 1128–1139.

Grünwald, N.J., Everhart, S.E., Knaus, B.J. & Kamvar, Z.N. (2017) Best practices for population genetic analyses. *Phytopathology*, 107, 1000–1010.

Grünwald, N.J., Goodwin, S.B., Milgroom, M.G. & Fry, W.E. (2003) Analysis of genotypic diversity data for populations of microorganisms. *Phytopathology*, 93, 738–746.

Hernandez-Escribano, L., Iturritxa, E., Elvira-Recuenco, M., Berbegal, M., Campos, J.A., Renobales, G. et al. (2018) Herbaceous plants in the understory of a pitch canker-affected *Pinus radiata* plantation are endophytically infected with *Fusarium circinatum*. *Fungal Ecology*, 32, 65–71.

Herron, D.A., Wingfield, M.J., Fru, F.F., Wingfield, B. & Steenkamp, E.T. (2020) Grasses as a refuge for *Fusarium circinatum* L. – evidence from South Africa. *Southern Forests*, 82, 253–262.

Hurlbert, S.H. (1971) The nonconcept of species diversity: a critique and alternative parameters. *Ecology*, 52, 577–586.

Jombart, T., Devillard, S. & Balloux, F. (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, 11, 94.

Kalinowski, S.T. (2005) hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes*, 5, 187–189.

Kamvar, Z.N., Tabima, J.F. & Grünwald, N.J. (2014) Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ*, 2, e281.

Kimura, M. & Crow, J.F. (1964) The number of alleles that can be maintained in a finite population. *Genetics*, 49, 725–738.

Kopelman, N.M., Mayzel, J., Jakobsson, M., Rosenberg, N.A. & Mayrose, I. (2015) Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, 15, 1179–1191.

Leslie, J.F. & Summerell, B.A. (2006) The Fusarium laboratory manual. Ames, IA, USA: Wiley-Blackwell Publishing.

Martin, S.H., Wingfield, B.D., Wingfield, M.J. & Steenkamp, E.T. (2011) Structure and evolution of the *Fusarium* mating type locus: new insights from the *Gibberella fujikuroi* complex. *Fungal Genetics and Biology*, 48, 731–740.

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

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

Milgroom, M.G. (1996) Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology*, 34, 457–477.

Mitchell, R.G., Steenkamp, E.T., Coutinho, T.A. & Wingfield, M.J. (2011) The pitch canker fungus, *Fusarium circinatum*: implications for South African forestry. *Southern Forests*, 73, 1–13.

Morris, A.R., Fourie, G., Greyling, I., Steenkamp, E.T. & Jones, N.B. (2014) Re-use of seedling containers and *Fusarium circinatum* association with asymptomatic *Pinus patula* planting stock. *Southern Forests*, 76, 177–187.

Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the United States of America*, 70, 3321–3323.

Nei, M. (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89, 583–590.

Peakall, R.O.D. & Smouse, P.E. (2006) GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288–295.

Pérez-Sierra, A., Landeras, E., León, M., Berbegal, M., García-Jiménez, J. & Armengol, J. (2007) Characterization of *Fusarium circinatum* from *Pinus* spp. in northern Spain. *Mycological Research*, 111, 832–839.

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

Putman, A.I. & Carbone, I. (2014) Challenges in analysis and interpretation of microsatellite data for population genetic studies. *Ecology and Evolution*, 4, 4399–4428.

R Core Team. (2013) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.R-project.org/ [Accessed 7 September 2022]

Santana, Q.C., Coetzee, M.P., Steenkamp, E.T., Mlonyeni, O.X., Hammond, G.N., Wingfield, M.J. et al. (2009) Microsatellite discovery by deep sequencing of enriched genomic libraries. *BioTechniques*, 46, 217–223.

Santana, Q.C., Coetzee, M.P.A., Wingfield, B.D., Wingfield, M.J. & Steenkamp, E.T. (2016) Nursery-linked plantation outbreaks and evidence for multiple introductions of the pitch canker pathogen *Fusarium circinatum* into South Africa. *Plant Pathology*, 65, 357–368.

Schurko, A.M., Neiman, M. & Logsdon, J.M., Jr. (2009) Signs of sex: what we know and how we know it. *Trends in Ecology & Evolution*, 24, 208–217.

Simpson, E.H. (1949) Measurement of diversity. *Nature*, 163, 688.

Smith, J.M., Smith, N.H., O'Rourke, M. & Spratt, B.G. (1993) How clonal are bacteria? *Proceedings of the National Academy of Sciences of the United States of America*, 90, 4384–4388.

Steenkamp, E.T., Makhari, O.M., Coutinho, T.A., Wingfield, B.D. & Wingfield, M.J. (2014) Evidence for a new introduction of the pitch canker fungus *Fusarium circinatum* in South Africa. *Plant Pathology*, 63, 530–538.

Stoddart, J.A. & Taylor, J.F. (1988) Genotypic diversity: estimation and prediction in samples. *Genetics*, 118, 705–711.

Swett, C.L. & Gordon, T.R. (2015) Endophytic association of the pine pathogen *Fusarium circinatum* with corn (*Zea mays*). *Fungal Ecology*, 13, 120–129.

Swett, C.L., Reynolds, G.J. & Gordon, T.R. (2018) Infection without wounding and symptomless shoot colonization of *Pinus radiata* by *Fusarium circinatum*, the cause of pitch canker. *Forest Pathology*, 48, e12422.

Taylor, J.W., Hann-Soden, C., Branco, S., Sylvain, I. & Ellison, C.E. (2015) Clonal reproduction in fungi. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 8901–8908.

Tibayrenc, M., Kjellberg, F., Arnaud, J., Oury, B., Brenière, S.F., Dardé, M.L. et al. (1991) Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. *Proceedings of the National Academy of Sciences of the United States of America*, 88, 5129–5133.

Viljoen, A., Wingfield, M.J., Gordon, T.R. & Marasas, W.F.O. (1997) Genotypic diversity in a South African population of the pitch canker fungus *Fusarium subglutinans* f. sp. *pini*. *Plant Pathology*, 46, 590–593.

Weir, B. & Cockerham, C. (1996) Genetic data analysis II: Methods for discrete population genetic data. Sunderland, MA, USA: Sinauer Assoc. Inc.

Wikler, K. & Gordon, T. (2000) An initial assessment of genetic relationships among populations of *Fusarium circinatum* in different parts of the world. *Canadian Journal of Botany*, 78, 709–717.

Wikler, K., Gordon, T.R., Clark, S.L., Wingfield, M.J. & Britz, H. (2000) Potential for outcrossing in an apparently asexual population of *Fusarium circinatum*, the causal agent of pitch canker disease. *Mycologia*, 92, 1085–1090.

Wingfield, M.J., Hammerbacher, R.J., Ganley, R.J., Gordon, T.R., Wingfield, B.D. & Coutinho, T.A. (2008) Pitch canker caused by *Fusarium circinatum* – a growing threat to pine plantations and forests worldwide. *Australasian Plant Pathology*, 37, 319–334.

Yeh, F., Yang, R.-C. & Boyle, T. (1999) .PopGene version 1.31: Microsoft Window-based freeware for population genetic analysis. University of Alberta, Edmonton, Canada, pp. 11–23.

Zhao, L., de Hoog, S., Hagen, F., Kang, Y. & Al-Hatmi, A.M. (2019) Species borderlines in *Fusarium* exemplified by *F. circinatum*/*F. subglutinans*. *Fungal Genetics and Biology*, 132, 103262.