Plant Pathology (2014) **63**, 530–538 Doi: 10.1111/ppa.12136

Evidence for a new introduction of the pitch canker fungus *Fusarium circinatum* in South Africa

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Fusarium circinatum causes pitch canker of *Pinus* species in many parts of the world. The fungus was first recorded in South Africa in 1990 as a pathogen of *P. patula* seedlings and emerged later as a pathogen of established plantation trees, especially *P. radiata* in the Western Cape Province (WCP). In this study the population biology of *F. circinatum* in the WCP was explored. The aim was to determine the possible origin and reproductive mode of the pathogen, with the ultimate intention of informing disease management strategies in the region. Vegetative compatibility assays, sexual mating studies and amplified fragment length polymorphism analyses were used. For comparative purposes, an isolate collection obtained from diseased *P. radiata* seedlings in a commercial nursery in the region, as well as a set of isolates from commercial seedling nurseries in the central and northern parts of South Africa, were included. The results showed that the WCP population of *F. circinatum* employs a predominantly asexual mode of reproduction and that it is highly differentiated from populations of the fungus elsewhere in South Africa. However, limited genetic structure was found within the respective WCP isolate collections. Overall these findings suggest that pitch canker in the WCP originates from one or more separate introductions of the pathogen and that its movement in the region is not restricted. More effective strategies are thus required to limit and manage the effects of *F. circinatum* in plantations in this region of South Africa.

Keywords: AFLP, *Fusarium circinatum*, sexual compatibility, vegetative compatibility

Introduction

The ascomycete fungus *Fusarium circinatum* is the causal agent of pitch canker of numerous *Pinus* species and *Pseudotsuga menziesii* (reviewed by Wingfield *et al.*, 2008). On trees, symptoms include discoloration and wilting of needles, branch die-back and the development of resinous cankers that can occur on branches, stems, shoots and exposed roots. When the pathogen infects seedlings, it can cause damping off, root and collar rot, tip dieback and often large-scale seedling mortality (Mitchell *et al.*, 2011). *Fusarium circinatum* has been reported from most regions where *Pinus* either occurs naturally or is grown commercially (Wingfield *et al.*, 2008; Steenkamp *et al.*, 2012). In almost all these regions, the fungus is associated with significant losses in yield and productivity, making the pitch canker pathogen one of the most important limitations to commercial forestry (Wingfield *et al.*, 2008; Mitchell *et al.*, 2011).

In an attempt to understand the observed global distribution of *F. circinatum*, various studies have sought to clarify the population biology of this fungus (reviewed by Wingfield *et al.*, 2008). Based on the levels of genetic diversity among isolates obtained from Mexico, it is now widely believed that Central America and Mexico represents the centre of origin of *F. circinatum*, where the fungus probably co-evolved with its *Pinus* host (Wikler & Gordon, 2000; Britz *et al.*, 2005). From here, the pathogen was introduced to other parts of world, either directly or indirectly via other geographic locations (Wikler & Gordon, 2000). Although the pathogen may in some regions be spread by wind, water splash and insects, much of the observed distribution of the fungus is probably as a result of the movement of infected plant material, seed and soil (Wingfield *et al.*, 2008). However, the application of stringent quarantine measures has in some instances prevented spread of the fungus to pitch canker-free areas.

In South Africa, *F. circinatum* was first detected on seedlings of *Pinus patula* in a single nursery in the Mpumalanga Province (Viljoen *et al.*, 1994). Subsequent to this initial outbreak in 1990, the pathogen has been found in most commercial nurseries in the country (Britz *et al.*, 2005; Wingfield *et al.*, 2008). Fifteen years after its initial discovery in South Africa, *F. circinatum* was identified for the first time on established plantation trees (Coutinho *et al.*, 2007). In this field outbreak of

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Published online 18 September 2013

pitch canker, the pathogen was isolated from 5- and 9-year-old *Pinus radiata* trees in the Western Cape Province (WCP) of South Africa (Coutinho *et al.*, 2007). Subsequently, the disease has been detected on *P. radiata* trees that are 12–15 years old in the George area (WCP), as well as on *Pinus greggii* in plantations in the Eastern Cape Province and KwaZulu-Natal (J. Roux, M. J. Wingfield, E. T. Steenkamp, unpublished data). Therefore, more effective control strategies are needed to limit pitch canker-related losses in these areas.

Knowledge regarding the origin, spread and evolution of *F. circinatum* populations responsible for the pitch canker outbreaks on established plantation trees in South Africa is fundamental to the development and/or implementation of effective management protocols. This is particularly true in terms of the origin of the inoculum that has given rise to these outbreaks and the reproductive behaviour of the fungus in those areas. For example, *F. circinatum* is dimictic (Burnett, 1956; Britz *et al.*, 1999), and thus capable of reproducing both asexually and sexually depending on the availability of fertile isolates of opposite mating types (Leslie & Summerell, 2006). The outcomes of these reproductive strategies are markedly different because predominantly asexual populations will be less diverse than those reproducing sexually.

The aim of this study was to investigate the possible origin(s) and reproductive mode(s) of the *F. circinatum* population(s) associated with pitch canker in the WCP. For this purpose a collection of *F. circinatum* isolates was obtained from diseased *P. radiata* seedlings in a nursery as well as from plantation trees. To determine their possible origin(s), this collection of isolates was compared with representative isolates obtained from diseased seedlings elsewhere in the country (Viljoen *et al.*, 1997; Britz *et al.*, 2005), as well as the USA and Mexico (Wikler & Gordon, 2000). Comparisons were made using vegetative compatibility group (VCG) assays (Leslie & Summerell, 2006) and amplified fragment length polymorphism (AFLP) analysis (Vos *et al.*, 1995). To assess the importance of the sexual cycle in the reproduction of the WCP population of the fungus, sexual compatibility and fertility tests were performed (Leslie & Summerell, 2006).

Materials and methods

Fusarium circinatum isolates

A total of 63 isolates of *F. circinatum* were obtained from tissue with symptoms on *P. radiata* trees growing at the site of the first outbreak in the Tokai plantation (WCP) (Coutinho *et al.*, 2007). Of these isolates, 21 were obtained from the galleries of, or damaged tissue caused by, the pine weevil *Pissodes nemorensis*. Each was from a different tree. Twenty-two isolates originated from diseased tissue associated with cankers on 22 different trees sampled randomly throughout the site of the outbreak. For 10 trees with multiple cankers, two distinct infection centres were sampled, resulting in 20 isolates. In the George area of the same Province, 37 isolates were obtained from cankers on different *P. radiata* trees. An additional collection of 70 isolates was obtained from diseased *P. radiata* seedlings collected in a commercial nursery in the region (designated 'WCP nursery'), which was previously suggested to have been a possible source of inoculum for the WCP pitch canker outbreaks (Coutinho *et al.*, 2007). Six isolates (FCC1844, FCC1845, FCC1928, FCC1985, FCC2154 and FCC2155) that were obtained from seedlings in the same nursery in 1996 were also included. The WCP nursery isolates were collected by B. Porter and the isolates from the George area were collected by J. Roux, M. Kvas and O. M. Makhari (Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa). All the isolates from the WCP used in this study were allocated isolate numbers prefixed with FCC or CMWF and are maintained in the FABI culture collection.

For all the isolations, approximately 2 mm^2 pieces of infected plant tissue were cut from the leading edges of lesions and plated onto fusarium selective medium (Leslie & Summerell, 2006). Following incubation for 7 days at 25°C under cool white fluorescent illumination, colonies resembling *Fusarium* were transferred to half-strength potato dextrose agar (PDA; Merck). After incubation (25°C), pure cultures were prepared by inoculating single germinating conidia onto fresh PDA (Leslie & Summerell, 2006). These were then confirmed to represent *F. circinatum* by making use of a procedure involving the specific amplification of a diagnostic portion of the intergenic spacer region of the ribosomal RNA cistron (Schweigkofler *et al.*, 2004). For this purpose, genomic DNA was extracted (Iturritxa *et al.*, 2011) from each isolate and then used as template in PCRs with the *F. circinatum*-specific primers CIRC1A and CIRC4A (Schweigkofler *et al.*, 2004). The PCR mixture contained 2·5 mM each dNTP, 25 mM $MgCl_2$, 10 μM each primer, 50 ng μL^{-1} template DNA, 0·03 U μL^{-1} *Taq* DNA polymerase and reaction buffer (Roche). Following denaturation at 95°C for 30 s, the amplification conditions were 30 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The PCR products were visualized using agarose gel electrophoresis (Sambrook *et al.*, 1989) to confirm the presence of the 360-bp diagnostic fragment of *F. circinatum* (Schweigkofler *et al.*, 2004).

For comparative purposes, two additional sets of *F. circinatum* isolates were included. The first set of 27 isolates represented the known VCGs originally identified from nurseries elsewhere in South Africa (Viljoen *et al.*, 1997; Britz *et al.*, 2005). The second set of 42 isolates represented known VCGs from California (10 isolates), Florida (25 isolates) and Mexico (seven isolates) (Wikler & Gordon, 2000).

Vegetative compatibility

To sort the isolates into VCGs, assays based on genetic complementation of *nit* (nitrate non-utilizing) mutants that carry lesions in either the structural or regulatory loci of their nitrate reduction pathway were used (Leslie & Summerell, 2006). Briefly, the *nit* mutants were generated by incubating isolates on medium containing 1·5% (w/v) of the toxic compound $KClO_3$, after which the mutants were phenotyped by testing their growth on medium containing differential nitrogen sources. Compatible *nit* mutants (e.g. *nit1* and *nit3*, or *nit1* and *nitM*) were then used in complementation assays to detect formation of heterokaryons between individuals of the same VCG. In all cases, the compatible *nit* mutants of a specific isolate were also complemented to control for self-incompatibility.

Genetic diversity based on the VCGs of the WCP collection of isolates was determined by using the simple *V*/*N* measure, where *N* is the number of isolates examined or sample size and *V* is the number of VCGs encountered. As an indication of population diversity, the Shannon diversity index (*SI*) was used, while the normalization of this index allowed comparisons among populations (Sheldon, 1969). *SI* was calculated with the equation $SI = -\Sigma\ p_i \ln p_i$, where p_i is the frequency of the *i*th VCG, while the normalized *SI* was calculated with the equation $H_S = SI/\ln N$. Genotypic diversity (*G*) was estimated as suggested by Stoddart & Taylor (1988) using $G = 1/\Sigma p_i^2$, while comparisons between populations used the maximum percentage of genotypic diversity ($\hat{G}$), which was obtained by multiplying *G* with *N*. To evaluate the distribution of phenotypes (VCGs) within a population, the equation $E = (G-1)/(e^{Hs}-1)$ was used where *E* is the evenness index (Grünwald *et al.*, 2003).

Sexual compatibility and fertility

The mating types for all isolates were determined by using a diagnostic PCR approach (Steenkamp *et al.*, 2000) which targets the respective conserved α-domain and the HMG (high mobility group) domain in the genomes of *mat-1* and *mat-2* individuals (Martin *et al.*, 2011). These PCRs were performed as described previously, making use of the diagnostic PCR primers MAT 1a and MAT 1b for the *mat-1* individuals, and primers MAT 2c and MAT 2d for the *mat-2* individuals (Steenkamp *et al.*, 2000). The components of the PCR mixture were the same as described earlier, including either the *mat-1* or *mat-2* primer sets. Following an initial denaturation at 94°C for 30 s, the cycling conditions included 35 cycles of 94°C for 30 s, 65°C for 45 s and 72°C for 30 s, which were concluded with a final extension at 72°C for 10 min. The mating types of the isolates were then scored using agarose gel electrophoresis of the amplified products (Sambrook *et al.*, 1989).

For the isolates from the Tokai plantation and the WCP nursery, sexual reproductive potential was evaluated by determining the extent to which isolates can act as females in crosses. For this purpose, the methods described in Leslie & Summerell's (2006) laboratory manual were used. All *mat-1* individuals were crossed with *mat-2* individuals. Because all of the Tokai isolates were *mat-1*, these were crossed with two *mat-2* laboratory isolates that are known to be female-fertile or hermaphrodites. These crosses were repeated such that all the Tokai and WCP nursery isolates were used at least once as both a female and a male. For control purposes, female fertile tester strains (MRC 7488 and MRC 6312) of opposite mating type were also crossed (Britz *et al.*, 1998).

When used as females, isolates were grown on carrot agar and when used as males they were grown on complete medium (Leslie & Summerell, 2006). Following incubation at 25°C for 7 days, the spores produced on complete medium were suspended in 2·5% (v/v) Tween 60 (Merck) and used to fertilize female isolates on carrot agar. All crosses were incubated at 17°C under cool white and near-ultraviolet lights (Leslie & Summerell, 2006). After the development of sexual fruiting structures (perithecia), the viability of ascospores was confirmed. This was accomplished by plating water-suspensions of the spores onto water agar and examining them for germination using light microscopy.

AFLP analysis

A set of isolates representing each of the different VCGs detected at each location in the WCP were subjected to AFLP analysis. The analysis also included representative isolates for VCGs from other parts of South Africa (Viljoen *et al.*, 1997; Britz *et al.*, 2005) and those from California, Florida and Mexico (Wikler & Gordon, 2000). The analysis was carried out as described by De Vos *et al.* (2007), using a set of five two-base-addition *Eco*RI and *Mse*I adapter-specific selective primers (*Eco*RI-AA + *Mse*I-AT, *Eco*RI-TC + *Mse*I-AG, *Eco*RI-AA + *Mse*I-AG, *Eco*RI-TC + *Mse*I-AT, *Eco*RI-AA + *Mse*I-CC). The *Eco*RI selective primers were labelled with either the IRDyeTM 700 or IRDyeTM 800 infrared dye (LI-COR). The amplified fragments, together with a 50–700 bp sizing standard (LI-COR), were separated and visualized with a 4200 LI-COR automated DNA sequencer. Electronic gel images were manually scored based on the presence or absence of clear and well-resolved bands. To ensure that the AFLP profiles generated were reproducible, the AFLP procedure was repeated using DNA isolated from 20 individuals. Any uncertainty in the scoring process was eliminated by excluding bands with low intensity, low molecular weight, and those with sizes inordinately similar, to allow accurate scoring (Bagley *et al.*, 2001). The scoring process was also repeated several times.

The scored AFLP data were subjected to neighbour-joining distance analysis (Saitou & Nei, 1987) in BIONUMERICS (Applied Maths) using the simple matching similarity coefficient (Kosman & Leonard, 2005), and in PHYLIP (J. Felsenstein, University of Washington; http://evolution.gs.washington.edu/phylip.html) using RESTDIST and the restriction fragment model of Nei & Li (1979) with a restriction site length of 46 nucleotides. For analysis of population structure, MULTILOCUS v. 1.3 (Agapow & Burt, 2001) was used to calculate theta (θ), which is a formulation of Wright's F_{ST} for haploids (Wright, 1978; Weir, 1996). For this purpose, θ was calculated across defined subpopulations by making use of 500 000 randomizations to test the null hypothesis of no differentiation between or among them. To determine the distribution of genetic variance within and among the different sets of isolates, ARLEQUIN v. 3.5 (Excoffier & Lischer, 2010) was used with 10 000 permutations to perform analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992).

Results

Vegetative compatibility

For the set of 170 isolates of *F. circinatum* that was obtained from the WCP, pairing of suitable *nit* mutants revealed that 143 were able to establish stable heterokaryons with at least one other isolate, which allowed their separation into 11 VCGs (VCO1–VCO11). None of the remaining 27 isolates was vegetatively compatible with any other isolate. This was not due to self-incompatibility between *nit* mutants, as the pairing of compatible *nit* mutants for a specific isolate always resulted in dense heterokaryotic growth. Each of these isolates was, therefore, considered to represent a distinct VCG (VCO12–VCO34, VCO38–VCO41) of which they were each the sole representative. Thus, the 170 *F. circinatum* isolates obtained from *P. radiata* in the WCP represent 38 distinct VCGs. However, none of these WCP isolates were vegetatively compatible with any of the isolates representing VCGs from previous studies elsewhere in South Africa or from the USA and Mexico.

Different numbers of VCGs were recovered from the three regions in the WCP (Table 1). A total of nine VCGs were identified among the Tokai isolates. Among the 37 isolates from George, 16 VCGs were identified, while the WCP nursery isolates separated into 19 VCGs. The Tokai and WCP nursery populations shared two VCGs (VCO2 and VCO5), the Tokai and George populations shared three VCGs (VCO1, VCO3 and VCO5), whilst the WCP nursery and George populations also shared two VCGs (VCO5 and VCO7).

Among the isolates obtained from Tokai, 21 isolates were associated with *P. nemorensis* feeding activity (Table 1). Fifteen of these isolates represented VCO1 and two represented VCO4, while the remaining four isolates represented VCO3 and VCO32–VCO34. In eight of the 10 instances where isolates were recovered from different cankers on a single tree, the isolates represented the same VCG. For six trees, the two isolates from different cankers represented VCO1, and for two trees, the two isolates from each represented VCO3 and VCO5. In the two instances where different VCGs were recovered from a single tree, VCO1 and VCO4 isolates were obtained from the one tree, and VCO1 and VCO5 isolates from the other.

Frequencies of individual VCGs for the three collections of isolates were not evenly distributed. For example, isolates from Tokai were dominated by VCO1, which represented 73% of the isolates collected (Table 1). Only one of the George isolates belonged to this VCG and it was not present in the collection from the commercial nursery. Among the George isolates, two other VCGs (VCO9 and VCO11) that were not recovered from any other location appeared to be dominant and were represented by 22 and 27%, respectively, of the isolates collected at that location. The dominant VCGs among the nursery isolates were VCO5 and VCO6, which represented 27 and 21% of the isolates, respectively. This patchy distribution of VCGs was also reflected by the indices for evenness, *E*, (Grünwald *et al.*, 2003) obtained for the three populations (Table 2).

Regardless of the diversity estimate employed (Table 2), the population collected in the Tokai plantation was the least diverse. This held true even when only one representative of each of the six sets of same-VCG isolates originating from different cankers on a single tree was included in the analysis. Despite the fact that a greater number of VCGs (19) were detected among the isolates obtained from diseased seedlings in the WCP nursery than in the population in George (16 VCGs), the diversity estimates for the latter population were always higher.

Table 1 The number of isolates representing the vegetative compatibility groups (VCGs) and mating types of the *Fusarium circinatum* population(s) collected in the Western Cape Province (WCP) of South Africa

Geographic location	VCG[a]	Mating type	Number of isolates[b]
Tokai (*n* = 63)	VCO1	*mat-1*	46 (15)
	VCO2	*mat-1*	1
	VCO3	*mat-1*	4 (1)
	VCO4	*mat-1*	5 (2)
	VCO5	*mat-1*	3
	VCO31–34	*mat-1*	1 each (3)
George (*n* = 37)	VCO1	*mat-1*	1
	VCO3	*mat-1*	1
	VCO5	*mat-1*	5
	VCO7	*mat-1*	1
	VCO9	*mat-1*	8
	VCO10	*mat-1*	2
	VCO11	*mat-1*	10
	VCO12–20	*mat-1*	1 each
WCP nursery (*n* = 70)	VCO2	*mat-1*	9
	VCO5	*mat-1*	19
	VCO6	*mat-2*	13
	VCO6	*mat-1*	2
	VCO7	*mat-2*	7
	VCO8	*mat-1*	4
	VCO8	*mat-2*	2
	VCO22–30	*mat-1*	1 each
	VCO21	*mat-2*	1
	VCO38	*mat-2*	1
	VCO39–41	*mat-1*	1 each

[a]VCO1 from Tokai includes isolates FCC5051 and FCC5052, which were originally isolated by Coutinho *et al.* (2007). VCO5 from the WCP nursery includes isolates FCC1844 and FCC1845 that were isolated in 1996 during a *F. circinatum* outbreak in that nursery. VCO38–VCO41 represent isolates FCC1928, FCC1985, FCC2154 and FCC2155, respectively, that were also collected during that outbreak.

[b]The number of isolates obtained from *Pissodes nemorensis* larval galleries is indicated in parentheses.

Table 2 Parameters for describing genetic diversity based on vegetative compatibility group (VCG) assignments for the different sets of isolates of *Fusarium circinatum* collected in the Western Cape Province (WCP)

	Isolate collection			
Parameter[a]	Tokai	George	WCP nursery	Combined
Population size (*N*)	63 (55)	37	70	170 (162)
Number of VCGs (*V*)	9	16	19	38
V/*N*	0·14 (0·16)	0·43	0·27	0·22 (0·23)
Shannon diversity index (*SI*)	1·08 (1·09)	2·28	2·24	2·69 (2·65)
Normalized *SI* (H_S)	0·26 (0·27)	0·63	0·53	0·52 (0·52)
Genotypic diversity (*G*)	1·77 (1·84)	6·68	6·40	7·92 (9·46)
Maximum percentage genotypic diversity ($\hat{G}$)	2·80 (3·35)	18·05	9·14	4·68 (5·84)
Evenness index (*E*)	0·40 (0·42)	0·64	0·64	0·51 (0·64)

[a]The *SI* and H_S values were calculated according to Sheldon (1969). The respective *G* and $\hat{G}$ values were determined as suggested by Stoddart & Taylor (1988) and McDonald *et al.* (1994). *E* was determined as proposed by Grünwald *et al.* (2003). Student's *t*-tests were used to show that the $\hat{G}$-values for the Tokai, George and WCP nursery populations are significantly different at $P > 0{\cdot}05$ (Chen *et al.*, 1994). The values in parentheses were calculated by including only one representative of the same-VCG isolates that were obtained from the same *Pinus radiata* tree in the Tokai plantation.

Sexual compatibility and fertility

Application of the diagnostic PCR for scoring mating type (Steenkamp *et al.*, 2000) resulted in the amplification of a *c.* 800-bp fragment from *mat-2* isolates and a *c.* 200-bp fragment from *mat-1* isolates. The mating type for all of the isolates from the George and Tokai *P. radiata* trees was *mat-1* (Table 1). Among the isolates obtained from the *P. radiata* seedlings in the WCP nursery, 24 were *mat-2* and 46 were *mat-1*. In most cases, isolates representing a single VCG were of the same mating type (Table 1). The only exceptions were VCO6 and VCO8, both of which occurred only in the nursery population, and VCO7 that were represented by *mat-2* isolates in the WCP nursery and a single *mat-1* isolate in the population from the George area.

In all cases, successful crosses (i.e. development of perithecia) were observed approximately 14 days after the isolates of opposite mating type were placed on carrot agar. In addition to the crosses between *F. circinatum* tester strains, perithecia were observed only for crosses among two isolates from the WCP nursery population. However, these perithecia were not abundant and neither matured nor exuded ascospores from their ostioles. Among the crosses between the Tokai isolates and the known hermaphrodite strains, perithecia were observed only when the latter strains were used as females. Therefore, none of the isolates from Tokai and the WCP nursery were regarded as female-fertile. They were, however, all able to act as males, because fertilization of the hermaphrodite isolates resulted in formation of perithecia from which viable ascospores were extruded.

AFLP analysis

Together with *F. circinatum* isolates representing the known VCGs in South Africa (Viljoen *et al.*, 1997; Britz *et al.*, 2005), the AFLP analysis included an isolate representing each of the VCGs obtained from the WCP. In cases where a VCG was associated with both mating types or occurred in more than one location, representative isolates for both mating types and from all the locations were also included.

The number of distinct fragments generated by the various AFLP primer sets differed. Of the five primer sets used, *Eco*RI-AA + *Mse*I-AG generated the most fragments (11–24) per isolate, whilst primer set *Eco*RI-AA + *Mse*I-AT generated the fewest fragments (7–13). For the selected isolates examined, a total of 152 distinct AFLP fragments were scored (i.e. 22 for primer set *Eco*RI-AA + *Mse*I-AT, 23 for *Eco*RI-TC + *Mse*I-AG, 39 for *Eco*RI-AA + *Mse*I-AG, 34 for *Eco*RI-TC + *Mse*I-AT, and 34 for primer set *Eco*RI-AA + *Mse*I-CC). For the isolates for which the entire AFLP procedure was repeated, identical profiles were generated.

In some cases, isolates representing distinct VCGs generated identical AFLP profiles. For example, the representatives of VCO1 (isolate CMWF609), VCO2 (isolate FCC5564), VCO3 (isolate CMWF585), VCO5 (isolate CMWF544) and VCO28 (isolate CMWF617) all displayed the same AFLP profile. In other cases, isolates representing a single VCG had different AFLP profiles. This was most pronounced for the opposite-mating-type isolates of VCO6 (CMWF621 and CMWF644) that differed at 26 of the 152 scored AFLP fragments and VCO8 (CMWF631 and CMWF594) that differed at 70 of the 152 scored bands. In addition, some isolates representing a single VCG, but that were isolated from different locations, also had different AFLP profiles. For example, the representatives of VCO2 (isolates FCC5564 and CMWF639) and VCO5 (isolates FCC5565, CMWF544 and FCC5563) from different locations differed at four and five of the scored fragments, respectively.

Analysis of the scored AFLP fragments with the distance-based approaches implemented in BIONUMERICS and PHYLIP consistently separated the majority of the VCGs from established *P. radiata* trees in the WCP into one of two groups (Clusters 1 and 2; Fig. 1). Of the 152 scored bands, isolates in Cluster 1 had more than 134 bands in common whilst those in Cluster 2 had more than 131 bands in common. Neither of the clusters included isolates representing VCGs from locations other than the WCP. These two clusters included all representatives from the Tokai population and some of the high-frequency VCGs from the WCP nursery and some VCGs from the George area. However, the majority (74%) of the isolates obtained from the plantation trees in the WCP were represented by VCGs in Clusters 1 and 2. To some extent, this was also true for the 70 isolates from the WCP seedlings, because 67% were represented by VCGs included in Clusters 1 and 2. The remaining isolates from the George area and those representing VCGs obtained from the WCP nursery were related to one another and/or to those from elsewhere in South Africa or the world.

Analysis of the AFLP data with MULTILOCUS suggested that the population in the WCP was significantly differentiated from the one represented by isolates from commercial nurseries elsewhere in South Africa ($\theta = 0{\cdot}122$; $P < 0{\cdot}0001$; Table 3). The null hypothesis of no population differentiation could not be confidently rejected ($\alpha = 0{\cdot}01$) for the remaining pairwise analyses. In other words, no significant population differentiation was detected between the Tokai and the George isolates and the Tokai and WCP nursery isolates, nor between the George and the WCP nursery isolates.

The differentiation between the WCP population and the collection of isolates from elsewhere in South Africa, was also evident from the AMOVA results (Table 3). About 13% of the observed AFLP-based genetic diversity was distributed between the WCP and non-WCP populations of *F. circinatum* isolates. Based on the AMOVA results, the only other isolate partition that showed significant ($P < 0{\cdot}01$) population structure was between the Tokai and George isolates. For this partition, about 86% of the genetic variation was distributed among individuals in

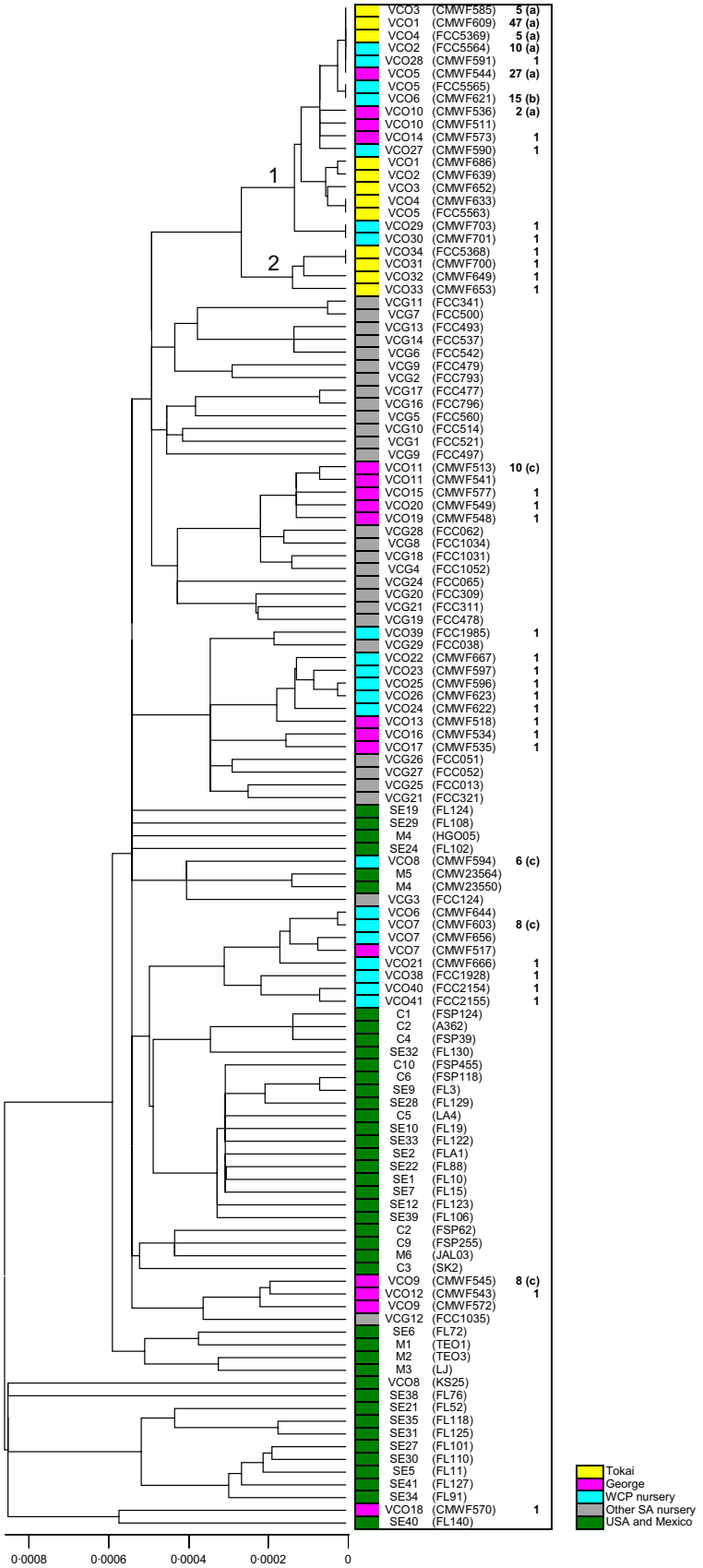

Figure 1 Dendogram depicting the distance-based relationships among the various vegetative compatibility groups (VCGs) of *Fusarium circinatum* that were examined in this study. This dendogram was inferred using PHYLIP and RESTDIST by applying a model that accounts for the evolutionary behaviour of AFLP fragments (Nei & Li, 1979). Clusters 1 and 2 (indicated by the digits at the nodes) were also recovered with the distance analysis employing the simple matching similarity coefficient (Kosman & Leonard, 2005). The Western Cape Province (WCP) isolates from the three collection areas are indicated in yellow (Tokai), pink (George area) and blue (WCP nursery), whilst those from nurseries elsewhere in South Africa (SA; Britz *et al.*, 2005; Viljoen *et al.*, 1997) or from the USA and Mexico (Wikler & Gordon, 2000) are indicated in grey and green, respectively. In each case, the VCG designation is followed in parentheses by the representative isolate used to generate the AFLP data. For the WCP isolates, the numbers of isolates in each VCG are indicated in bold. For the latter, 'a' in parentheses indicates those VCGs that are associated with more than one AFLP phenotype in either Clusters 1 or 2, 'b' indicates those VCGs associated with more than one AFLP phenotype both inside and outside Cluster 1 + 2, or 'c' indicates those VCGs associated with more than one AFLP phenotype that did not form part of Cluster 1 + 2.

Table 3 Parameters for describing population differentiation based on AFLP data for representative isolates of *Fusarium circinatum* from the three collection areas in the Western Cape Province (WCP) (Tokai, George and the WCP nursery) and commercial nurseries elsewhere in South Africa (designated non-WCP)[a]

Partitions tested	Theta (θ)[b]	Analysis of molecular variance (AMOVA)[c]				
		Source of variation	d.f.[d]	Sum of squares[e]	% of total[f]	*P*-value
Tokai and George	0·319 (0·117)	Between partitions	1	25·881 (1·32840)	14·03	<0·001
		Within partitions	26	211·583 (8·13780)	85·97	
Tokai and WCP nursery	0·239 (0·538)	Between partitions	1	18·333 (0·57238)	5·45	0·033
		Within partitions	31	308·091 (9·93842)	94·55	
George and WCP nursery	0·071 (0·012)	Between partitions	1	23·142 (0·71616)	7·07	0·014
		Within partitions	37	348·037 (9·40642)	92·93	
WCP and non-WCP sites	0·122 (<0·0001)	Between partitions	1	59·899 (1·39084)	12·24	<0·001
		Within partitions	76	757·857 (9·97180)	87·76	

[a]For the three WCP isolate collections, representatives for each vegetative compatibility group (VCG) and mating type were used, whilst the non-WCP collection included known representatives from elsewhere in South Africa (Viljoen *et al.*, 1997; Britz *et al.*, 2005).
[b]θ represents Wright's F_{ST} for haploids (Weir, 1996) and was calculated with MULTILOCUS v. 1.3 (Agapow & Burt, 2001) using 500 000 randomizations. *P*-values are indicated in parentheses.
[c]AMOVA was performed with ARLEQUIN v. 3.5 (Excoffier & Lischer, 2010) using 10 000 permutations.
[d]Degrees of freedom.
[e]Variance components are indicated in parentheses.
[f]Percentage of the total molecular variance.

the two populations, whilst 14% was distributed between populations, also suggesting that some connectivity exists among these collection areas.

Discussion

The evidence presented in this study indicates that pitch canker on *P. radiata* in the WCP is associated with a genetically distinct population of *F. circinatum*. All the VCGs identified within the WCP collection of isolates were different from those known to occur in *Pinus* seedling nurseries elsewhere in South Africa (Viljoen *et al.*, 1997; Britz *et al.*, 2005). Furthermore, AFLP-based genetic analysis of these VCGs showed that the differentiation of the WCP populations from those outside this region is highly significant ($P < 0·0001$). The value for the F_{ST}-estimate θ (Weir, 1996) was 0·122, which, according to Wright's (1978) guidelines, is indicative of substantial differentiation between the WCP and non-WCP populations of the fungus. The genetic make-up of *F. circinatum* isolates in the WCP is thus markedly different from those that were isolated from diseased *Pinus* seedlings in other regions in South Africa.

The notion that the WCP population of *F. circinatum* is genetically distinct from other South African populations of the fungus is consistent with a unique origin for pitch canker of *P. radiata* in this region. Previous studies of populations collected outside the WCP and primarily associated with diseased *P. patula* seedlings, showed that the initial introduction of the pathogen into the country was probably in the form of multiple VCG phenotypes (Viljoen *et al.*, 1997; Britz *et al.*, 2005). This was because the initial outbreak was characterized by 23 distinct VCGs that were identified among 69 isolates of the pathogen (Viljoen *et al.*, 1997). Later, after the disease had spread to other nurseries in the country, Britz *et al.* (2005) reported the presence of 29 VCGs among the isolates examined. Of these, six VCGs were shared between the isolates originating from the initial outbreak in 1990 and the collections made during the 1990–1998 period (Britz *et al.*, 2005). However, none of those VCGs were found in the current study, suggesting that the disease in the WCP is as a result of a separate introduction(s) into the area.

A high level of relatedness was observed among the VCGs identified in this study, particularly those that were represented by isolates from diseased *P. radiata* trees in Tokai. The AFLP data revealed that isolates in certain VCGs in the three sampling areas were clonally related (i.e. the isolates from a single VCG were characterized by identical AFLP profiles), whilst AFLP profiles for a number of isolates were identical, despite their representing distinct VCGs. Distance-based analyses of these data further suggested that the large majority of the representatives of the VCGs associated with *P. radiata* were more closely related to one another than to isolates that were obtained elsewhere in the country or the world. This was particularly true for all of the representatives of the pathogen in Tokai and the high-frequency VCGs in the WCP nursery. The isolates representing these VCGs formed part of two clusters in which they shared more than 86% of their scored AFLP fragments with one another. Pitch canker on *P. radiata* in the WCP, especially in the Tokai plantation, thus appears to be the result of a number of genetically distinct, but closely related, *F. circinatum* individuals.

Isolates of *F. circinatum* originating from plantation trees in the WCP have a predominantly asexual mode

of reproduction. For the Tokai and George populations all the isolates examined belonged to a single mating type (*mat-1*). This also has been observed for the population of the pitch canker pathogen in the Basque country of Spain, where all of the examined isolates represented *mat-2* (Iturritxa *et al.*, 2011). However, in terms of *F. circinatum*-associated seedling disease in South Africa and pitch canker in other parts of the world, previous work has shown that the pathogen is usually represented by both mating types (Wikler *et al.*, 2000; Britz *et al.*, 2005; Pérez-Sierra *et al.*, 2007). Furthermore, none of the isolates from either the diseased trees in the Tokai region or those from the WCP nursery were capable of acting as females in sexual crosses. This is in contrast to what has been reported previously for other South African populations of *F. circinatum* (Britz *et al.*, 1998), despite the fact that these previous female fertility assays were conducted at suboptimal conditions (Britz *et al.*, 2005). It thus seems improbable that an isolate associated with diseased plantation trees in the WCP will encounter a fertile isolate of opposite mating type.

The three collections of WCP isolates that were examined represent interconnected groups that form part of the same relatively unstructured population of *F. circinatum*. A number of common VCGs were identified among the three collections of isolates. High levels of gene flow were also evident from the AFLP-based analysis of population differentiation and molecular variance. These findings indicate that isolates are being spread in the WCP, either through human activity in the form of the movement of infected material or as a result of insect vectors such as *P. nemorensis* (Coutinho *et al.*, 2007). The occurrence of VCGs that are shared among isolates originating from plantation trees and a commercial seedling nursery further supports the hypothesis that the source of the inoculum that gave rise to the first outbreaks of pitch canker in WCP *P. radiata* plantations, originated from commercial pine seedling nurseries in the region (Coutinho *et al.*, 2007).

The low genetic diversity within the Tokai collection of *F. circinatum* suggests that pitch canker in this plantation originated from a small founder population (McDonald & Linde, 2002). As a result, the Tokai population probably has a small effective population, which is a contrast to what is expected for the *F. circinatum* populations in the WCP nursery and the George area. Future studies should thus address the possibility that the dominant genotypes in the Tokai plantation (and potentially some of those in the WCP nursery and George area) represent isolates with specific fitness properties enabling their successful survival. This is especially true in light of the fact that one of the dominant VCGs occurring in the George and Tokai plantations was also isolated from diseased nursery seedlings during both the recent sampling as well as from the sampling in 1996.

The predominance of asexual reproduction in the WCP *Pinus* plantation setting might account for the stable preservation of favourable or well-adapted genotypes, as well as the apparent relatedness among isolates with distinct VCGs and AFLP profiles. AFLP phenotype and vegetative compatibility are both multilocus traits (Vos *et al.*, 1995; Leslie & Summerell, 2006). In the absence of sexual reproduction, the members of a VCG or those that share the same AFLP profile are probably clonally related (Leslie & Summerell, 2006), whereas sexual reproduction would generate new combinations of alleles, thus giving rise to new VCGs or AFLP phenotypes. However, in the absence of sexual recombination, new AFLP profiles or VCGs can also be generated through mutation (Loubradou & Turcq, 2000). For example, Wikler & Gordon (2000) suggested that some closely related sets of *F. circinatum* isolates from the California and Florida populations potentially lost their vegetative compatibility identities through mutation, which resulted in isolates that originally belonged to the same VCG now representing distinct VCGs (Petersen & Gordon, 2005). It is, therefore, possible that mutation contributed to the generation of some of the VCG and AFLP diversity observed in this study. However, a more complete understanding of the genetic and demographic processes underlying the population biology of the pitch canker pathogen in the WCP will require more extensive analyses using suitable co-dominant markers (e.g. Santana *et al.*, 2009).

The results of this study have two important implications for the management of pitch canker in the WCP. First, the general lack of structure within the WCP population of *F. circinatum* suggests that there are no barriers to the movement of the pathogen in this region. Secondly, the structure and mode of reproduction of the WCP population of the pathogen potentially provide opportunities for the development and deployment of planting stock that is tolerant to infection by *F. circinatum*. Despite being regarded as one of the most susceptible species to pitch canker (Hodge & Dvorak, 2000), *P. radiata* has been shown to harbour sufficient genetic variation to allow improvement in its tolerance to pitch canker (Matheson *et al.*, 2006).

Acknowledgements

The authors thank the South African foresters who contributed to this study by collecting and supplying diseased *Pinus* samples for isolation of the pitch canker fungus. This work is based on the research supported in part by a number of grants from the National Research Foundation of South Africa (includes Grant specific unique reference number (UID) 83924), members of Tree Pathology Co-operative Programme (TPCP) and the National Department of Agriculture, Forestry and Fisheries. The Grant holders acknowledge that opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF supported research are that of the author(s), and that the NRF accepts no liability whatsoever in this regard.

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