Nursery-linked plantation-outbreaks and evidence for multiple introductions of the pitch canker pathogen *Fusarium circinatum* into South Africa

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Running title: Fusarium circinatum in South Africa

Abstract

In recent years, *Pinus* plantation forestry has been significantly hampered by outbreaks of pitch canker caused by the fungus *Fusarium circinatum*. In this study we investigated the role of *Pinus* host, geographic origin and reproductive mode in structuring the *F. circinatum* populations in plantations. For this purpose, 159 isolates originating from diseased plantation trees in the Western and Eastern Cape Provinces of South Africa, were genotyped using 10 microsatellite markers. Analyses of these data revealed 30 multilocus haplotypes and that the populations were distinct based on geographic origin as well as host. However, shared haplotypes were observed between populations, showing that these populations are connected possibly through the movement of haplotypes. A second aim was to determine whether the genetic variation found in these populations of the fungus could be attributed to outbreaks of

the seedling disease caused by this pathogen in *Pinus* nurseries. To achieve this goal, an additional set of 43 isolates originating from pine seedling nurseries were genotyped and analysed. The results showed that the populations of *F. circinatum* in plantations most likely originated from the nursery outbreaks that occurred prior to the plantation outbreak. Inferences regarding reproductive mode further showed that sexual reproduction has little impact on the genetic makeup of the *F. circinatum* populations and that they primarily reproduce asexually. Overall the results of this study showed that the *F. circinatum* diversity in South Africa has arisen due to multiple introductions of the pathogen and is not due to sexual reproduction.

Keywords: *Fusarium circinatum*, population genetics, pitch canker, Pine plantations

Introduction

Fusarium circinatum Nirenberg and O'Donnell is a pathogen of numerous *Pinus* L. species (Wingfield *et al.*, 2008). This fungus is the causal agent of pitch canker, which is typically associated with the presence of resinous (pitch-soaked) cankers on stems, trunks and exposed roots of susceptible trees. The pathogen can also infect seedlings, where it causes symptoms such as tip dieback, root and collar disease and damping off. From a commercial forestry point of view, *F. circinatum* infection is almost always associated with high levels of seedling mortality, reduced establishment of plants in the field and reduced wood quality and yield (Mitchell *et al.*, 2011, Wingfield *et al.*, 2008). The fungus is consequently regarded as one of the most important pathogens of *Pinus* species, particularly where susceptible species of these trees are cultivated for commercial purposes.

The pitch canker fungus is well adapted for both short- and long-distance dispersal. Spores of the pathogen can be dispersed by wind and insect vectors, as well as human activity through the movement of contaminated soils and plant material (Wingfield *et al.*, 2008). On suitable substrates, *F. circinatum* is also capable of saprophytic survival. For example, the pathogen can survive in soil for at least three years (Wingfield *et al.*, 2008) and it can be recovered from old felled branches and wood chips (McNee *et al.*, 2002). In addition, the pathogen is capable of colonizing various grass species without causing any apparent symptoms (Swett *et al.*, 2014). It is, therefore, not surprising that *F. circinatum* has been found in many parts of the world as an invasive alien (Wingfield *et al.*, 2008). These include reports from both natural forests and commercial plantations (Bragança *et al.*, 2009, Carlucci *et al.*, 2007, Wingfield *et al.*, 2008) where eradication of the pathogen using conventional control strategies has been virtually impossible (Mitchell *et al.*, 2011, Wingfield *et al.*, 2008).

In South Africa, *F. circinatum* has hampered commercial *Pinus* production since 1990 (Viljoen *et al.*, 1994). It was initially discovered on diseased *Pinus patula* Schiede:Schltdl. & Cham. seedlings in a single nursery in the Mpumalanga Province (South Africa), from which it has apparently spread to other nurseries (Wingfield *et al.*, 2008). The pine nurseries in South Africa cultivate various *Pinus* spp. for the establishment of commercial plantations. Nurseries are not limited to growing only single species of *Pinus* and the species cultivated is determined by the requirements of plantation establishment. These seedlings are distributed both regionally and nationally to satisfy the demand of the plantation operations.

Fusarium circinatum is now regularly recorded from nurseries across the country where it represents the primary obstacle to seedling production and plantation establishment, especially of *P. patula* (Mitchell *et al.*, 2011). The first record of the pathogen affecting established plantation trees was in 2005, when the typical pitch canker symptoms were

observed on 5-9 year old *Pinus radiata* D. Don trees in a plantation in the Western Cape Province (WCP) (Coutinho *et al.*, 2007). Pitch canker has subsequently also been reported from 12-15 year old *P. radiata* trees in other plantations in the Province, as well as on *Pinus greggii* trees in the Eastern Cape Province (ECP) and the KwaZulu-Natal Province of South Africa (Steenkamp *et al.*, 2013).

Various studies have investigated the movement and distribution of *F. circinatum* in South Africa. Population genetic analyses revealed high levels of diversity among the isolates originating from diseased seedlings (Viljoen *et al.*, 1997). This apparent diversity was attributed to sexual reproduction (Viljoen *et al.*, 1997), while an overall lack of genetic structure within the seedling populations suggested wide spread movement of *F. circinatum* among nurseries after the initial nursery outbreak of the disease in the country (Britz *et al.*, 2005). This involvement of human activity in the spread of the pathogen in South Africa was also reflected by the results of population genetic studies investigating the origin of the pathogen in the country (Britz *et al.*, 2001, Wikler *et al.*, 2000). The results of these previous studies on *F. circinatum* associated with diseased nursery seedlings suggested that *F. circinatum* in South African was introduced accidently into the country on contaminated seed collected in Central America or Mexico (Britz *et al.*, 2001, Wikler *et al.*, 2000).

Little is known regarding the population biology of *F. circinatum* responsible for the pitch canker outbreaks in South African *Pinus* plantations. The results of a single previous study revealed significant diversity among isolates originating from diseased *P. radiata* trees in WCP plantations, but unlike the situation in *Pinus* nurseries, these populations appeared to be more structured and to reproduce clonally (Steenkamp *et al.*, 2013). This previous study also suggested that the WCP plantations originated from distinct introductions of the pathogen into the region (Steenkamp *et al.*, 2013). Whether *F. circinatum* populations associated with

pitch canker outbreaks in other parts of the South Africa are related to the WCP population and also structured similarly remains to be determined. This seems unlikely, however, given the fact that pitch canker outbreaks in South African plantations appear be geographically and climatically diverse and that seedlings are sourced from different nurseries in the country. For example, the pitch canker-affected *P. greggii* plantations in the ECP are located in the Ugie and Maclear region, which is approximately 600 and 1000 km from the respective WCP sites where isolates from *P. radiata* were previously examined (Coutinho *et al.*, 2007, Steenkamp *et al.*, 2013). In the WCP, these plantations usually occur near the coast at elevations not exceeding 500 m above sea level, while those in the ECP are located in the southernmost end of the Drakensberg mountain range at altitudes higher than 1200 m. Also, the *P. radiata* plantations in the WCP typically experience Mediterranean to Oceanic climates with mild winters, warm summers and most rain in the winter or spring. In contrast, the *P. greggii* stands usually experience a subtropical highland climate with mild summers, cold winters and most precipitation during the summer (Schulze, 1997).

Most previous investigations into the population biology of *F. circinatum* have used dominant genetic markers to study diversity. These include vegetative compatibility group (VCG) assays (Britz *et al.*, 2005, Iturritxa *et al.*, 2011, Steenkamp *et al.*, 2013, Viljoen *et al.*, 1997, Wikler and Gordon, 2000, Wikler *et al.*, 2000) and amplified fragment length polymorphisms (AFLPs) (Iturritxa *et al.*, 2011, Steenkamp *et al.*, 2013). However, VCG and AFLP analyses provide limited value for inferences of gene flow and reproductive biology (Majer *et al.*, 1996, Vekemans *et al.*, 2002). In the few studies that have employed *F. circinatum*-specific co-dominant markers (Britz *et al.*, 2002, Wikler and Gordon, 2000), population genetic inferences were hampered by high levels of conservation or large numbers of null alleles among the loci examined (Chapuis and Estoup, 2007). Despite the fact that microsatellites represent one of the most powerful tools for population genetic studies (Jarne

and Lagoda, 1996), these markers have been utilized only in one previous study on the population biology of *F. circinatum* (Berbegal *et al.*, 2013, Santana *et al.*, 2009).

In this study we utilized microsatellite markers to study the population biology of *F.circinatum* collected from plantation trees South Africa. For this purpose we specifically targeted the *P. greggii* and *P. radiata* pitch canker outbreaks in the ECP and WCP for which extensive isolate collections could be obtained or were available from previous studies (Coutinho *et al.*, 2007, Steenkamp *et al.*, 2013). Our overall aim was to evaluate the impact that extrinsic factors such as plant host and geographic origin, and intrinsic factors such as reproductive mode could have had in structuring the *F. circinatum* populations in these regions. A secondary aim was to determine if the *F. circinatum* genetic variation found in these plantation outbreaks could be linked to outbreaks of the *Pinus* seedling disease occurring in nurseries in the WCP and elsewhere in the country. The results of this study would thus be valuable for understanding and predicting how and why plantation outbreaks of pitch canker occur in South Africa.

Methods and Materials

Fusarium circinatum isolates

A total of 202 isolates of *F. circinatum* were used in this study. Of these, 64 isolates originated from diseased *P. greggii* trees from plantations in the Maclear (35 isolates) and Ugie (29 isolates) regions of the ECP (see below), while 95 isolates originated from pitch canker-affected *P. radiata* trees in WCP plantations. The latter set of isolates were from previous studies and were collected in the Tokai and George regions of the WCP (Coutinho *et al.*, 2007, Steenkamp *et al.*, 2013). To study the possible link between the plantation populations of the pathogen and those occurring on seedlings in nurseries, we also included 43 individuals of *F. circinatum* that were originally isolated from *Pinus* seedlings. Of these, 17 isolates represented distinct VCGs collected from diseased *P. radiata* seedlings in a

commercial forestry nursery in the WCP (Coutinho *et al*., 2007, Steenkamp *et al*., 2013), while the remaining 26 isolates represented unique VCGs obtained from diseased *P. patula* seedlings in nurseries elsewhere in the country (Britz *et al.*, 2005, Viljoen *et al.*, 1997). These two isolate collections from nurseries were designated the WCP and non-WCP nursery collections.

The *F. circinatum* isolates originating from the two ECP plantations were obtained as follows. Plant tissue samples were collected from the edges of resinous cankers on the stems and branches of 10-year-old *P. greggii* trees displaying symptoms typical of pitch canker. Small sections (ca. 4 mm^2) of the diseased tissue were cut and transferred to *Fusarium* selective medium (Nelson *et al.*, 1986) and incubated at 25 °C for one week under cool, white fluorescent light. Colonies resembling *Fusarium* were then transferred to half strength potato dextrose agar (PDA) (Biolab, Merck, South Africa) and incubated at 25°C for one week. To obtain pure cultures, single germinating conidia were transferred to PDA medium and incubated as described above. The identity of the isolates was confirmed as described before (Steenkamp *et al*., 2013) by using a PCR-based diagnostic method that employs *F. circinatum*-specific primers (Schweigkofler *et al.*, 2004). Each of the *F. circinatum* isolates recovered from *P. greggii* originated from a separate tree and have been deposited in the Fusarium culture collection (CMWF) maintained at the Forestry and Agricultural Biotechnology Institute (FABI).

Microsatellite analyses

Extraction of genomic DNA was performed using SDS (Sodium docecyl sulphate) and CTAB (N-cetyl-N,N,N-trimethyl-ammonium) as described by Iturritxa *et al.* (2011). Following successful DNA extraction, the alleles at 10 microsatellite loci were amplified for all isolates included in this study. The amplification used fluorescently labelled primers and

followed the protocol described by Santana *et al.* (2009). The amplicons were multiplexed into two panels and separated according to size using an ABI Prism 3100 Genetic analyser (Applied Biosystems). Amplicon sizes were determined by comparing the peaks of the four fluorescent dyes against the internal LIZ-500 size standard (Applied Biosystems). Allele sizes were estimated using GENEMAPPER 4.0 computer software (Applied Biosystems) and confirmed by visual inspection of the electropherogram for each isolate.

Genetic diversity of *F. circinatum* originating from *Pinus* plantations

A data matrix was generated by coding the alleles at the different loci using letters for each individual allele. The multilocus haplotype (MLH) for each isolate was inferred by combining its allele information for the respective loci into a 10 letter sequence (e.g. AFDCCDAAFB, where the first locus had allele A, the second locus had allele F, etc.). The frequency of each allele at a specific locus was then determined for the entire population, as well as for various pre-defined sub-populations (see below). To calculate genetic diversity parameters, a number of populations based on host and geographic origin were defined. Thus, the 159 individuals of *F. circinatum* isolated from pitch canker-affected plantation trees were separated into four collections representing the geographic region from which they originated (George, Tokai, Ugie and Maclear) and two collections representing their hosts (*P. greggii* and *P. radiata*). To account for biases associated with sampling the same genetic individual more than once (Chen *et al.*, 1994), all parameters for describing genetic diversity were calculated both with and without clone correction. For clone correction, duplicate MLHs were removed from the respective datasets before calculations were preformed or indices determined.

Allele diversity (*H*) was estimated with POPGENE (version 1.32) (Yei *et al.*, 1999) by using the equation $H = 1 - \sum x_k^2$ where x is the frequency of the k^{th} allele (Nei, 1973). Genotypic

diversity (*G*) was calculated using the equation $G = \frac{1}{\sum p_i^2}$. where p_i is the observed frequency of the i^{th} genotype in the population (Stoddart and Taylor, 1988). Population diversity was also evaluated using the Shannon diversity index (*SI*) using the equation $SI = -\sum p_i \ln p_i$, where p_i is the frequency of i^{th} genotype in the population (Sheldon, 1969). To allow for comparisons between populations we used a normalized *SI* (H_s) based on the equation $H_s = SI/\ln N$, where *N* is the number of individuals in the population. To evaluate the arrangement of MLHs within the populations, the Evenness index was employed using the equation $E_5 = (G - 1) / (e^{Hs} - 1)$ (Grünwald *et al.*, 2003).

Population differentiation was analysed using the equation $\theta = \frac{Q-q}{1-q}$ where θ is an estimate of Wright's F_{st} (Wright, 1978). Here, *Q* is the probability that two alleles in a single population are the same and *q* is the probability that two alleles from different populations are the same (Weir, 1996). These analyses was performed using the MLH datasets and MULTILOCUS (version 1.3b) (Agapow and Burt, 2001). To test the null hypothesis of no population differentiation, we calculated θ across a number of defined populations using 1 000 000 randomizations. The level of gene flow (*M*) between populations was calculated as $M = \frac{\left(\frac{1}{\theta}\right)-1}{2}$ (Cockerham and Weir, 1993).

Inference of the reproductive mode *F. circinatum* in *Pinus* plantations

Two approaches were used to make inferences regarding the reproductive mode(s) utilized by the populations of *F. circinatum* in the WCP and ECP, one approach tested for the random association of alleles which is a hallmark of sexual reproduction (Otto and Lenormand, 2002). A second, approach utilized mating compatibility among isolates to assess the potential for sexual recombination. The association of alleles was tested on the clone-corrected dataset using MULTILOCUS, where we utilized the Index of association (I_A) and

rBarD ($\bar{r}_d$), which provide estimates of linkage disequilibrium within a population that account for recombination among individuals and association between alleles at different loci (Smith *et al.*, 1993). In all cases, 10 000 randomized datasets were used to test the null hypothesis that the alleles in the population are randomly associated and that the population is expected to freely undergo recombination. Sexual compatibility among isolates was analysed as described before (Steenkamp *et al.*, 2013) by making use of a PCR-based approach to determine the mating type of individuals (Steenkamp *et al.*, 2000).

Comparison of the genetic diversity of *F. circinatum* occurring in *Pinus* plantations and seedling nurseries

To determine whether the *F. circinatum* diversity observed in the plantation outbreaks is in any way linked to the populations of the pathogen responsible for the nursery outbreaks of the *Pinus* seedling disease, we utilized 43 distinct VGC representatives previously isolated from diseased nursery seedlings (Britz *et al.*, 2005, Coutinho *et al.*, 2007, Steenkamp *et al.*, 2013, Viljoen *et al.*, 1997). To allow for a reasonable comparison between these two isolate collections, we compared the clone corrected MLH dataset generated for the isolates obtained from plantation trees with the MLH dataset generated for the 43 nursery isolates. The reproductive mode of the nursery collections was compared using the Index of Association and rBarD as described previously. GENALEX (version 6.41) (Peakall and Smouse, 2006) was used to conduct analyses of molecular variance (AMOVA) using 99999 permutations to identify the potential sources of genetic variation within and between the pre-defined populations.

The MLH datasets were also subjected to an analysis with STRUCTURE 2.3.4 (Hubisz *et al.*, 2009, Pritchard *et al.*, 2000). Runs utilized 750 000 Markov Chain Monte Carlo repetitions following a burn-in of 500 000, and the LOCPRIOR model (Hubisz *et al.*, 2009), where

collection origins were used as presumed populations of origin. The number of clusters (*K*) in the population was determined based on 20 independent runs for each *K* ranging from one to 10. The optimal number of clusters in the dataset was determined by computing L(*K*) (the mean log-likelihood of *K*) and Δ*K* as implemented in STRUCTURE HARVESTER v.06.94 (http://taylor0.biology.ucla.edu/structureHarvester/) (Earl and von Holdt, 2012). The 20 replicates for the optimal *K*-value were processed using CLUMPP 1.1 using the greedy algorithm (Jakobsson and Rosenberg, 2007), after which the results were graphically presented using DISTRUCT 1.1 (Rosenberg, 2004).

Results

Genetic diversity originating from pitch canker-affected plantation trees

High levels of polymorphism were detected at most of the microsatellite loci analysed in this study (Table 1). The only exception was for the isolate collection from Tokai (*P. radiata*) in which all but one of the 10 microsatellite loci were monomorphic. However, all of the loci were polymorphic within the Ugie (*P. greggii*) collection, and nine of the loci were polymorphic in each of the collections from George (*P. radiata*) and Maclear (*P. greggii*) (i.e., only one locus was monomorphic in each of these collections). No null alleles were identified in any of the isolate collections using these microsatellite markers.

A total of 49 alleles were identified using the 10 microsatellite markers across the 159 individuals originating from diseased plantation trees of either *P. radiata* or *P. greggii* (Tables 1 and 2). The greatest number of alleles were detected for microsatellite marker FCM-7 (11), while markers FCM-20 and FCM-24 had the least with only two alleles each. Within the various isolate collections examined, the number of alleles detected ranged from 11-38 alleles and the number of unique alleles ranged from 0 to 15 (Tables 1 and 2). The

Table 1. Allele frequencies at 10 microsatellite loci determined for the examined *F. circinatum* isolate collections obtained from pitch canker-affected *Pinus* plantations in the Western (George and Tokai) and Eastern (Ugie and Maclear) Cape provinces of South Africa.

		Isolate collections[a]						
Locus	**Allele**	**George**	**Tokai**	**Maclear**	**Ugie**	**WCP (*P. radiata*)**	**ECP (*P. greggii*)**	**Overall**
FCM-2	A	0.412	-	0.514	0.069	0.147	0.313	0.214
	B	-	-	0.057	0.862	-	0.422	0.170
	C	0.294	0.984	-	0.069	0.737	0.031	0.453
	D	0.235	0.016	-	-	0.095	-	0.057
	E	-	-	0.343	-	-	0.188	0.075
	F	0.059	-	0.086	-	0.021	0.047	0.031
FCM-3	A	0.382	-	0.543	0.103	0.137	0.344	0.220
	B	0.029	-	-	-	0.011	-	0.006
	C	0.294	-	0.314	0.897	0.105	0.578	0.296
	D	0.294	1.000	0.143	-	0.747	0.078	0.478
FCM-4	A	0.412	-	0.486	0.103	0.147	0.313	0.214
	B	0.294	1.000	0.200	-	0.747	0.109	0.491
	C	0.294	-	0.114	-	0.105	0.063	0.088
	D	-	-	0.029	-	-	0.016	0.006
	E	-	-	0.057	0.897	-	0.438	0.176
	F	-	-	0.114	-	-	0.063	0.025
FCM-6	A	0.235	-	-	-	0.084	-	0.050
	B	0.765	1.000	0.800	1.000	0.916	0.891	0.906

	C	-	-	0.200	-	-	0.109	0.044
FCM-7	A	-	-	0.200	-	-	0.109	0.044
	B	0.029	-	-	-	0.011	-	0.006
	C	-	-	0.057	-	-	0.031	0.013
	D	0.265	1.000	-	-	0.737	-	0.440
	E	0.059	-	0.229	0.034	0.021	0.141	0.069
	F	0.382	-	0.429	0.034	0.137	0.250	0.182
	G	0.029	-	-	-	0.011	-	0.006
	H	0.235	-	-	0.034	0.084	0.016	0.057
	I	-	-	0.057	0.793	-	0.391	0.157
	J	-	-	-	0.103	-	0.047	0.019
	K	-	-	0.029	-	-	0.016	0.006
FCM-16	A	0.676	-	0.800	1.000	0.242	0.891	0.503
	B	0.029	-	-	-	0.011	-	0.006
	C	-	-	0.114	-	-	0.063	0.025
	D	0.294	1.000	-	-	0.747	-	0.447
	E	-	-	0.086	-	-	0.047	0.019
FCM-20	A	1.000	1.000	0.943	0.069	1.000	0.547	0.818
	B	-	-	0.057	0.931	-	0.453	0.182
FCM-23	A	0.029	-	-	-	0.011	-	0.006
	B	0.500	1.000	0.114	-	0.821	0.063	0.516
	C	0.382	-	0.429	0.034	0.137	0.250	0.182
	D	0.029	-	0.114	0.069	0.011	0.094	0.044

	E	0.029	-	0.086	0.897	0.011	0.453	0.189
	F	0.029	-	0.057	-	0.011	0.031	0.019
	G	-	-	0.200	-	-	0.109	0.044
FCM-24	A	0.324	1.000	0.429	0.034	0.758	0.250	0.553
	B	0.676	-	0.571	0.966	0.242	0.750	0.447
FCM-25	A	0.853	1.000	0.743	0.931	0.947	0.828	0.899
	B	0.118	-	0.257	0.069	0.042	0.172	0.094
	C	0.029	-	-	-	0.011	-	0.006

[a] Isolates were obtained from diseased plantation trees of *P. radiata* (George and Tokai) and *P. greggii* (Ugie and Maclear). Isolates were grouped according to their plantation of origin and according to the province from which they were obtained (those originating from the Tokai and George areas were isolated from *P. radiata* in the Western Cape Province, while those originating from Ugie and Maclear were isolated from *P. greggii* in the Easten Cape Province).

corresponding allele diversity (*H*) for the respective collections ranged from 0.003 for the isolates from Tokai (*P. radiata*) to 0.496 for those from Maclear (*P. greggii*) (Table 2).

Among the 159 plantation isolates examined, a total of 30 MLHs were found (Table 2; Supplementary Table S1). Of these, 16 were identified only once, while 4 (MLH 2, 5, 38, and 44) were shared at least once between two collections. Although none of the MLHs were shared across all of the individual isolate collections considered, four MLHs were shared between collections: MLH 44 was detected in isolate collections from Tokai and George (*P. radiata*); MLH 2 was detected in those of Maclear and Ugie (*P. greggii*); and both MLH 38 and 5 were found in Maclear and George.

Table 2. Statistics for describing genetic diversity based on the analysis of 10 microsatellite loci for the different collections of *F. circinatum* isolates originating from *Pinus* hosts in the Eastern and Western Cape Provinces (ECP and WCP, respectively) of South Africa.

Isolate collections [a]	**Number of isolates**	**Number of alleles**	**Number of unique alleles**	**Number of MLHs** [c]	H [b]	G [d]	$\hat{G}$ [e]	SI [f]	H_s [g]	E_5 [h]
George (WCP)	34	34	7	10	0.486	4.587	13.492	1.778	0.504	0.729
Tokai (WCP)	61	11	0	2	0.003	1.033	1.693	0.084	0.020	0.382
Maclear (ECP)	35	35	10	16	0.496	11.239	32.110	2.575	0.724	0.844
Ugie (ECP)	29	22	0	6	0.137	1.840	6.345	1.001	0.297	0.488
WCP (*P. radiata*)	95	34	7	11	0.299	1.830	1.926	1.061	0.233	0.439
ECP (*P. greggii*)	64	38	15	21	0.298	7.211	11.268	2.529	0.608	0.538
Total isolates	159	46	N/A	30	0.510	4.509	2.836	2.252	0.444	0.413

[a] Isolates were obtained from diseased plantation trees of *P. radiata* (George and Tokai) and *P. greggii* (Ugie and Maclear). For the various comparisons, isolates were grouped according to their plantation of origin and according to the hosts from which they were obtained (those originating from the Tokai and George areas were isolated from *P. radiata*, while those originating from Ugie and Maclear were isolated from *P. greggii*).

[b] *H*: Allele diversity (Nei 1973); $H = 1 - \sum x_k^2$, where x_k is the frequency of the *k*th allele.

[c] MLH = multilocus haplotype.

[d] *G*: Genotypic diversity (Stoddart and Taylor 1998) of the *F. circinatum* populations in South Africa; $G = \frac{1}{\sum p_i^2}$, where p_i stands for the observed frequency of the *i*th genotype.

[e] $\hat{G}$: Maximum genotypic diversity ($\hat{G} = \frac{G}{n} \times 100$) in the population expressed as a percentage (%), where *G* is the Genotypic diversity of the population.

[f] *SI*: Shannon Diversity Index (Sheldon1969); $SI = -\sum p_i \ln p_i$, where p_i is the frequency of *i*th genotype in the population.

[g] H_s: Normalized Shannon Diversity Index; $H_s = SI/\ln N$, where N is the number of individuals in the population.

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

As reflected by the E_5-values, the frequencies of MLHs in two of the four isolate collections were not evenly distributed, especially in the WCP collections (Table 2, Figure 1). For example, in Tokai (*P. radiata*) (E_5 = 0.382) MLH 44 comprised 98% the population and in Ugie (*P. greggii*) (E_5 = 0.488) MLH 23 comprised 72% of the population. In contrast, the isolate collections from Maclear and George were characterized by higher E_5-values, which are suggestive of a more even within-population distribution of MLHs. The corresponding genotypic diversity (G) and maximum percentage genotypic diversity ($\hat{G}$) indices for these

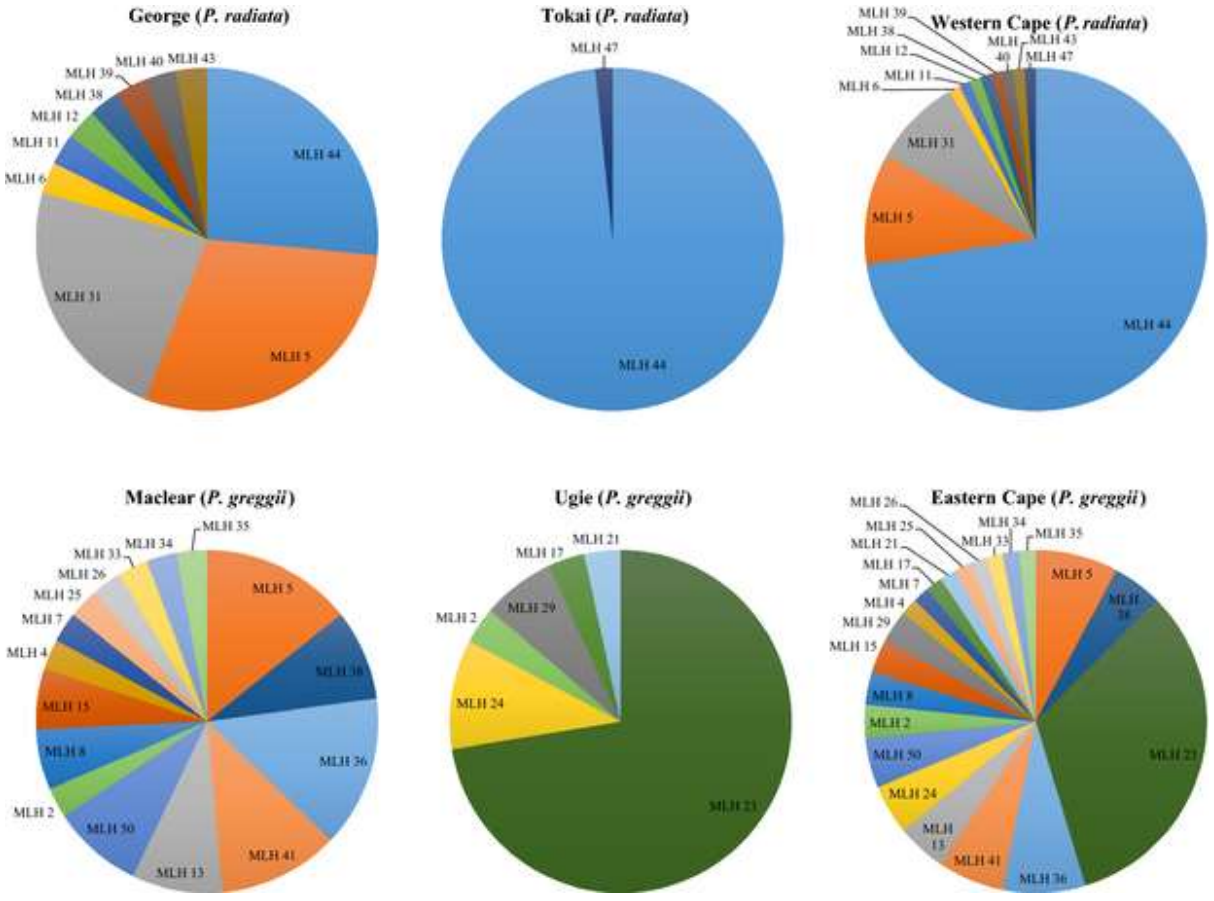

Figure 1. Distribution and frequency of multilocus haplotypes (MLHs) found in the South African isolate collections of *F. circinatum* (see supplementary table 1). Graphs indicate individual isolate collections as well as the combined collections based on geographic origin. Haplotypes are represented in different colours in each section of the pie graph. The size of each section is representative of the number of individuals found in each collection.

isolate collections were also much higher than those for the Tokai and Ugie collections (Table 2).

Analysis of the MLH data with MULTILOCUS generated significant *theta*-values (θ) ($P \leq 0.05$) for most of the pairwise comparisons of the different isolate collections (Table 3). In these instances, the null hypothesis that there are no population differences was thus rejected. Among the isolate collections grouped according to geographic origin, the collection from Tokai was most differentiated from the others, irrespective of whether the datasets were clone corrected (i.e., duplicate MLHs were removed prior to analysis) or not. The frequencies of the alleles in the Tokai isolates were thus dominated by a single allele at each locus, with limited alleles shared with the collections from the other locations. This was also reflected in the relatively low gene flow values estimated for the Tokai comparisons (Table 3). Although, the two ECP isolate collections displayed some differentiation, this disappeared when the clone corrected data were used. However, of the two ECP (*P. greggii*) collections, the one from Ugie was most different from the two WCP collections (*P. radiata*) using both the clone-corrected and uncorrected data (Table 3). When the isolates were grouped based on their host of origin, some level of differentiation (θ=0.425; $P \leq 0.05$) was observed between the collections, although this also disappeared when the data were clone-corrected.

Inference of the reproductive mode for isolates from plantations

Analyses of random mating in the collection of *F. circinatum* isolates obtained from pitch canker-affected plantation trees in the ECP (*P. greggii*) and WCP (*P. radiata*) resulted in I_A and $\bar{r}_d$ values of 3.525 and 0.394, respectively. Neither the I_A nor the $\bar{r}_d$ values fell within the distribution of values generated from a 10 000 times randomized datasets ($P < 0.001$ for all populations analysed). The I_A and $\bar{r}_d$ values for each of the individual isolate collections showed similar trends in which the values fell outside the distribution range of the

Table 3. Pairwise comparison of population differentiation (θ) and gene flow (M) [a] among the *F. circinatum* isolate collections originating from diseased *P. radiata* trees in the Western Cape Province [b] and from *P. greggii* plantation trees in the Eastern Cape Province [c].

Collections	**George**	**Tokai**	**Maclear**	**Ugie**	**WCP (*P. radiata*)**	**ECP (*P. greggii*)**
George		0.594: 0.341 *(0.262: 1.408)*	0.074: 6.257 *(-0.019^{NS}: -)*	0.499: 0.502 *(0.117: 3.774)*		0.168: 2.476 *(0.007^{NS}: -)*
Tokai			0.686: 0.229 *(0.377: 0.826)*	0.938: 0.033 *(0.515: 0.471)*		0.651: 0.268 (0.383: *0.805)*
Maclear				0.460: 0.587 *(0.084^{NS}: -)*	0.377: 0.826 *(0.022^{NS}: -)*	
Ugie					0.661: 0.256 *(0.149: 2.856)*	
WCP (*P. radiata*)						0.425: 0.676 *(0.048^{NS}: -)*

[a] Values are indicated as θ:M. Here θ is an estimate of Wright's Fst (Weir, 1996) and calculated using $\theta = \frac{Q-q}{1-q}$, where Q is the probability that two alleles in a single population are the same and q is the probability that two alleles from different populations are the same (Weir, 1996). M is the level of gene flow calculated using $M = \frac{\left(\frac{1}{\theta}\right)-1}{2}$ (Cockerham and Weir, 1993). Italicized values indicated in parenthesis are based on the clone corrected data for each isolate collection. Negative values for θ were treated as zero, meaning no population differentiation between isolate collections. Unless indicated with "NS" (for not significant), all θ values were significant at $P \leq 0.05$ based on 1 000 000 randomizations of the relevant dataset. Hyphen indicates M values not calculated due to the insignificant θ value.

[b] Collections from the George and Tokai regions.

[c] Collections from the Ugie and Maclear regions.

Table 4. Observed Index of Association (I_A) and $\bar{r}_d$ values, as well as, the mating type distribution for each of the isolate collections grouped according to whether they originate from *Pinus* hosts in plantations or seedling nurseries.

Grouping	**Isolate Collection**	I_A [a]	$\bar{r}_d$ [b]	***P*- value[c]**	**Mating type[d]**	
					Mat-1	***Mat-2***
Plantation	George[e]	3.216 *(1.836)*	0.403 *(0.233)*	< 0.001	34	-
	Tokai[e]	-	-	-	59	2
	Maclear	3.445 *(1.206)*	0.517 *(0.178)*	< 0.001	29	-
	Ugie	1.947 *(1.597)*	0.219 *(0.179)*	< 0.001	38	-
	P. radiata	5.320 *(2.185)*	0.681 *(0.277)*	< 0.001	95	2
	P. greggii	2.349 *(1.171)*	0.262 *(0.132)*	< 0.001	67	-
	Plantation combined	3.525 *(1.233)*	0.394 *(0.139)*	< 0.001	160	2
Nursery[f]	WCP Nursery	3.054	0.436	< 0.001	13	4
	Non-WCP nurseries	0.448	0.057	< 0.001	10	13
	Nursery combined	3.631	0.439	< 0.001	23	17
All isolate collections combined		**2.881**	**0.323**	**< 0.001**	**183**	**19**

[a] I_A: Index of association measure of multilocus linkage disequilibrium (Smith *et al*., 1993). Values in parenthesis are for clone corrected datasets. For Tokai neither the I_A or $\bar{r}_d$ could be calculated due to there being only 2 MLH in the collection.

[b] $\bar{r}_d$: Measure of multilocus linkage disequilibrium independent of sample size (Smith *et al*., 1993). Values in parenthesis are for clone corrected datasets.

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

[d] Number of individuals in each of the populations containing with either *mat1* or *mat2* mating type. The data presented for the isolates from George and Tokai were obtained from previous work (Steenkamp *et al*., 2013).

[e] Isolates from George and Tokai as described in Coutinho *et al*,. (2007) and Steenkamp *et al*,. (2013)

[f] Isolates from the nursery collections comprised different vegetative compatibility groups represented by a single isolate.

randomized dataset (Table 4). The null hypothesis that the alleles within the respective isolate collections are randomly associated was therefore rejected. This suggests a lack of recombination among individuals of *F. circinatum* associated with pitch canker of plantation trees in the WCP (*P. radiata*) and ECP (*P. greggii*). This apparent lack of sexual reproduction was also supported by the results of the sexual compatibility tests, where all of the isolates from the ECP (*P. greggii*) were diagnosed as having the *mat 1* mating type (Table 4), which is similar to what has been observed previously for the WCP plantation population (*P. radiata*) of the fungus (Steenkamp *et al*., 2013).

Comparison of isolate genetic diversity from plantations and seedling nurseries

Within the set of 43 isolates representing distinct VCGs and that were previously obtained from diseased nursery seedlings (Britz *et al.*, 2005, Coutinho *et al.*, 2007, Steenkamp *et al.*, 2013, Viljoen *et al.*, 1997), 29 MLHs were detected (Supplementary Table S1). No MLHs were shared between the WCP and non-WCP nursery collections. Two MLHs (MLH 6 and MLH 11) were shared between the WCP nursery collection and the plantation collection from George, while MLH 44 was found in the George and Tokai plantation collections. Comparison of the combined nursery collection to the isolate collections originating from plantations revealed the presence of one MLH (MLH 5) common to all the collections, and another three (i.e., MLH 6, 11 and 44) and four (i.e., MLH 2, 3 23 and 29) were common to the nursery collection and the respective WCP and ECP plantation populations of the pathogen (Supplementary Table S1 and Figure S1).

To determine if the source of genetic variation found in the plantation populations could be attributed to the nursery outbreaks of the seedling disease caused by *F. circinatum*, the data were subjected to AMOVA to investigate partitioning of diversity within and among the various isolate collections. The results indicated that none of the molecular variance in the

Table 5. Results of analysis of molecular variance (AMOVA)[a] based on the microsatellite data for different *F. circinatum* isolate collections obtained from disease nursery seedlings or established plantation trees of *Pinus*.

Source of Variation [b]	**Degrees of freedom**	**Sum of Squares**	**Variance component**	**% of the total molecular variance**	**Φ**	***P* value**
Between nurseries and plantations	1	2.216	0.000	0%	-0.046	1.000
Between collections	4	21.527	0.265	9%	0.0095	<0.0001
Within collections	71	180.205	2.538	91%	0.0530	0.008
Total	76	203.948	2.803			

[a] Statistics and parameters as described by Excoffier *et al*. (1992) and implemented in GeneAlEx 6.5 (Peakall and Smouse, 2006). The Φ-statistic is analogous to Weir and Cockerham's (1984) θ estimate and reflects the correlation of haplotypic diversity at different levels of hierarchical subdivision (Excoffier *et al*. 1992). Significance of the covariance components for different levels of subdivision was tested with a non-parametric involving 99999 permutations.

[b] The different levels of hierarchical subdivision evaluated in this study. This analysis included the clone corrected MLH datasets. For the "between nurseries and plantations", one collection included MLHs originating from plantations (i.e., in George and Tokai in the Western Cape Province [WCP] and Ugie and Maclear in the Eastern Cape Province) and the other included MLHs determined for the representative isolates obtained from seedling nurseries (i.e., the WCP and non-WCP nursery collections). For the "between collections" and "within collections" subdivisions, MLHs were grouped according to geographic origin and corresponded to six collections (i.e., the WCP and non-WCP nursery collections, as well as the George, Tokai, Ugie and Maclear plantation collections

data could be attributed to variation between the isolate collection from plantations and from seedling nurseries (Table 5). However, 91% of the total variance could be ascribed to the variation within isolate collections, while 9% of the variance was attributable to the variation between geographically defined isolate collections. This limited genetic differentiation, or lack thereof, among the various isolate collections from *Pinus* seedling nurseries and plantations was also evident from the results of the STRUCTURE analysis (Pritchard *et al.*, 2000). The latter analysis revealed that the examined *F. circinatum* collections are comprised of four genetic clusters (Figure 2), all four of which were distributed across all six of the geographically defined collections and in all of the individuals examined. The different isolate collections were however unique in terms of the association of individuals to the different clusters. For example, two of the clusters, indicated in grey and pink, were prevalent in the WCP-nursery collection, whereas in the non-WCP nurseries the most prevalent clusters were those coloured grey, blue and orange (Figure 2). Also, in contrast to the WCP plantation collections, one of the clusters (pink) was rare in the ECP plantation collections.

Results of the I_A and $\bar{r}_d$ analyses for the nursery collections revealed the same trend as in the plantation populations (i.e., no random association of alleles in the population). The I_A and $\bar{r}_d$ values for combined dataset of the nursery and plantation collections were 2.881 and 0.323 respectively. Both indices were not within the values of a 10 000 times randomised dataset ($P < 0.0001$).

Discussion

Analysis of the microsatellite data generated in this study revealed significant structure within the *F. circinatum* populations associated with pitch canker-affected plantation trees in the ECP (*P. greggii*) and the WCP (*P. radiata*) of South Africa. To some extent this structuring appeared to be driven by geography rather than host. Although definition of populations

according to host of origin produced a θ-value suggestive of strong population differentiation, this differentiation disappeared when the data were clone corrected. However, irrespective of clone correction, θ-values of >0.10 and >0.25 were obtained for most of the comparisons of isolate collections defined based on sampling locality. Such high values for Weir's (1996) estimate of F_{ST} are suggestive of moderate and strong population differentiation, respectively (Wright (1978). This was particularly true for the comparisons involving the isolate collections from Tokai (WCP) and Ugie (ECP). These findings are thus consistent with the results of a previous AFLP-based study of *F. circinatum* responsible for pitch canker in the WCP, where populations from different locations were also substantially differentiated (Steenkamp et al 2014).

Despite being significantly structured, the various plantation populations of *F. circinatum* appeared to be interconnected. This was evident from the relatively high levels of gene flow inferred for some population pairs, especially when the clone corrected data were used. For example, *M*-values indicative of considerable gene flow (Cockerham and Weir, 1993, Halliburton, 2004) were estimated for the populations from George and Tokai (M=1.4) and from George and Ugie (M=3.8). This connectivity among populations was also reflected in the various MLHs shared between and among the respective isolate collections and the general lack of unique alleles in some of the collections (particularly those from Ugie and Tokai). Based on the results for both gene flow and population differentiation analyses, it thus seems as if the *F. circinatum* populations responsible for the various pitch canker outbreaks are generally distinct (as reflected by significant θ-values), but that gametes or individuals may move among populations (as reflected by significant *M*-values). In other words, high proportions of the genetic diversity in each plantation are due to allele frequency differences among populations (Holsinger and Weir, 2009), although individual populations also contain "migrant" alleles shared with other populations (Cockerham and Weir, 1993,

Halliburton, 2004).These "migrants" might represent remnants from a recent establishment of the pathogen from a common source. They might also reflect poor hygiene and silvicultural practices. Both of these hypothesis are consistent the notion that distinct introductions gave rise to the various pitch canker outbreaks in South African *Pinus* plantations and that the presence of apparently migrant individuals or gametes are due to anthropogenic activity linked to nursery production (Steenkamp et al. 2014).

Overall, the population of *F. circinatum* in South African plantations appeared to be more diverse than anticipated. For example, the genotypic diversity (Stoddart and Taylor, 1988) for the overall plantation population (G=4.509) is comparable to what has been estimated for the *F. circinatum* population in the South Eastern United States where pitch canker has been known for more than seven decades (Wikler and Gordon, 2000). The genotypic diversity and allele diversity in plantations also seemed higher than had had previously been estimated for the *F. circinatum* populations responsible for the *Pinus* seedling disease in South Africa (Britz *et al.*, 2005). However, our data suggested that the diversity of the fungus is not evenly distributed across the plantation landscape, as individual isolate collections were characterized by G-values that ranged from 1.033 for Tokai to 11.239 for Maclear. The allele diversity (Nei, 1973) within the isolate collections was also dramatically different ranging from 0.003 for Tokai to 0.496 for Maclear, relative to the 0.510 of the combined isolate collection. In addition, the Ugie and Tokai populations were both dominated by single MLHs. These data therefore also support the notion that pitch canker in the various plantations originated from distinct introductions. But, the high diversity observed for certain locations (i.e., George and Maclear) suggests continued influx of gene genotypes/alleles into these areas and that the pathogen has likely been present in these environments for much longer than originally believed. This is similar to what has been reported for the chestnut blight

fungus *Cryphonectria parasitica* in North America and Europe, where multiple introductions have occurred over an extended time frame (Dutech *et al.*, 2012).

The most plausible hypothesis for the source of the pitch canker fungus in South African *Pinus* plantations is that it has originated from the commercial nurseries that are used to establish these plantations (Coutinho *et al.*, 2007, Steenkamp *et al.*, 2013). To test this hypothesis we compared the clone corrected MLH data for the plantation isolates to the MLH data generated for a set of isolates representing the VCGs previously identified in commercial seedling nurseries (Britz *et al.*, 2005, Steenkamp *et al.*, 2013, Viljoen *et al.*, 1997). *F. circinatum* isolates belonging to a specific VCG share the same alleles at most or all of the 6-10 loci determining heterokaryon compatibility (*het*) (Gordon *et al.*, 2006). Therefore, in the absence of sexual reproduction (as is the case for the isolate collections examined in this study; see below) individuals sharing a VCG are clonally related (Leslie and Summerell, 2006), although mutation might change the VCG of isolates that are otherwise clones (Gordon *et al.*, 2006, Wikler *et al.*, 2000). The latter likely explained why only 29 MLHs were detected among the 43 VCGs examined. Nevertheless, comparisons of the two sets of microsatellite data revealed the presence of various MLHs shared between the nursery and plantation collections. The results of the AMOVA and STRUCTURE analyses further supported the connectedness between the nursery and plantation populations of *F. circinatum*.

Overall, our data supports the nursery origin of the pitch canker fungus in *Pinus* plantations, although the actual timing and mechanisms of these introductions remains to be determined. For example, the pathogen could have been introduced during planting of infected, but apparently healthy seedlings in the field (Coutinho *et al.*, 2007, Mitchell *et al.*, 2011,

Steenkamp *et al.*, 2013), or via the feeding activity of insects such as *Pissodes nemorensis* that are known to carry spores of the fungus (Coutinho *et al.*, 2007, Steenkamp *et al.*, 2013). The potential role of human activity such as pruning and replanting when seedlings succumb to post-planting stresses (Wingfield et al 2008; Mitchell et al 2011) in the establishment of pitch canker in a plantation is also not well understood and could be involved in the establishment of pitch canker outbreaks in plantations.

Despite the fact that the sexual fruiting structures of *F. circinatum* have never been observed in nature, sexual reproduction and recombination have been proposed to be the source of the observed genetic diversity of the fungus in South African commercial nurseries. The basis for these claims were the large numbers of VCGs detected in the various studies (Britz *et al.*, 2005, Britz *et al.*, 1998, Viljoen *et al.*, 1997), as well as the occurrence of both mating types in most of the nurseries where the pathogen was detected (Britz *et al.*, 2005). However, both these approaches provide only indirect evidence for the hypothesis that sexual reproduction has driven diversity of the pathogen. By making use of the microsatellite data generated in this study, we could directly test for random rearrangement of alleles (Smith *et al.*, 1993). Different from previous suggestions, no evidence for sexual recombination could be detected in the set of isolates representative of the nursery populations of *F. circinatum*. Furthermore, evidence for sexual reproduction could not be detected in any of the plantation isolate collections examined. This was also reflected by the fact that all of the plantation isolates represented a single mating type (i.e., *mat 1*; the only exception was a two *mat 2* isolates from Tokai) (this study; Steenkamp et al 2014). The diversity observed in the South African population of *F. circinatum* can therefore be ascribed mainly to the genotypic diversity associated with the initial introduction(s) of the fungus and subsequent mutationThis overall lack of or rare sexual recombination appears to be a hallmark of other *F. circinatum* populations, including Spain and the United States (Berbegal *et al.*, 2013).

Although the asexual reproductive mode is likely to have had a significant influence on structuring the genetic diversity of *F. circinatum* in the plantation setting, other factors could have also been involved. For example, in these *mat 1* dominated populations, mating type might be linked to increased fitness properties. Morphological differences between *mat 1* and *mat 2* isolates of *F. circinatum* in Spain have been reported (Pérez-Sierra *et al.*, 2007) and it is conceivable that such differences might also extend to other phenotypes such as growth rate and pathogenicity. In the case of *Cryptococcus neoformans*, an association between virulence and the mating type of individuals was observed, where individuals carrying the α mating type were more virulent than their a mating type counterparts (Kwon-Chung *et al.*, 1992). A similar argument can also be made for the occurrence of predominant MLHs in the various populations. These MLHs might represent genotypes of the fungus that are particularly well adapted at surviving and thriving in the plantation environment. Likewise, they could be involved in overcoming the defence mechanisms of the specific *P. radiata* and *P. greggii* planting stock cultivated in the various plantations because both species are known to have some genetic variation in terms of their be susceptibility to *F. circinatum* (Hodge and Dvorak, 2000, Roux *et al.*, 2007). In *Alternaria alternata*, for example, certain strains produce toxins that make them more successful in causing diseases on their preferred hosts (Hatta *et al.*, 2002). Such potential associations between genotype/mating type and fitness is actively being researched as their existence would not only explain why *F. circinatum* populations are structured in a specific ways, but also how they are likely to behave in future.

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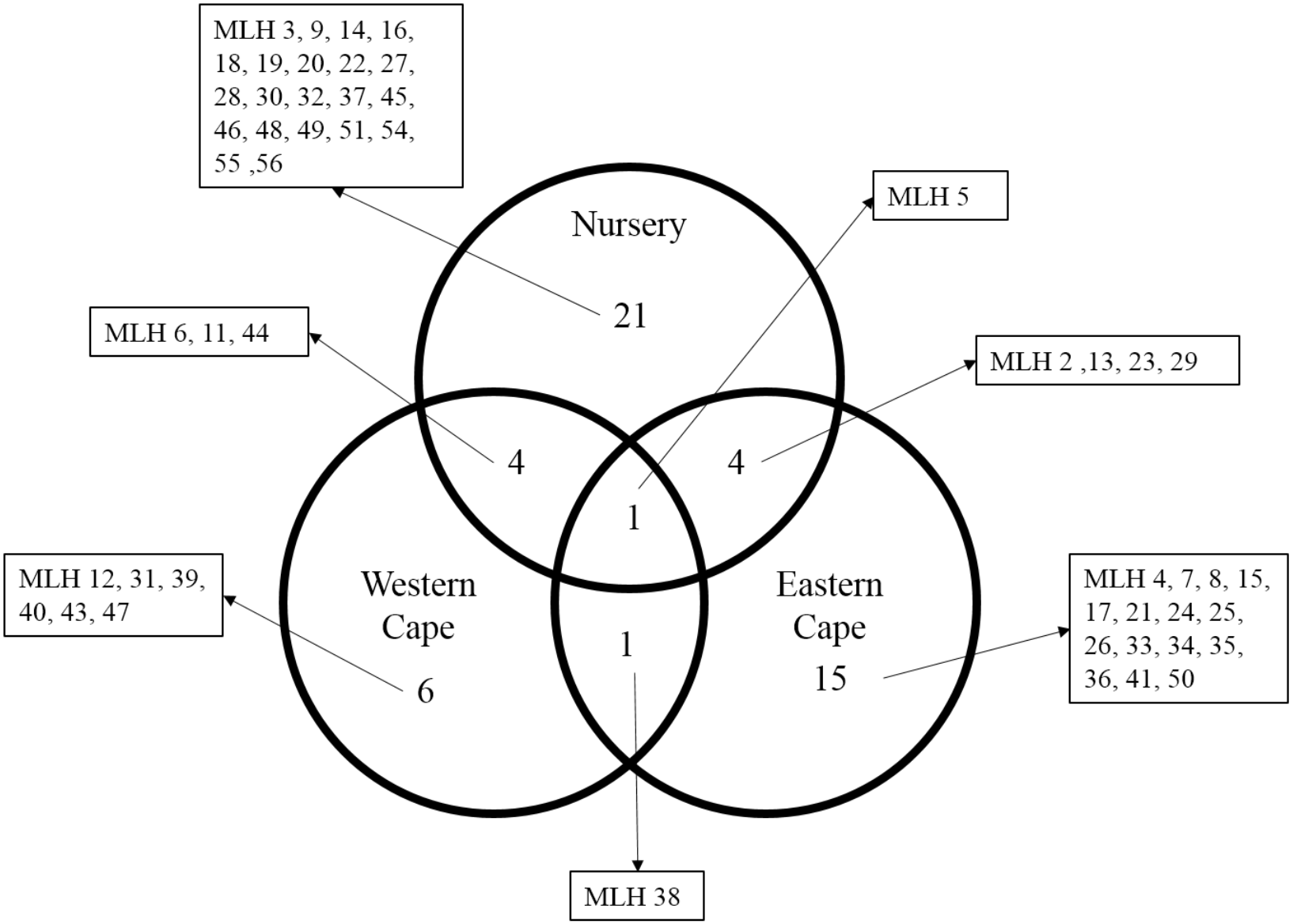
Supplementary Figure S1. Venn diagram indicating the unique and shared multilocus haplotypes (MLHs) based on geographical isolate groupings of F. circinatum in South Africa.



Supplementary table S1. Composition of multilocus haplotypes found in the *F. circinatum* collections.

Location	Isolate	MLH #
George	L53A	5
George	L53A1	5
George	L53A3	5
George	L53A4	5
George	L53A6	5
George	L53A7	5
George	L53A9	5
George	L53H	5
George	WT1	5
George	WT1.2	5
George	B2H3	6
George	B2H1	11
George	O6	12
George	B1-5	31
George	B1-6	31
George	B1-7	31
George	B1-8	31
George	L53A2	31
George	L53A5	31
George	L53A8	31
George	O19	31
George	CBH11	38
George	CBH19	39
George	BG-2	40
George	B2H6	43
George	B2H2	44
George	B2H5	44
George	CBH10	44
George	CBH12	44
George	CBH13	44
George	CBH15	44
George	CBH18	44
George	CBHA	44
George	CBHA1	44
WCP nursery	KS51	3
WCP nursery	KS1	6
WCP nursery	KS21	6
WCP nursery	KS26	6
WCP nursery	KS4	6
WCP nursery	KS39	11
WCP nursery	KS40	11
WCP nursery	KS41	11

WCP nursery	KS43	11
WCP nursery	KS44	11
WCP nursery	KS52	44
WCP nursery	KS81	44
WCP nursery	KS87	44
WCP nursery	KS47	45
WCP nursery	KS49	45
WCP nursery	KS22	46
WCP nursery	KS23	46
Maclear	7.1.1	2
Maclear	8.1.2	4
Maclear	14.4	5
Maclear	16.2	5
Maclear	17.6	5
Maclear	2.1.2	5
Maclear	U5	5
Maclear	12.2	7
Maclear	17.5	8
Maclear	2.1.2	8
Maclear	20.2	13
Maclear	3.1.4	13
Maclear	7.1.2	13
Maclear	11.2	15
Maclear	12.3	15
Maclear	6.1.1	25
Maclear	6.2.1	26
Maclear	8.1.6	33
Maclear	14.1	34
Maclear	13.8	35
Maclear	11.1	36
Maclear	12.8	36
Maclear	15.4	36
Maclear	3.1.3	36
Maclear	8.1.1	36
Maclear	20.5	38
Maclear	1.4.1	38
Maclear	U1	38
Maclear	5.1.1	41
Maclear	5.2.1	41
Maclear	8.2.1	41
Maclear	U4	41
Maclear	3.1.2	50
Maclear	4.1.1	50
Maclear	U2	50
Non-WCP nursery	FCC537	2
Non-WCP nursery	FCC1031	5

Non-WCP nursery	FCC542	9
Non-WCP nursery	FCC309	13
Non-WCP nursery	FCC311	13
Non-WCP nursery	FCC478	13
Non-WCP nursery	FCC52	14
Non-WCP nursery	FCC479	16
Non-WCP nursery	FCC341	18
Non-WCP nursery	FCC500	18
Non-WCP nursery	FCC477	19
Non-WCP nursery	FCC124	20
Non-WCP nursery	FCC65	22
Non-WCP nursery	FCC793	23
Non-WCP nursery	FCC62	27
Non-WCP nursery	FCC51	28
Non-WCP nursery	FCC38	29
Non-WCP nursery	FCC13	30
Non-WCP nursery	FCC560	32
Non-WCP nursery	FCC493	37
Non-WCP nursery	FCC514	48
Non-WCP nursery	FCC521	49
Non-WCP nursery	FCC321	51
Non-WCP nursery	FCC1034	54
Non-WCP nursery	FCC1052	55
Non-WCP nursery	FCC1035	56
Tokai	25-2	44
Tokai	28-2	44
Tokai	38-2	44
Tokai	9-1I	44
Tokai	T10-1I	44
Tokai	T1-1	44
Tokai	T11-2	44
Tokai	T1-2	44
Tokai	T12-2I	44
Tokai	T17-1I	44
Tokai	T19-1	44
Tokai	T20-2I	44
Tokai	T2-1	44
Tokai	T27-2	44
Tokai	T28-2	44
Tokai	T29-1I	44
Tokai	T30-2I	44
Tokai	T31	44
Tokai	T3-2	44
Tokai	T32-1	44
Tokai	T32-2	44
Tokai	T33-1	44

Tokai	T33-2	44
Tokai	T34-1	44
Tokai	T34-2	44
Tokai	T37-1	44
Tokai	T37-2	44
Tokai	T40-2	44
Tokai	T43-2	44
Tokai	T46-1	44
Tokai	T46-2I	44
Tokai	T47-1I	44
Tokai	T47-2I	44
Tokai	T48-1	44
Tokai	T48-2	44
Tokai	T49-1I	44
Tokai	T5	44
Tokai	T50-1I	44
Tokai	T54-1	44
Tokai	T54-2	44
Tokai	T57	44
Tokai	T58	44
Tokai	T59-1	44
Tokai	T59-2	44
Tokai	T6-1I	44
Tokai	T6-2I	44
Tokai	T63	44
Tokai	T64-1	44
Tokai	T64-2	44
Tokai	T65-1	44
Tokai	T65-2	44
Tokai	T69-2	44
Tokai	T7-1I	44
Tokai	T72-1	44
Tokai	T74-1	44
Tokai	T80	44
Tokai	T8-1I	44
Tokai	T8-2I	44
Tokai	T9-1I	44
Tokai	14	44
Tokai	T20-1	47
Ugie	UG17	2
Ugie	UG27	17
Ugie	UG18	21
Ugie	UG1	23
Ugie	UG10	23
Ugie	UG11	23
Ugie	UG12	23

Ugie	UG13	23
Ugie	UG19	23
Ugie	UG2	23
Ugie	UG20	23
Ugie	UG21	23
Ugie	UG26	23
Ugie	UG3	23
Ugie	UG30	23
Ugie	UG31	23
Ugie	UG37	23
Ugie	UG4	23
Ugie	UG42	23
Ugie	UG5	23
Ugie	UG6	23
Ugie	UG7	23
Ugie	UG8	23
Ugie	UG9	23
Ugie	UG22	24
Ugie	UG23	24
Ugie	UG24	24
Ugie	UG32	29
Ugie	UG43	29