Mycol. Res. **108** (12): 1399–1406 (December 2004). © The British Mycological Society
DOI: 10.1017/S0953756204001443 *Printed in the United Kingdom.*

Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*

Treena I. BURGESS[1,3]*, Thomas R. GORDON[4], Michael J. WINGFIELD[1] and Brenda D. WINGFIELD[2]

[1] *Forestry and Agriculture Biotechnology Institute and* [2] *Department of Genetics, University of Pretoria, Pretoria 0002, Republic of South Africa.*
[3] *School of Biological Sciences and Biotechnology, Murdoch University, Perth, WA 6150, Australia.*
[4] *Department of Plant Pathology, University of California, Davis, 95616, USA.*
E-mail: *tburgess@central.murdoch.edu.au*

Received 17 May 2004; accepted 30 August 2004.

Diplodia pinea (syn. *Sphaeropsis sapinea*) is a well-known latent pathogen of *Pinus* spp. with a worldwide distribution. As such, this fungus is native where pines are endemic in the northern hemisphere and it has been introduced into all countries of the Southern Hemisphere where pines are exotic. The newly described *D. scrobiculata* (formerly known as the B morphotype of *D. pinea*) is thought to have a much more limited distribution. *D. scrobiculata* was first reported as an endophyte and weak pathogen of *P. banksiana*, where it was found to coexist with *D. pinea*. *Diplodia scrobiculata* is now known to have a much broader distribution in Northern America and Europe. In this study, seven Simple Sequence Repeat (SSR) markers were used to evaluate genetic diversity and gene flow between populations of *D. scrobiculata*. Results indicate a strong geographic isolation between populations of *D. scrobiculata* from different regions in North America, with unique alleles fixed in the different populations. The data fits the isolation by distance model indicating limited dispersal. Geographic isolation in combination with isolation by distance suggests prolonged reproductive isolation. Intensive collections of endophytes from native *P. radiata* in California have yielded only *D. scrobiculata* and not the significantly more pathogenic *D. pinea*. SSR analysis of three populations of *D. scrobiculata* from native *P. radiata* identified many shared alleles among the populations and moderate to high gene flow between them. The three Californian populations are distant and distinct from populations of *D. scrobiculata* from elsewhere. Under stress conditions, *P. radiata* is known to be very susceptible to *D. pinea* in plantations in the Southern Hemisphere. Native *P. radiata* is currently experiencing severe stress due to pitch canker caused by *Fusarium circinatum*. Such stress would provide ideal conditions for an associated outbreak of *D. pinea*. Thus, it is critical to prevent the movement of *D. pinea* into the last remaining native stands of *P. radiata*.

INTRODUCTION

Monterey pine (*Pinus radiata*) is native to California and two islands in the Pacific Ocean off the west coast of Baja California (Fig. 1), where less than 7000 ha now exist in five discrete populations (Lavery 1986, Moran, Bell & Eldridge 1988, Libby 1997). In its native range, *P. radiata* is not an impressive timber species. However, *P. radiata* has been extremely successful as an exotic in the southern hemisphere with 3.4 M ha of commercial plantations in South Africa, Australia, New Zealand, and Chile (Balocchi, Ahumada & Ramirez 1998).

Conservation of the genetic diversity of *P. radiata* is important because of its limited natural distribution and the economic importance of this species in exotic plantation forestry. *P. radiata* has high interpopulation genetic diversity (Moran *et al.* 1988) and it is, therefore, important to conserve genetic material from all populations. *Fusarium circinatum* (the pitch canker pathogen) has recently emerged as the causal agent of a devastating disease of *P. radiata* in its natural environment in California (Devey, Matheson & Gordon 1999). The loss of susceptible trees may ultimately limit the genetic diversity of native *P. radiata*, especially if only the small percentage of resistant trees survive to populate these forests in the future.

Diplodia pinea (syn. *Sphaeropsis sapinea*) is a well-known pathogen of *Pinus* spp. in many parts of the world (Punithalingam & Waterson 1970, Swart, Wingfield & Knox-Davies 1985, Zwolinski, Swart & Wingfield 1990, Burgess & Wingfield 2002). In Southern Hemisphere plantations, *P. radiata* is known to be

* Corresponding author.

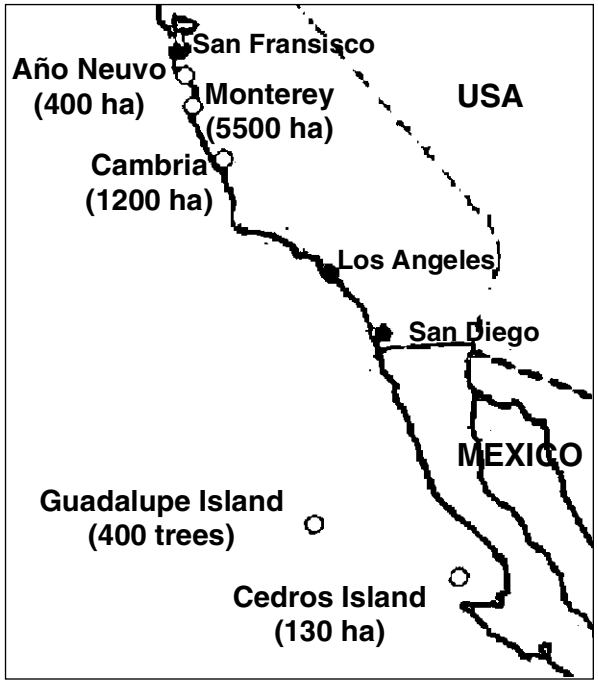

Fig. 1. Natural distribution of *Pinus radiata* (after Moran *et al*. 1988).

particularly susceptible to *D. pinea* when planted off-site in regions with summer rainfall, hail, drought or waterlogging (Marks & Minko 1969, Thomson 1969, Gibson 1979, Lundquist 1987, Zwolinski *et al*. 1990, Davison, Tay & Peroni 1991, Burgess & Wingfield 2001).

Diplodia pinea occurs commonly on native *Pinus* spp. and the relatively recent discovery of its endophytic nature suggests it is present in pines wherever they grow (Petrini & Fisher 1988, Stanosz *et al*. 1995, Smith *et al*. 1996, Burgess, Wingfield & Wingfield 2001a, Flowers *et al*. 2001), although it is not known if it is native to all regions or if it has been introduced by the activities of man. *D. pinea* was thought to be a single species consisting of at least three morphotypes (Palmer, Stewart & Wingfield 1987, Hausner *et al*. 1999, Stanosz, Swart & Smith 1999, de Wet *et al*. 2000, Burgess, Wingfield & Wingfield 2001b, Zhou, Smith & Stanosz 2001). However, a recent study using multiple gene genealogies, has led to the description of a new species, *D. scrobiculata*, to accommodate the 'B' morphotype (de Wet *et al*. 2003). This separation of cryptic species fits well with what is known of the biology of the two species. *D. pinea* is a virulent pathogen found throughout the world (Burgess & Wingfield 2002, Burgess, Wingfield & Wingfield 2004), whereas *D. scrobiculata* has a more limited range, principally the North-Central USA, and has low levels of pathogenicity (Palmer *et al*. 1987, Blodgett & Stanosz 1997). Where their ranges overlap, the two morphotypes have been found to coexist on the same pine species and trees (Palmer *et al*. 1987).

Burgess *et al*. (2001b) developed and tested 11 polymorphic SSR markers on 40 isolates of *D. pinea*, representing what were then known as four different morphotypes. At each of the loci, different alleles were amplified from what are now known as the two discrete species, *D. pinea* and *D. scrobiculata*. Further studies were conducted to determine the origin of *D. pinea* in southern hemisphere plantations of *P. radiata* (Burgess *et al*. 2004). However, the allelic diversity of this pathogen was found to be extremely limited and genotypes were shared across continents and between the northern and southern hemispheres. This result raises important questions about the biological role of endophytes as latent pathogens and their associations with their hosts in native forests.

Due to the extensive association of *D. pinea* with *P. radiata*, both as an endophyte and a pathogen, in exotic Southern Hemisphere plantations, it was assumed that this pathogen would also be associated with *P. radiata* in its native range in California. However, initial investigations resulted in isolation of only *D. scrobiculata* from *P. radiata* in California (Burgess *et al*. 2001a), suggesting that *D. pinea* may not be a resident in native populations. This is of concern, because *P. radiata* in its native range is already threatened by loss of habitat to development, diminished regeneration due to fire suppression and accelerated mortality due to pitch canker. If *D. pinea* represents another potential exotic pathogen of *P. radiata*, its introduction could add significantly to the challenges already facing an economically important species with a limited natural distribution. To clarify the association between *D. pinea* and *P. radiate* a more intensive survey was undertaken in California. Molecular markers were used firstly to identify *Diplodia* sp. isolated from *P. radiata* in California and secondly to compare these populations with those from elsewhere.

MATERIALS AND METHODS

Fungal isolates

Isolates from three populations of *Diplodia scrobiculata* were collected from within the native range of this fungus in North America. From northern California, 22 isolates were collected in 1998 (CAL3) and 12 isolates were collected in 2000 (CAL2) from *Pinus radiata* at Pebble Beach, Monterey, and 18 isolates were from the Cambria population of *P. radiata* (CAL1). In addition there were 20 isolates from *P. patula* in Southern Mexico (MEX) and 20 isolates were collected from various *Pinus* spp. around the Great Lakes region of eastern USA and Canada (NCA). An additional six isolates of a *Diplodia* sp. were obtained from diseased *P. ponderosa* at Igo, California.

SSR-PCR

DNA was extracted following the protocol of Raeder & Broda (1985) as described previously (Burgess *et al*. 2001b). SSR-PCR was performed on all isolates with seven of the 12 fluorescent-labelled markers, SS1, SS2,

SS3, SS4, SS7, SS8, SS9 described previously (Burgess *et al*. 2001b), except that 0.25 U Expand High^{TM} Fidelity *Taq* polymerase (Roche Biochemicals, Alameda, CA) was used following a PCR protocol also described previously (de Wet *et al*. 2003). SS6 and SS10 were omitted because they are monomorphic among isolates of *D. scrobiculata* (Burgess *et al*. 2001b). SS5, SS11 and SS12 were excluded as they are polymorphic in single repeat regions, giving rise to 1 bp polymorphisms that are difficult to distinguish (Burgess *et al*. 2001b).

Fluorescent-labeled SSR PCR products were separated on an ABI Prism 377 DNA sequencer as previously described (Burgess *et al*. 2001b). Allele size was estimated by comparing the mobility of the SSR products to that of the TAMRA internal size standard as determined by GeneScan 2.1 analysis software (Perkin Elmer, Applied Biosystems, Forster City, CA) in conjunction with Genotyper 2 (Perkin Elmer).

The identity of the isolates collected from *P. ponderosa* was determined by amplifying locus SS9 and visualizing amplicons on a 1% agarose gel along with PCR products from reference strains of *D. pinea* and *D. scrobiculata*. *D. pinea* yields a region 30 bp larger than *D. scrobiculata* and this can clearly be seen on the gel.

Gene and genotypic diversity

For each isolate, a data matrix of multistate characters was compiled by assigning each allele at each of the seven loci, a different letter (e.g. EDBACLA). The frequency of each allele at each locus was calculated, and gene diversity determined, using the program POPGENE (Yeh, Yang & Boyle 1999) and the equation $H = 1 - \sum x_k^2$, where x_k is the frequency of the kth allele (Nei 1973). Chi-square tests for differences in allele frequencies were calculated for each locus across clone corrected populations (Workman & Niswander 1970). The Bonferroni correction was applied to significance levels of all χ^2 tests (Weir 1997).

Each genotype was assigned a number and genotypic diversity (G) was estimated (Stoddart & Taylor 1988) using the equation $G = 1/\sum p_i^2$ where p_i is the observed frequency of the ith phenotype. To compare G between populations, the maximum percentage of genotypic diversity was obtained using the equation $\hat{G} = G/N * 100$ where N is the population size (Chen, Boeger & McDonald 1994).

Population differentiation

Population differentiation, theta (θ), was calculated for clone corrected populations in Multilocus (Agapow & Burt 2000) using an estimate of Wright's F_{ST}, as $\theta = Q - q/1 - q$ where Q is the probability that two alleles from the same population are the same and q is the probability that two alleles from different populations are the same (Burt *et al*. 1997, Weir 1997). Two populations with the same allele frequencies, $\theta = 0$, are considered to be identical, whereas populations sharing no alleles ($\theta = 1$) are considered completely isolated. The statistical significance of θ was determined by comparing the observed value to that of 1000 randomized datasets in which individuals were randomized among populations. A significant P value ($P < 0.05$) means that the null hypothesis of no population differentiation can be rejected. Gene flow (M) or the number of migrants exchanged between populations each generation to give the observed θ value, where $M = (1/\theta)/2$ (Cockerham & Weir 1993).

Isolation by distance

To test for isolation by distance (Slankin 1993) estimates of genetic distance between clone corrected poulations (Nei 1978) in POPGENE (Yeh *et al*. 1999) were correlated with geographical distance (measured in approximate km) and log geographic distance using matrix correlation methods based on the Mantel test (Manly 1997) in the IBU 1.2 program (Bohonak 2002). The analysis was conducted using all five populations. The strength of the isolation by distance relationship was determined with reduced major axis (RMA) regression using 10 000 replications (Bohonak 2002).

Mode of reproduction

The index of association (I_A) was used to measure multilocus linkage disequilibrium for each population and for all populations combined (Maynard Smith *et al*. 1993). I_A was calculated for the clone corrected population containing only one representative of each genotype. The tests were performed on a data matrix of 7 multistate characters using the program Multilocus (Agapow & Burt 2000). The expected data for a 1000 randomly recombining data sets was calculated and compared with the observed data. Where the observed data fall within the distribution range of the recombined data, then the hypothesis that the population was undergoing recombination cannot be rejected. If the observed data fall outside the distribution range with a significant P value ($P < 0.05$), the population is most likely clonal.

RESULTS

Segregation of SSR alleles

The SSR markers produced 41 alleles across the 7 loci examined (Table 1). Of these, only 3 were shared by all populations. There were 15 alleles in the CAL1 population, 13 alleles in the CAL2 population, 13 alleles in the CAL3 population, 17 alleles in the NCA population and 21 alleles in the MEX population (Table 1). Some alleles were unique to specific populations of *Diplodia scrobiculata* (Table 1). There were 13 unique amongst the Californian populations, 8 unique alleles in the MEX population and 7 unique alleles in the NCA population (Table 1).

Table 1. Allele size (bp) and frequency at 7 loci (SS1-4, 7-9) for *Diplodia scrobiculata* populations collected from California (CAL1, CAL2 and CAL3), Mexico (MEX) and north-central America (NCA).

Locus	Allele	CAL1	CAL2	CAL3	MEX	NCA
SS1	326	–	–	0.136	0.050	0.050
	361	–	–	–	0.250	0.950
	342	–	–	0.046	0.050	–
	444	0.055	–	–	–	–
	468	0.945	1.000	0.818	0.250	–
	508	–	–	–	0.400	–
SS2	200	0.222	0.083	–	–	–
	204	–	–	–	0.250	–
	206	0.778	0.916	1.000	0.750	1.000
SS3	182	–	–	–	0.150	0.450
	184	1.000	1.000	1.000	0.800	0.550
	186	–	–	–	0.050	–
SS4	406	1.000	0.916	0.773	0.950	0.150
	409	–	0.076	0.227	0.050	0.850
SS7	383	0.667	–	–	–	–
	387	0.056	–	–	–	–
	394	0.277	0.667	0.909	–	–
	396	–	0.333	0.091	0.050	–
	401	–	–	–	–	0.650
	404	–	–	–	–	0.300
	409	–	–	–	–	0.050
	411	–	–	–	0.500	–
	415	–	–	–	0.250	–
	419	–	–	–	0.150	–
	423	–	–	–	0.050	–
SS8	283	0.111	–	–	–	–
	288	0.111	–	–	0.850	–
	293	–	–	–	0.100	0.150
	295	–	–	–	–	0.500
	298	–	–	–	–	0.100
	301	–	–	–	–	0.050
	305	–	–	–	0.050	0.100
	317	0.556	–	–	–	–
	322	0.056	–	–	–	–
	330	–	0.250	–	–	–
	333	0.167	0.583	0.864	–	–
	337	–	0.083	0.091	–	–
	349	–	0.083	0.046	–	–
SS9	236	1.000	1.000	1.000	–	0.050
	237	–	–	–	–	0.950
	238	–	–	–	1.000	–
No. Isolates		18	12	22	20	20
No. Alleles		15	13	13	21	17
No. Unique Alleles		5	1	0	8	7
Polymorphic loci		4	4	4	6	6
H		0.223	0.191	0.153	0.349	0.303

Table 2. *Diplodia scrobiculata* genotypes as estimated from multilocus profiles generated from the 7 SSR loci. Genotypes were distributed among populations collected from California (CAL1, CAL2 and CAL3), Mexico (MEX) and north-central America (NCA).

Genotype	CAL1	CAL2	CAL3	MEX	NCA
CMS1	1	4	13		
CMS2–5			1		
CMS6		1	1		
CMS7			1		
CMS8		1	1		
CMS9		1			
CMS10		2			
CMS11–13		1			
CMS14	2				
CMS15–16	1				
CMS17	5				
CMS18	3				
CMS19–22	1				
CMS23			2		
CMS24			1		
MMS25–40				1	
MMS41				2	
MMS42–43				1	
NMS44–55					1
NMS56–57					4
N	18	12	22	20	20
N(g)	10	8	10	19	14
G	7.2	5.6	2.7	18.2	9.1
$\hat{G}$ (%)	40	46	12	90	46

N, number of isolates.
N(g), number of genotypes.
G, Genotypic diversity (Stoddart & Taylor 1988).
$\hat{G}$, % maximum diversity.

The presence of unique alleles influences the gene diversity of the populations. The mean total gene diversity across the all populations of *D. scrobiculata* was 0.518. This was much higher than the values observed for the individual populations; 0.223 for the CAL1 population, 0.191 for CAL2, 0.153 for CAL3, 0.349 for the NCA population and 0.303 for the MEX population (Table 1).

In general, the genotypic diversity ($\hat{G}$) of the *D. scrobiculata* populations was moderate (40% for CAL1, 46% for CAL2 and 46% for NCA) or high (90% for MEX) (Table 2). Diversity in the CAL3 population was low (12%) due to the predominance of a single genotype (13 of the 22 isolates). Genotypes were shared among the three populations from California (Table 2). However, no genotypes were shared among populations of *D. scrobiculata* from different regions (Table 2).

The six isolates from *P. ponderosa* in California amplified a band equivalent to that of known reference isolates of *D. pinea* and obviously larger than those observed for *D. scrobiculata*. Thus, these isolates were found to be *D. pinea* (data not shown) and they were not studied further.

Population differentiation and gene flow

Amongst clone corrected Californian populations of *Diplodia scrobiculata*, the values obtained for χ^2 tests indicate significant differences ($P<0.05$) in gene diversity at only two of the loci, SS7 and SS8 (Table 3). On closer examination, gene diversity does not differ significantly at any loci between the populations collected in different years at Pebble Beach (CAL2 and CAL3)

Table 3. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 7 polymorphic SSR loci across clone corrected populations of *Diplodia scrobiculata*, from (A) all three California populations, (B) populations CAL2 and CAL3.

	Gene diversity (H)			A		B	
Locus	CAL1	CAL2	CAL3	χ^2	df	χ^2	df
SS1	0.18	0.00	0.49	8.3	6	3.2	2
SS2	0.42	0.22	0.00	3.4	4	1.2	2
SS3	0.00	0.00	0.00	–	–	–	–
SS4	0.00	0.22	0.49	9.1	2	3.4	1
SS7	0.54	0.47	0.35	16.9*[a]	9	0.3	1
SS8	0.78	0.69	0.49	25.6*	14	4.8	3
SS9	0.00	0.00	0.00	–	–	–	–
N	10	8	10				
Mean	0.27	0.23	0.25				

[a] Stars indicate significant ($P<0.05$) χ^2 values.

Table 4. Gene diversity (*H*) and contingency χ^2 tests for differences in allele frequencies for the 7 polymorphic SSR loci across clone corrected populations of *Diplodia scrobiculata* from different regions.

	Gene diversity (*H*)					
Locus	CAL1	CAL2/3	MEX	NCA	χ^2	df
SS1	0.18	0.36	0.70	0.13	68.5*[a]	15
SS2	0.42	0.13	0.39	0.00	23.2*	6
SS3	0.00	0.00	0.34	0.49	14.3*	6
SS4	0.00	0.49	0.10	0.34	25.8*	3
SS7	0.54	0.46	0.68	0.56	143.1*	33
SS8	0.78	0.60	0.28	0.80	121.0*	39
SS9	0.00	0.00	0.00	0.13	107.8*	6
N	10	14	19	14		
Mean	0.27	0.25	0.26	0.28		

[a] Stars indicate significant ($P<0.05$) χ^2 values.

(Table 3). A comparison of populations from different regions (CAL2 and CAL3 combined), indicate highly significant differences in gene diversity at all seven SSR loci (Table 4).

As indicated by results of χ^2 tests, theta values between the two Pebble beach populations (CAL2 and CAL3) were not significant, indicating little population differentiation. Alternatively, θ values between all other populations are highly significant ($P<0.05$) (Table 5).

Isolation by distance

Significant isolation by distance (Mantel test, $P<0.05$) was observed between the five populations of *D. scrobiculata* when genotypic diversity was compared with log geographic distance. The strength of the relationship was moderate with the RMA regression accounting for 72% of the variance.

Reproductive mode

For the entire data set including all genotypes across all populations, the I_A of the observed data was significantly greater than that for the randomized data set (Fig. 2E) giving the appearance of clonality. Conversely, the I_A of the observed data did not differ significantly from the values obtained for the recombined data set for all the individual *Diplodia scrobiculata* populations (Fig. 2A–D). Thus, the null hypothesis that alleles are freely recombining within each of these reproductively isolated populations, either now or historically, could not be rejected.

Table 5. Pairwise comparisons of population differentiation (θ) (above the diagonal) and gene flow (below the diagonal) among *Diplodia scrobiculata* populations from California (CAL1, 2 and 3), Mexico (MEX) and north-central America (NCA). Values obtained are for clone corrected populations.

	CAL1	CAL2	CAL3	MEX	NCA
CAL1	–	0.128*[a]	0.257***	0.441***	0.555***
CAL2	3.40	–	0.092	0.477***	0.554***
CAL3	1.45	4.93	–	0.500***	0.502***
MEX	0.63	0.55	0.50	–	0.499***
NCA	0.40	0.40	0.50	0.50	–

[a] For θ values, stars indicate significance of χ^2 values; * $P<0.05$, *** $P<0.001$.

DISCUSSION

Populations of *Diplodia scrobiculata* had high allelic diversity, and they separated into groups based upon their geographic origin, within which there seems to be a recent history of recombination. Two populations collected from the same location in Pebble Beach, California, in different years were very similar and χ^2 tests indicate no differences in allelic frequencies at any loci. A population from another location in California, did vary significantly from the Pebble Beach population at two loci, and could not be combined with the other Californian populations. However, this population was more similar to the other Californian population than it was to populations from other regions.

Genotypic diversity was high and no multilocus genotypes were shared between populations from different geographic regions. Unique alleles are fixed in the various regions, gene flow was low and there is considerable genetic distance between populations from the different regions. Rare alleles are important indicators of isolated populations and genetic drift and low gene flow (Leung, Nelson & Leach 1993). The data fits the isolation by distance model indicating that the further apart two populations, the greater the difference between them, as would be expected by limited long-distance dispersal.

For the entire data set, including all genotypes of *D. scrobiculata* across all populations, the I_A of the observed data was significantly greater than that for the randomized data set giving the appearance of clonality. This is a classic example of the importance of first dividing data sets for a species into individual populations before conducting such tests as the I_A (Taylor,

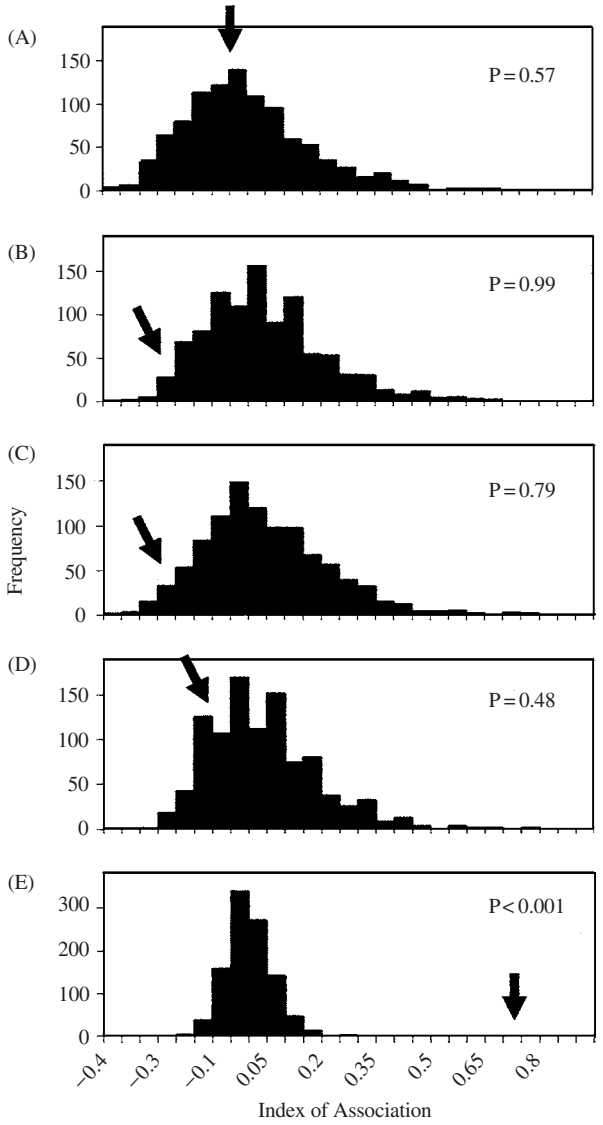

Fig. 2. Histograms of the frequency distribution representing multilocus disequilibrium estimate I_A for 1000 randomized datasets. (A) CAL1, (B) CAL2/3 (C) Mexico, (D) north-central America and (E) all populations combined. Results were compared with the observed dataset (arrows).

Jacobson & Fisher 1999). In the combined data, the predominance of unique alleles and the very different gene frequencies in the different populations biases the test giving the impression that the species is clonal. However, when divided into the separate populations based on geographic origin, each population of *D. scrobiculata* was found to have a history of recombination. There has been limited genetic exchange between populations of *D. scrobiculata*, as reflected by the fixation of alleles in different populations. This suggests a history of recombination and/or mutation, and perhaps the presence of a cryptic sexual stage. No sexual structures have been reported for this fungus, but we are not aware of any concerted attempts to find them. In contrast, considerable efforts have not succeeded in finding the sexual state of *D. pinea* anywhere in the world (M. J. Wingfield, unpublished). This may indicate that *D. pinea* has forsaken sexual reproduction, which would be consistent with the clear evidence of clonality in this species (Burgess *et al.* 2004). Alternatively, the data presented here argue for recent recombination in *D. scrobiculata* and consequently a focused effort in California could very well lead to the discovery of a sexual state in this species.

In its native range, *P. radiata* appears to be exclusively associated with *D. scrobiculata*. However, only *D. pinea* is known to have been introduced to southern hemisphere pine plantations (Swart *et al.* 1991, Stanosz *et al.* 1999, de Wet *et al.* 2000, Burgess & Wingfield 2001, Burgess *et al.* 2001a, Burgess *et al.* 2004). The origin of *D. pinea* populations in exotic plantations was impossible to determine due to the lack of equivalent unique alleles in native populations (Burgess *et al.* 2004). In studies on human fungal pathogens, it has been possible to determine the origin of isolates due to unique alleles, which characterize reproductively isolated populations (Burt *et al.* 1997, Geiser, Pitt & Taylor 1998, Koufopanou *et al.* 2001). This approach would be applicable to *D. scrobiculata*, for which unique alleles exist in geographically isolated populations. However, *D. scrobiculata* appears not to have moved with its host *P. radiata* to southern hemisphere plantations. *D. scrobiculata* has been reported in Europe (Stanosz *et al.* 1999), but it has yet to be determined whether or not this is part of its native range.

In north central USA, *D. pinea* and *D. scrobiculata* coexist on *Pinus banksiana* (Palmer *et al.* 1987, Palmer 1991, Blodgett & Stanosz 1997). *P. radiata* forms associations with *D. pinea* in exotic plantations but apparently only with *D. scrobiculata* in its native range. As both species are present in California, it is puzzling that *P. radiata* in its native range is apparently exclusively associated with *D. scrobiculata*. Perhaps in California, the *P. radiata* niche is occupied by *D. scrobiculata* to the exclusion of *D. pinea*. Alternatively, *D. pinea* might be a more recent introduction to California and as such it has not yet spread to native *P. radiata*. It is also possible that our sampling was insufficient to discover infections of *D. pinea* on native *P. radiata*. However, this seems unlikely, given that elsewhere *D. pinea* is recovered from virtually every tree sampled. Consequently, if *D. pinea* was present on *P. radiata*, we would have expected to encounter it, at least occasionally.

Many endophytes, including *D. pinea*, are thought to be seed borne and are consequently present within the plant from the time it germinates (Redlin & Carris 1996). A recent study of endophytic associations of pines indicated that, although *D. pinea* is an endophyte, it is seldom present in nursery stock, but rather enters the tree after out-planting (Ganley, Brunsfeld & Newcombe 2003). In a native environment, seeds generally germinate and seedlings grow in close proximity to adult trees and endophytic associations would probably form earlier than observed for nursery stock. This could explain how *D. scrobiculata*, a known endophyte of adult *P. radiata*, by its proximity to native germinating

seedling, preferentially infects and inhabits the endophytic niche of these seedlings, thus excluding *D. pinea*.

Although we have confirmed the presence of *D. pinea* in California, it may a recent introduction. Prior to the intervention of humans, natural barriers such as the Rocky Mountains and the arid south-western US may have resticted *D. pinea* to eastern US, as appears to have been the case for *F. circinatum* (Gordon, Storer & Wood 2001). Likewise, *D. pinea* may not be a native to Mexico, which would be consistent with the fact that the native Mexican pine, *P. patula* is very susceptible to die-back caused by *D. pinea* (Gibson 1979, Zwolinski *et al.* 1990). Similarly, *P. radiata* which appears not to be associated with *D. pinea* in its native range, is highly susceptible to this pathogen in the exotic environment (Laughton 1937, Marks & Minko 1969, Thomson 1969, Lundquist 1987).

Why *D. pinea* was widely introduced to exotic plantations, whilst another endophyte, *D. scrobiculata*, was not, is an intriguing question. If *D. pinea* is a seed endophyte and *D. scrobiculata* is not, then *D. pinea* could have been introduced to the southern hemisphere on seed of commonly planted species such as *P. patula*. However, the only evidence of *D. pinea* being isolated from seed is when the cones have been lying on the ground (Fraedrich, Miller & Zarnoch 1994). Alternatively, it has been well documented that the first pines introduced by European colonialists were species from Europe, such as *P. pinea* and *P. sylvestris*, which, for example, were well established in South Africa by 1714 (Legat 1930). These initial pine introductions were not as seed but as seedlings and may have been infected with *D. pinea* when they were introduced. Importantly, seedlings of these same European pine species were also planted by colonialists to North America, which could have served to introduce *D. pinea* from what may have been its aboriginal home in Europe. In principle, this hypothesis could be tested using molecular markers, but those currently available reveal insufficient diversity in populations of *D. pinea* to be useful in this application (Burgess *et al.* 2004).

Although *D. pinea* appears not to be associated with native *P. radiata*, it is found elsewhere in California on species such as *P. ponderosa*. Thus, the introduction of *D. pinea* to *P. radiata* forests from elsewhere in California should be considered a threat to this species, which is already severely impacted by the pitch canker pathogen, *Fusarium circinatum* (Devey *et al.* 1999). Currently, *D. pinea* is regarded as a resident pathogen in California and as such it is not subject to quarantine restrictions. Thus, there are no regulatory barriers to movement of infected hosts or other inoculum sources to areas that may currently be free of the pathogen.

In summary, *D. scrobiculata* was found associated with pines across North America, alone or in combination with the closely related species *D. pinea*. *Diplodia pinea* is more pathogenic than *D. scrobiculata* and may originally have been confined by geographical barriers, such as the Rocky Mountains, to Eastern North America or even Europe. *Diplodia pinea* is widespread with high gene flow between populations, probably assisted by human movement of exotic pines around the world, whereas populations of *D. scrobiculata* are reproductively isolated with little gene flow between them, indicating barriers to the movement of the pathogen. In its native environment, *P. radiata* appears to be exclusively associated with *D. scrobiculata*, most likely because of the proximity of germinating seedlings to adult trees. Thus, *D. scrobiculata* may occupy this niche to the exclusion of *D. pinea* in native *P. radiata* forests. In exotic locations, where *D. scrobiculata* is absent, *P. radiata* is almost uniformly infected by *D. pinea* and trees subject to stress are often damaged or killed by this pathogen. It is tempting to speculate that the presence of *D. scrobiculata* in exotic plantations would moderate the impact of *D. pinea*, but it is not clear this has occurred in the US where both species are found.

ACKNOWLEDGEMENTS

The project was funded by the University of Pretoria, the National Research Foundation (NRF), the THRIP support programme of the Department of Trade and Industry (South Africa) and members of the Tree Pathology Co-operative Programme (TPCP), South Africa. We thank Brenna Aegerter for collection of *Diplodia pinea* from California. We are most grateful to Robert A. Blanchette who enabled us to make collections of *D. scrobiculata* in Minnesota. Joyce Jakavula is thanked for technical support.

REFERENCES

Agapow, P.-M. & Burt, A. (2000) *Multilocus 1.2*. Department of Biology, Imperial College, Silwood Park, Ascot.

Balocchi, C., Ahumada, R. & Ramirez, O. (1998) Present and future of radiata pine in Chile. *Proceedings of the IMPACT Monterey Workshop, Monterey, CA, USA, 30 Nov. to 3 Dec. 1998*: 1–4. CSIRO, Canberra.

Blodgett, J. T. & Stanosz, G. R. (1997) *Sphaeropsis sapinea* morphotypes differ in aggressiveness but both infect nonwounded red or jack pines. *Plant Disease* **81**: 143–147.

Bohonak, A. J. (2002) IBD (Isolation by Distance): a program for analysis of isolation by distance. *Journal of Heredity* **93**: 153–154.

Burgess, T. & Wingfield, M. J. (2001) Exotic pine forestry in the Southern Hemisphere: a brief history of establishment and quarantine practices. *South African Forestry Journal* **192**: 79–84.

Burgess, T. & Wingfield, M. J. (2002) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the southern hemisphere. *International Forestry Review* **4**: 56–65.

Burgess, T., Wingfield, B. D. & Wingfield, M. J. (2001a) Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*. *Mycological Research* **105**: 1331–1339.

Burgess, T., Wingfield, M. J. & Wingfield, B. D. (2001b) Simple sequence repeat (SSR) markers distinguish between morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology* **67**: 354–362.

Burgess, T. I., Wingfield, B. D. & Wingfield, M. J. (2004) Global distribution of *Diplodia pinea* genotypes as revealed using SSR markers. *Australasian Plant Pathology* **33**: in press.

Burt, A., Dechairo, B. M., Koenig, G. L., Carter, D. A., White, T. J. & Taylor, J. W. (1997) Molecular markers reveal differentiation among isolates of *Coccidiodes immitis* from California, Arizona and Texas. *Molecular Ecology* **6**: 781–786.

Chen, R. S., Boeger, J. M. & McDonald, B. A. (1994) Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology* **3**: 209–218.

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

Davison, E. M., Tay, F. C. S. & Peroni, D. (1991) *Sphaeropsis sapinea* on pines in Western Australia. *Australasian Plant Pathology* **20**: 31.

de Wet, J., Burgess, T., Slippers, B., Presig, O., Wingfield, B. D. & Wingfield, M. J. (2003) Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and identify a new species of *Diplodia*. *Mycological Research* **107**: 557–566.

de Wet, J., Wingfield, M. J., Coutinho, T. A. & Wingfield, W. D. (2000) Characterization of *Sphaeropsis sapinea* isolates from South Africa, Mexico and Indonesia. *Plant Disease* **84**: 151–156.

Devey, M., Matheson, C. & Gordon, T. E. (eds) (1999) *Current and Potential Impacts of Pitch canker in Radiata Pine*. Proc. IMPACT Monterey Workshop, Monterey, CA, USA, 30 Nov. to 3 Dec. 1998. CSIRO, Canberra.

Flowers, E., Nuckles, E., Hartman, J. & Vaillancourt, L. (2001) Latent infection of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. *Plant Disease* **85**: 1107–1112.

Fraedrich, S. W., Miller, T. & Zarnoch, S. J. (1994) Factors affecting the incidence of black seed rot in slash pine. *Canadian Journal of Forest Research* **24**: 1717–1725.

Ganley, R. J., Brunsfeld, S. J. & Newcombe, G. (2003) Species diversity of fungal endophytes in *Pinus monticola*. *8th International Congress of Plant Pathology, Christchurch, New Zealand, Sunday 2–Friday 7 February 2003, Offered Papers*: 152.

Geiser, D. M., Pitt, J. I. & Taylor, J. W. (1998) Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proceedings of the National Academy of Sciences, USA* **95**: 388–393.

Gibson, I. A. S. (1979) *Diseases of Forest Trees widely planted as Exotics in the Tropics and Southern Hemisphere*. Part 2. *The genus Pinus*. Commonwealth Mycological Institute, Kew.

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

Hausner, G., Hopkin, A. A., Davis, C. N. & Reid, J. (1999) Variation in culture and rDNA among isolates of *Sphaeropsis sapinea* from Ontario and Manitoba. *Canadian Journal of Plant Pathology* **21**: 256–264.

Koufopanou, V., Burt, A., Szaro, T. & Taylor, J. E. (2001) Gene geneologies, cryptic species and molecular evolution in the human pathogen *Coccidioides immitis* and relatives (Ascomyta, Onygenales). *Molecular Biology and Evolution* **18**: 1246–1258.

Laughton, E. M. (1937) The incidence of fungal diseases of timber trees in South Africa. *South African Journal of Science* **33**: 337–382.

Lavery, P. B. (1986) *Pinus radiata* – an introduction to the species. In *Plantation Forestry with Pinus radiata* (P. B. Lavery, ed.): 5–12. [Review Papers No. 12.] School of Forestry, University of Canterbury, Christchurch.

Legat, C. E. (1930) The cultivation of exotic conifers in South Africa. *Empire Forestry Journal* **9**: 32–63.

Leung, H., Nelson, R. J. & Leach, J. E. (1993) Population structure of plant pathogenic fungi and bacteria. *Advances in Plant Pathology* **10**: 157–205.

Libby, W. J. (1997) Native origins of domesticated Radiata Pine *IUFRO '97 – Genetics of Radiata Pine, 1–4 December, Rotorua, New Zealand, New Zealand Forest Research Institute*: 9–21.

Lundquist, J. E. (1987) A history of five forest diseases in South Africa. *South African Forestry Journal* **140**: 51–59.

Manly, B. F. J. (1997) *Randomization and Monte Carlo Methods in Biology*. Chapman & Hall, New York.

Marks, G. C. & Minko, G. (1969) The pathogenicity of *Diplodia pinea* to *Pinus radiata* D. Don. *Australian Journal of Botany* **17**: 1–12.

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

Moran, G. F., Bell, J. C. & Eldridge, K. G. (1988) The genetic structure and the conservation of the five natural populations of *Pinus radiata*. *Canadian Journal of Forest Research* **18**: 506–514.

Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA* **70**: 3321–3233.

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

Palmer, M. A. (1991) Isolate types of *Sphaeropsis sapinea* associated with main stem cankers and top kill of *Pinus resinosa* in Minnesota and Winsconsin. *Plant Disease* **75**: 507–510.

Palmer, M. A., Stewart, E. L. & Wingfield, M. J. (1987) Variation among isolates of *Sphaeropsis sapinea* in North Central United States. *Phytopathology* **77**: 944–948.

Petrini, O. & Fisher, P. J. (1988) A comparative study of fungal endophytes in xylem and whole stem of *Pinus sylvestris* and *Fagus sylvatica*. *Transactions of the British Mycological Society* **91**: 233–238.

Punithalingam, E. & Waterson, J. M. (1970) *Diplodia pinea*. *CMI Descriptions of Plant Pathogenic Fungi and Bacteria* **273**: 1–2.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17–20.

Redlin, S. C. & Carris, L. M. (1996) *Endophytic Fungi in Grasses and Woody Plants*. American Phytopathological Society Press, St Paul, MN.

Slankin, M. (1993) Isolation by distance in equilibrium and non-equilibrium populations. *Evolution* **47**: 264–279.

Smith, H., Wingfield, M. J., Crous, P. W. & Coutinho, T. A. (1996) *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany* **62**: 86–88.

Stanosz, G. R., Smith, D. R., Guthmiller, M. A. & Stanosz, J. C. (1995) Persistence of *Sphaeropsis sapinea* on or in asymptomatic stems of red pine nursery seedlings. *Phytopathology* **85**: 1196.

Stanosz, G. R., Swart, W. J. & Smith, D. R. (1999) RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. *Mycological Research* **103**: 1193–1202.

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

Swart, W. J., Wingfield, M. J. & Knox-Davies, P. S. (1985) *Sphaeropsis sapinea*, with special reference to its occurrence on *Pinus* spp. in South Africa. *South African Forestry Journal* **35**: 1–8.

Swart, W. J., Wingfield, M. J., Palmer, M. A. & Blanchette, R. A. (1991) Variation among South African isolates of *Sphaeropsis sapinea*. *Phytopathology* **81**: 489–493.

Taylor, J. W., Jacobson, D. J. & Fisher, M. C. (1999) The evolution of asexual fungi: reproduction, speciation and classification. *Annual Review of Phytopathology* **37**: 197–246.

Thomson, A. P. (1969) New Zealand's expanding forest resources. *Commonwealth Forest Review* **48**: 289–301.

Weir, B. S. (1997) *Genetic Data Analysis II*. Sinauer Associates, Sunderland, MA.

Workman, P. L. & Niswander, J. D. (1970) Population studies in South-western Indian tribes. II. Local genetic variation in the Papago. *American Journal of Human Genetics* **22**: 24–49.

Yeh, F. C., Yang, R.-C. & Boyle, T. (1999) *POPGENE*. Version 1.31. *Microsoft windows based freeware for population genetic analysis*. University of Alberta, Alberta.

Zhou, S., Smith, D. R. & Stanosz, G. R. (2001) Differentiation of *Botryosphaeria* species and related anamorphic fungi using Inter Simple or Short Sequence Repeat (ISSR) fingerprinting. *Mycological Research* **105**: 919–926.

Zwolinski, J. B., Swart, W. J. & Wingfield, M. J. (1990) Intensity of die-back induced by *Sphaeropsis sapinea* in relation to site conditions. *European Journal of Forest Pathology* **20**: 167–174.

Corresponding Editor: G. W. Griffith