Mycol. Res. **105** (11): 1331–1339 (November 2001). © The British Mycological Society
DOI: 10.1017/S0953756201005056 *Printed in the United Kingdom.*

Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*

Treena BURGESS[1,2]*†, Brenda D. WINGFIELD[2] and Michael J. WINGFIELD[1]

[1] *Forestry and Agriculture Biotechnology Institute (FABI),* [2] *Department of Genetics, University of Pretoria, Pretoria,* 0002, *South Africa.*
E-mail: tburgess@central.murdoch.edu.au

Received 23 January 2001; accepted 18 July 2001.

Sphaeropsis sapinea is an endophyte and latent pathogen of pines, assumedly introduced to the Southern Hemisphere along with its host. There are at least three recognised forms of *S. sapinea* that differ from each other morphologically and can also be separated based on molecular characteristics. *Pinus radiata* is a native to California but has been used extensively for afforestation in the Southern Hemisphere. For this study, populations of *S. sapinea* were collected from exotic *P. radiata* plantations in South Africa, South and Western Australia, Tasmania and New Zealand and from native *P. radiata* in California. The genotypic diversity of the populations was assessed and compared using vegetative compatibility tests. SSR markers were used to determine the morphotype of isolates from each vegetative compatibility group. All Californian isolates of *S. sapinea* were found to be of the 'B' morphotype, while all introduced isolates in the Southern Hemisphere were of the 'A' morphotype. The genotypic diversities of *S. sapinea* populations ranged from extremely low in Australia to very high in South Africa with New Zealand having an intermediate genotypic diversity. *S. sapinea* is an asexual fungus and, therefore, different genotypes in an exotic population represent separate introductions. Our results suggest there have been very few introductions into Australia and multiple introductions into South Africa. In addition, it appears that *S. sapinea* isolates from *P. radiata* in the Southern Hemisphere did not originate from native *P. radiata*, but rather the widely planted exotic *P. radiata* has acquired this fungal endophyte from other *Pinus* within the exotic environment.

INTRODUCTION

Sphaeropsis sapinea is a ubiquitous endophyte and latent pathogen of *Pinus* (Smith & Stanosz 1995, Smith *et al.* 1996). Thus, the natural distribution of *S. sapinea* mimics that of *Pinus*. The fungus is thought to be seed borne or present in chaff associated with consignments of seed. (Anderson, Belcher & Miller 1984, Fraedrich, Miller & Zarnoch 1994). As such, *S. sapinea* would have reached the Southern Hemisphere along with *Pinus* introduced into this region during the early stages of European colonisation.

Pinus radiata is native to California and two islands in the Gulf of Mexico (Lavery 1986, Libby 1997). Overall there is less than 7000 ha of native *P. radiata* remaining in natural stands. However, as an exotic, *P. radiata* has been extremely successful, especially in the Southern Hemisphere where there are now over 3.2 Mha under afforestation. (Balocchi, Ahumada & Ramirez 1998). In California, where native *P. radiata* grows, *S. sapinea* is not known to be an important pathogen. In contrast, exotic plantations of *P. radiata* are often situated 'off-site' and subject to stress and are commonly damaged by this pathogen. For example, severe losses of susceptible *Pinus* are regularly recorded following hail damage in summer rainfall areas of South Africa (Laughton 1937, Poynton 1977, Swart, Wingfield & Knox-Davies 1985, Wingfield 1999). In addition, *P. radiata* growing on poor or badly drained sites, susceptible to water logging and drought, were also vulnerable to damage by *S. sapinea* in winter rainfall areas of South Africa (Laughton 1937). Similar drought-associated losses have been experienced in New Zealand (Thomson 1969) and Australia (Marks & Minko 1969, Davison, Tay & Peroni 1991).

S. sapinea is generally recognised to undergo only asexual reproduction (Sutton 1980) resulting in clonal lineages within a population (McDonald 1997). Four morphotypes of the fungus have been described. The 'A' and 'B' morphotypes were the first described and were initially separated based on conidial morphology and cultural characteristics (Wang *et al.* 1985, Palmer, Stewart & Wingfield 1987). The existence of two discrete morphotypes was confirmed using randomly amplified polymorphic DNA (RAPD) markers (Smith & Stanosz 1995). De Wet *et al.* (2000) described a 'C' morphotype of *S. sapinea* from a collection of isolates from

* Corresponding author.
† Current address: Division of Science and Engineering, Murdoch University, Perth 6150, Australia.

Northern Sumatra in Indonesia and supported this finding based on spore morphology, RAPDs and ITS sequence data. Likewise, Hausner *et al*. (1999), using restriction fragment length polymorphism (RFLP) fingerprinting of rDNA, reported the presence of an 'I' morphotype among Canadian isolates. Simple sequence repeat (SSR) markers have recently been developed for *S. sapinea* (Burgess, Wingfield & Wingfield 2001). These markers clearly distinguish between isolates that have been assigned to the four morphotypes of *S. sapinea*, however those assigned to the 'I' morphotype were found to be identical to the closely related species, *Botryospheria obtusa*.

Vegetative incompatibility is a phenotypic characteristic that has often been used to study genotypic diversity in fungi (Anagnostakis 1983, Leslie 1993, Cortesi & Milgroom 1998). Isolates capable of merging vegetatively and forming confluent lawns mycelium when paired are referred to as being vegetatively compatible. Vegetative compatibility of isolates is governed by similarity or differences in so called *vic* loci (Leslie 1993). Thus, vegetatively compatible isolates are considered to be identical at a number of *vic* loci. The number of *vic* loci in *S. sapinea* is unknown, but at least 5 *vic* loci exist for *Cryphonectria parasitica* (Anagnostakis 1988) and up to 10 *vic* loci for *Fusarium moniliforme* (Leslie 1993).

Using vegetative compatibility (VC) tests, Smith *et al*. (2000) found the genotypic diversity of an introduced *S. sapinea* population in South Africa was high. This was in contrast to the low diversity of a purported indigenous population from Indonesia. The aim of the current study was to use VC tests to determine the genotypic diversity of populations of *S. sapinea* from stands of exotic *P. radiata* in Western Australia, South Australia, Tasmania, New Zealand and South Africa. The genotypic diversity of these isolates was compared with that of an indigenous population of *S. sapinea* collected from *P. radiata* in Monterey, California. These results were confirmed using SSR markers developed previously for *S. sapinea*.

MATERIALS AND METHODS

Collection of isolates

Sphaeropsis sapinea was isolated, using a hierarchical sampling strategy, from *Pinus radiata* cones collected in Australia, New Zealand and South Africa. The Western Australian collection consisted of 28 isolates from different trees across the range of *P. radiata* plantations in Western Australia (Fig. 1A), 32 isolates from different trees within a 25 km^2 of Lewana plantation near Balingup (Fig. 1B), 29 isolates from different trees within a 100 m^2 sampling plot at Lewana plantation (Fig. 1C); and 24 isolates from different cones from the same tree at Lewana plantation. The South Australian collection consisted of 26 isolates from different trees in a 1918 plantation in the Adelaide Hills. In addition, eight isolates were obtained from individual cones from Seven Mile Beach in south-east Tasmania. The South African collection consisted of 29 isolates from individual trees within a 100 m^2 sampling plot at George in the Eastern Cape and 25 isolates from individual *P. radiata* trees across South Africa. The New Zealand collection consisted of 29 isolates from individual trees within a 100 m^2 sampling plot at the Matakana Island seed orchard in the Bay of Plenty. An indigenous population of 22 *S. sapinea* isolates was obtained from individual *P. radiata* shoots showing dieback symptoms, collected at Pebble Beach near Monterey, California.

Isolation of Sphaeropsis sapinea

Isolates from California and some isolates collected in South African (not including those from George) were from dying shoots or other disease symptoms. All isolates from Australia, New Zealand and George in South Africa were from cones. For cone isolations, bracts were removed from cones using a pair of pliers, surface sterilised and cut into 3–4 segments. These pieces were placed on malt extract agar (MEA; 20 g l^{-1} malt extract, 5 g l^{-1} peptone). The Californian isolates were obtained by placing small segments of surface sterilised diseased twig tissue onto MEA. *Sphaeropsis sapinea* grew out from cones or diseased twigs within 3–4 d, producing fluffy white colonies. A plug (5 mm^2) of mycelium was removed from these colonies and placed on the surface of tap water agar between two sterilised pine needles. Plates were incubated under a mixture of fluorescent and near ultraviolet light at 25 °C until pycnidia developed (usually 2 wk). Individual pycnidia (containing conidia) were then suspended in sterile water in Eppendorf tubes, shaken using a vortex mixer and plated onto tap water agar plates and incubated overnight at 25 °. From these plates, single germinating conidia were transferred onto MEA and incubated at 25 ° for 7 d. Cultures were then stored at 4 °. Positive identification of isolates was based on conidial and cultural characteristics (Sutton 1980, de Wet *et al*. 2000).

The 24 South African isolates of *S. sapinea*, other than those collected at George, originated from the culture collection at Forestry and Agriculture Biotechnology Institute (FABI). Fifteen of these isolates were from cones and the rest were from stem cankers collected during outbreaks across South Africa. All isolates used in this study have been preserved in the culture collection at FABI, University of Pretoria, Pretoria, South Africa.

Vegetative compatibility tests

Genotypic diversity of isolates collected from *Pinus radiata* across the Southern Hemisphere was estimated using VC tests as described previously (Anagnostakis 1983). Tests were performed on oatmeal agar as described previously (Smith *et al*. 2000). Isolates were initially compared in every possible combination at each level of hierarchy, for each location. Six isolates were compared on a single Petri dish. Small plugs of mycelium from 7–10 d-old cultures were placed approximately 1 cm apart in a predetermined order. After 4–6 d in the dark at 25 °, plates were assessed for the formation of barrage lines between incompatible isolates (Fig. 2). After the initial comparisons, representative VC groups from each population were compared in all possible combinations on large square assay plates (20 cm^2), where 11 isolates were compared simultaneously.

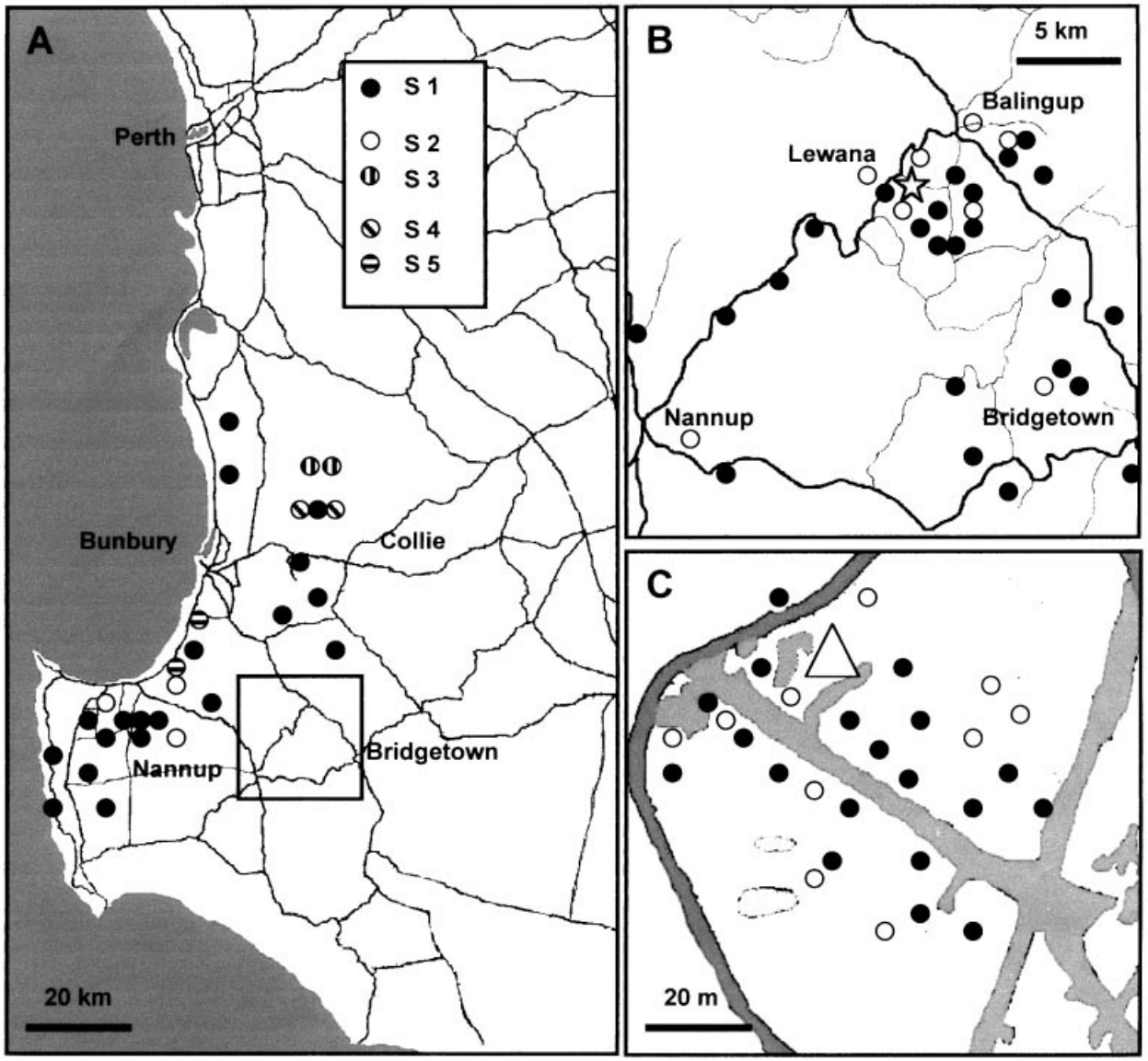

Fig. 1. Distribution and vegetative compatibility groups of *Sphaeropsis sapinea* isolates collected in Western Australia at the different levels of hierarchy: (A) the outliers with the position of the Balingup-Nannup region designated by a square; (B) 25 km² around Lewana plantation which is indicated by a star; and (C) 100 m² at Lewana plantation with the position of tree 1 indicated by a triangle. The shaded areas in (C) are cleared patches and roads.

Assessment of diversity

Each VC group was assigned a number. Isolates belonging to the same VC group were treated as separate phenotypes. This phenotypic characteristic was used to estimate genotypic diversity using 3 separate statistical parameters. First was a simple estimate of V/N where V = number of VC groups and N = population size. Secondly, the genotypic diversity (G) was estimated according to Stoddart & Taylor (1988) using the equation $G = 1/\Sigma p_i^2$, where p_i is the observed frequency of the ith phenotype. To compare G between populations, the maximum percentage of genotypic diversity was obtained according to the equation $\hat{G} = G/N \times 100$ where N is the population size. The significant difference between the maximum diversity for different populations was calculated using a t-test. Thirdly, the Shannon index (SI) was calculated as $SI = -\Sigma p_i \ln p_i$, where p_i is the observed frequency of the ith phenotype. (Bowman *et al.* 1971). To compare between populations the normalised Shannon index was calculated using the equation $H_s = SI/\ln N$, where N is the population size.

Pathogenicity of Western Australian isolates

The pathogenicity of representative isolates from VC groups, S1-5, was tested using an apple technique (Fulbright 1984), described previously as useful to assess the relative aggressiveness of *Sphaeropsis sapinea* isolates by de Wet *et al.* (2000). A cork borer (10 mm) was used to make a wound 5 mm deep on one side of large Granny Smith apples. A similarly sized agar plug of mycelium growing on MEA was placed into this wound and covered with masking tape. Control inoculations were made using plugs of sterile MEA.

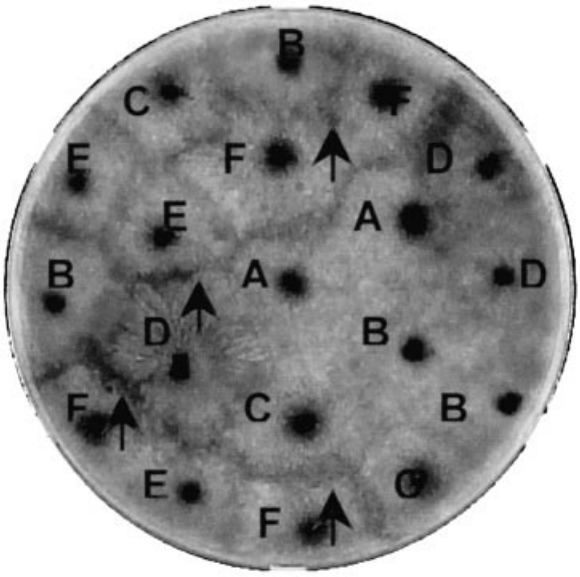

Fig. 2. *Sphaeropsis sapinea* Vegetative Compatibility groups on oatmeal agar. VC group S5 is represented by an isolate from: (A) Western Australia; (B) South Australia; (C) Tasmania; and (D) New Zealand. VC group S6 is represented by an isolate from (E) South Australia and (F) Tasmania. These barrages (↑) are indicative of a moderate incompatible reaction.

Each isolate was replicated on 3 apples and the entire experiment was repeated. After 2 weeks the diameter of the lesion on the inoculated apples was measured, the area calculated and data analysed by ANOVA (SYSTAT, version 7.0.1).

Molecular identification of morphotypes

DNA was extracted from representative isolates from each VC group according to the protocol of Raeder & Broda (1985). SSR–PCR was conducted using markers SS8, SS10 and SS11 (Burgess *et al*. 2001), previously shown to provide a robust technique to distinguish *S. sapinea* morphotypes. The reaction mixture contained 2 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 100 μM of each deoxynucleotide triphosphate, 300 nM of each primer, 2 ng of DNA template, 0.25 U Expand™ High Fidelity *Taq* polymerase (Roche Biochemicals) and water to a final volume of 25 μl. The reactions were carried out in an Eppendorf (Germany) thermocycler programmed for an initial denaturation of 2 min at 95 °, followed by 10 cycles of 30 s at 95 °, 40 s at 62 ° and 45 s at 72 °. The last three temperature intervals were repeated for another 25 cycles with a 5 s increase per cycle for the elongation step at 72 °. This was followed by a final elongation step of 7 min at 72 °.

Phosphoramidite-labelled SSR PCR products (0.5 μl containing approximately 1.5 ng DNA for each amplification product) and 0.4 μl of the internal standard GS-500 TAMRA (Perkin–Elmer) were added to 1.1 μl of loading buffer. The mixture was heated to 95 ° for 3 min. One μl of this mixture was separated by PAGE (4.25%) on an ABI Prism 377 DNA sequencer. Allele size was estimated by comparing their mobility to that of the internal size standard as determined by GeneScan 2.1 analysis software (Perkin–Elmer) in conjunction with Genotyper 2 (Perkin–Elmer).

RESULTS

Vegetative compatibility tests

Isolates that formed barrages representing incompatibility were assigned to separate VC groups, whereas isolates that were compatible grew into each other with no barrage formation (Fig. 2). Among the 252 isolates compared in this study, 67 VC groups were identified (Table 1).

There were 4 levels of hierarchy among the Western Australian isolates of *Sphaeropsis sapinea* (Table 1). Three VC groups (S1-3) were found among isolates collected from individual cones from a single tree in Western Australia. Only two of these VC groups (S1-2) were found among 29 isolates collected from individual trees within 100 m^2 (Fig. 1C). The same two VC groups (S1-2) were found among 32 isolates collected within 25 km^2 of each other (Fig. 1B). Across the range of *Pinus radiata* in Western Australia, a total of 5 VC groups (S1-5) were found among 28 isolates (Fig. 1A). A total of 113 isolates were collected in Western Australia and only 5 VC groups were found (Fig. 1, Table 1). The most common VC group, S1, accounted for 70% of the isolates, the next common group, S2, for 24% of the isolates and the remaining 3 VC groups, S3-5, for approximately 6% of the isolates. Only 2 VC groups (S5-6) were found among 26 isolates collected in South Australia (Table 1). VC group S6 accounted for 88% of the isolates. The same VC groups, S5-6, were also found among the 8 isolates collected in Tasmania (Table 1).

Thirteen VC groups were identified among the 29 isolates from New Zealand. The most common VC group, S7, accounted for 21% of the isolates, but there were also 7 VC groups (S12-18) accounting for less than 25% of the isolates (Table 1).

There were two levels of hierarchy among the South African isolates. At the lower level, there were 19 VC groups (S19-38) among 29 isolates collected within 100 m^2 of each other at George. The most common VC group, S19, accounted for only 14% of the isolates and 11 VC groups, S27-37, accounted for 40% of the isolates. For the South African isolates collected across the range of *P. radiata* growing areas, almost every isolate represented a different genotype with 23 VC groups among 25 isolates (S19, S38-59). In total for South Africa there were 42 VC groups from among a collection of 54 isolates. Only one of these VC groups, S19, was shared between the two levels of hierarchy and this group accounted for 10% of the isolates (Table 1).

There were 8 VC groups among the 22 isolates from California (S60-67) (Table 1). The distribution of isolates across the groups was fairly even although VC groups S60 and S61 accounted for 50% of isolates and S66 and S67 were represented by only one isolate.

VC group S5 was present in Western Australia, South Australia, Tasmania and New Zealand (Fig. 2; Table 1). It was the dominant VC group found among the 8 Tasmanian isolates, but in the other populations it accounted for less than 10% of the isolates (Table 1). VC group S6 was found in South

Table 1. Summarised vegetative compatibility groups and population data for *Sphaeropsis sapinea* isolates collected from introduced *Pinus radiata* in Western Australia (WA), South Australia (SA), Tasmania (TAS), New Zealand (NZ), South Africa (RSA) and native *Pinus radiata* California (USA). The levels of hierarchy are described in the Materials and Methods.

VC group	WA 1 tree	WA 100 m²	WA 25 km²	WA outliers	WA total	SA 100 m²	TAS* 100 m²	NZ 100 m²	RSA 100 m²	RSA outliers	RSA total	USA
S1	17	19	24	19	79							
S2	6	10	8	3	27							
S3	1			2	3							
S4				2	2							
S5				2	2	3	5	4				
S6						23						
S7								6				
S8-10								3				
S11								2				
S12-18								1				
S19									4	2	6	
S20-26									2		2	
S27-37									1		1	
S38										2	2	
S39-59										1	1	
S60												6
S61												5
S62												3
S63-65												2
S66-67												1
N	24	29	32	28	113	26	8	29	29	25	54	22
V	3	2	2	5	5	2	2	13	19	23	41	8
V/*N*	0.13	0.07	0.06	0.18	0.04	0.08		0.41	0.66	0.92	0.76	0.36
SI	0.72	0.64	0.57	1.05	0.82	0.36		2.28	2.85	3.10	3.65	1.89
H_s	0.23	0.19	0.16	0.31	0.17	0.11		0.68	0.85	0.97	0.92	0.62
G	1.77	1.83	1.6	2.04	1.80	1.25		8.17	15.28	21.55	38.56	5.81
$\hat{G}$	7.4	6.3	5.0	7.3	1.6	4.8		28.2	52.7	86.2	71.4	26.4

N = population size.
V = number of vegetative compatibility groups.
SI = Shannon diversity index (Bowman *et al.* 1971).
H_s = normalised Shannon diversity index.
G = Genotypic diversity (Stoddart & Taylor 1988).
$\hat{G}$ = G/*N*% = percent maximum diversity.
* = population data not estimated from the Tasmanian collection as the sample size is too small.

Australia and Tasmania and was the dominant group in South Australia. Only two VC groups, S5 and S6, were found in South Australia and Tasmania (Table 1). Of these groups, S6 was exclusive to these regions while S5 was also found elsewhere. No VC groups found in Australia and New Zealand were present among the South African isolates studied. All the VC groups of *S. sapinea* isolates from *P. radiata* in California were different from those on introduced *P. radiata* in the Southern Hemisphere (Table 1).

Assessment of diversity

The *V*/*N* ratios for different *Sphaeropis sapinea* populations ranged from 4–92%. The lowest ratios (< 10%) were obtained for the introduced populations in Western and South Australia. The highest ratios (66 and 92%) were found in South Africa. Populations of isolates from New Zealand and California had *V*/*N* ratios of 41 and 36%, respectively, and thus were intermediate between those in Australia and South Africa (Table 1).

For all populations, the normalised Shannon diversity index (H_s) was higher than the *V*/*N* ratio, but followed the same pattern. The lowest H_s was found for the Western Australian (0.16–0.31) and South Australian (0.11) populations. The highest H_s accrued to the South African populations (0.85–0.97), while New Zealand (0.68) and California (0.62) populations had intermediate values (Table 1).

The maximum percentage of genotypic diversity ($\hat{G}$) in all populations was lower than either the *V*/*N* ratios or H_s but also followed the same patterns as observed for these parameters. It was extremely low (< 8%) for the all Western Australian and South Australian populations and intermediate for the New Zealand (28%) and Californian (26%) populations. In contrast, genotypic diversity was very high for the South African populations (53–86%) (Table 1).

Estimates of population diversity differed depending on the statistical parameter used for assessment. Of the various statistics, $\hat{G}$ gave the lowest diversity, *V*/*N* intermediate diversity, and H_s the highest diversity for each population. All the data, however, showed the diversity was highest in South Africa, intermediate in New Zealand and California and low in Australia. *V*/*N* is the least reliable estimate of diversity, as it does not take into account the frequency of any given genotype. H_s and $\hat{G}$ statistics both use the frequency of a phenotype. H_s is specific for phenotypes and probably gives the most reliable data (Cortesi, Milgroom & Bisiachi 1996).

Table 2. Identification of morphotypes using SSR markers.

Genotype				
SS7	SS9	SS10	VC Groups	Morphotype
384	252	313	S8, S11-12, S39, S48-49, S59	A
384	253	313	S2, S15-17, S21, S26-28, S30, S32, S35-37, S45, S52, S54, S57	A
384	256	313	S5-7, S10, S19-20, S25, S29, S40, S44, S47, S50, S55	A
384	257	313	S1, S9, S13-14, S18, S23-24, S31, S34, S41, S58	A
384	252	315	S3-4, S22, S42-43, S46, S51, S53	A
384	253	315	S33	A
384	257	315	S38, S56	A
397	236	288	S60-67	B

Even so, all statistics followed the same pattern and therefore genotypic diversity ($\hat{G}$) was used in the following discussion.

Pathogenicity of isolates

Lesions produced by Western Australian isolates representing VC group S1 (43.6 ± 2.94 mm^2), S2 (42.8 ± 1.19 mm^2) and S4 (45.8 ± 1.52 mm^2) were significantly larger than those produced by S3 (33.4 ± 2.30 mm^2) and S5 (37.2 ± 2.12 mm^2). Among the more aggressive pathogens were isolates belonging to VC groups, S1 and S2, the most common VC groups found in Western Australia. However, isolates in VC group S4 were also aggressive pathogens and these isolates were rare, accounting for only 1% of isolates collected. One of the less aggressive pathogens, isolates belonging to VC group S5, had a wide geographic distribution and was found in Western and South Australia, Tasmania and New Zealand.

Molecular characterisation of isolates

All *Sphaeropsis sapinea* isolates representing different VC groups produced alleles with the three SSR markers known to separate morphotypes (Burgess *et al*. 2001). All introduced isolates (S1-59) of *S. sapinea* from Australia, New Zealand and South Africa belonged to the 'A' morphotype. Indigenous isolates (S60-67) of *S. sapinea* from California belonged to the 'B' morphotype (Table 2).

For isolates of the 'A' morphotype, marker SS7 amplified one dominant allele (384 bp) with a frequency of 98%, marker SS10 also amplified one dominant allele (313 bp) with a frequency of 90%, and marker SS9 amplified four alleles (252, 253, 256 and 257 bp). For isolates of the 'B' morphotype, each of the markers amplified only one allele (Table 2).

DISCUSSION

This study produced two significant findings. Firstly, the genotypic diversity of *Sphaeropsis sapinea* on exotic *Pinus radiata* varied considerably, probably reflecting the number of introduction events. Secondly, the morphotype of *S. sapinea* found on *P. radiata* differed between indigenous and introduced populations. These findings will be discussed separately.

Genotypic diversity reflects the number of introduction events

In this study, the genotypic diversity of *Sphaeropsis sapinea* populations collected from *Pinus radiata* in Australia and New Zealand was lower than that of an indigenous Californian population, while the diversity of populations from South Africa was higher. It is generally accepted that the diversity of an introduced population will be lower than the diversity of an indigenous population. For example, the diversity of introduced populations of *Cryphonectria parasitica* in Europe (Anagnostakis, Hau & Kranz 1986, Cortesi *et al*. 1996) was much lower than the diversity of introduced populations in North America, which in turn was lower than that of an indigenous population from China (Milgroom, Lipari & Powell 1992). Similarly, the genotypic diversity of indigenous *C. cubensis* populations in Brazil (van Zyl *et al*. 1998), Indonesia, and Venezuela (van Heerden *et al*. 1997) is much higher than that of an introduced population in South Africa (van Heerden & Wingfield 2001). In contrast, a purported indigenous population of *S. sapinea* from Indonesia had much lower diversity than an introduced population in South Africa (Smith *et al*. 2000). For the latter unusual finding, the authors suggested that the high level of diversity in South Africa arose from multiple introductions of the pathogen. The indigenous origin of the Indonesian *S. sapinea* isolates also could be disputed. The isolates were collected from newly introduced, exotic *P. patula* growing in close association with native *P. merkussii*. The isolates belonged to the new 'C' morphotype and were more pathogenic than those of the 'A' and 'B' morphotype (de Wet *et al*. 2000). This led the authors to propose that they had moved from native *P. merkussii* to exotic *P. patula*, however the low diversity could also be attributed to a small number of introductions.

The sampling of *S. sapinea* conducted in Western Australia was extensive and the number of VC groups obtained should reflect the diversity of the region adequately. The New Zealand population originated from a seed orchard of elite selected trees collected from many parts of New Zealand and is probably also a representative population. The South Australian population from an old plantation near Adelaide might under represent the diversity in South Australia as most of recent afforestation with *P. radiata* has been in the Mt Gambier region. The South African populations were very diverse at both levels of hierarchy and our results compare well with previous estimates of diversity in South Africa Smith *et al*. 2000).

S. sapinea is an asexual fungus and as such, VC groups are useful for detecting clonal lineages (Leslie 1993). The genotypic diversity of an introduced population estimated using VC tests should thus reflect the number of introductions

into a given region. This hypothesis is based on two assumptions. Firstly, there is no selective pressure on particular VC phenotypes that could account for low diversities such as those observed in Australia. Secondly, there is no asexual form of recombination, such as parasexuality, that could account for high diversities such as those observed in South Africa (Leslie 1993, Milgroom 1996). Single mutations at *vic* loci, would also give rise to new clonal lineages. However, it must be assumed that the probability of this occurring is equal in different populations and could not, as such, account for the large differences in diversity observed between South Africa and Australia.

The *vic* loci are seen as selectively neutral (Leslie 1993), however, if *vic* loci are linked to a trait such as pathogenicity, then selection pressure would favour more aggressive genotypes. This would result in the more aggressive VC groups becoming dominant in a population, while the less aggressive VC groups would be lost. Pathogenicity tests, conducted on apples in this study, indicated that the most common VC groups in Western Australia (S1-2) were more aggressive than the less common groups. However, the most widespread VG group (S5), distributed throughout Australia and New Zealand, was a less aggressive pathogen than VC groups, S1-2. Consequently, there does not appear to be any correlation between aggressiveness and the fate of a given VC group. Thus, the low diversity observed in Australia probably reflects limited introductions.

VC data can be used to test recombination, provided controlled crosses can be made (Cortesi & Milgroom 1998). This is not possible with asexual fungi such as *S. sapinea*. However, it is possible to construct a similar diversity to that observed in South Africa using genotypes from different regions within the native range of *Pinus*. In a recent experiment, 18 isolates of the 'A' morphotype from indigenous populations in the USA, Mexico and Switzerland, known to be from individual VC groups within each population, were compared with each other (unpubl.). There were no shared VC groups among isolates from different regions. It is therefore probable that the diversity observed in South Africa is due to multiple introductions from many regions where *Pinus* are native as previously suggested by Smith *et al*. (2000).

The presence of the same VC group in Western Australia, Tasmania, South Australia and New Zealand could be explained in one of two ways. Isolates of this genotype could have been introduced from the same location in the Northern Hemisphere. A more probable explanation would be a movement of material within these Southern Hemisphere countries. Interestingly, only 2 VC groups were found in South Australia and Tasmania and they were the same. Thus, while there have been very few introductions into Australia, there must have been germplasm exchanged between Tasmania and South Australia.

Origin of Sphaeropsis sapinea *in the Southern Hemisphere*

Sphaeropsis sapinea isolates from native *Pinus radiata* in California belonged to the 'B' morphotype, whilst all of the 230 isolates from exotic *P. radiata* in the Southern Hemisphere belonged to the 'A' morphotype. This was established from culture characteristics and spore morphology, as well as by SSR markers. Other isolates of *S. sapinea* collected from exotic pines throughout the world also belong to the 'A' morphotype (Wang *et al*. 1985, Stanosz, Swart & Smith 1999, de Wet *et al*. 2000, Smith *et al*. 2000). If the 'B' morphotype had been introduced into the Southern Hemisphere with *P. radiata*, it would have been observed in the extensive collection of isolates from such a diverse environmental range. Thus, it is likely that the isolates of *S. sapinea* found on exotic *P. radiata* did not originate from imported seed collected from native *P. radiata* stands. Isolates of *S. sapinea* recovered from exotic *P. radiata* could only have originated from other pine species introduced into these regions, then moved onto *P. radiata*, either in nurseries or in the field.

S. sapinea isolates of the 'A' morphotype were first recognised from many coniferous hosts throughout the world. This contrasts with the 'B' morphotype isolates originally found only on *P. resinosa* and *P. banksiana* in the North-Central United States (Wang *et al*. 1985, Palmer *et al*. 1987). Subsequent investigations found that both the 'A' and 'B' morphotypes are associated with *Pinus* and *Cedrus* across their native range in Europe, North and Central America (Morelet & Chandelier 1993, Smith & Stanosz 1995, Hausner *et al*. 1999, Stanosz *et al*. 1999, de Wet *et al*. 2000). In the present study, isolates from native *P. radiata* in California were collected from diseased shoots and not as endophytes in cones. It was originally thought that the 'B' morphotype isolates were opportunistic pathogens that could only colonise wounds, while isolates of the 'A' morphotype could infect non-wounded tissues (Wang *et al*. 1985). However, endophytic isolates of the 'B' morphotype have been routinely recovered from cones of Mexican pines (de Wet *et al*. 2000) and have more recently been isolated in *P. radiata* cones from California (unpubl.). The 'B' morphotype isolates are also less aggressive pathogens than those of the 'A' morphotype (Blodgett & Stanosz 1997, de Wet *et al*. 2000). Thus, the 'B' morphotype is not only associated with disease, but can also be an endophyte in asymptomatic tissue.

The 'B' morphotype isolates of *S. sapinea* have never been found on exotic *Pinus* in the Southern Hemisphere. This suggests that the epidemiology of the two forms of the fungus differ. The 'A' morphotype has been isolated from seed and seed chaff (Anderson *et al*. 1984, Fraedrich *et al*. 1994), and would be present in the host throughout its life-cycle. Alternatively, the 'B' morphotype is not known to be seed transmitted, but rather infects the host post-germination. Thus, 'B' morphotype isolates would not have been transported around the world in seed consignments.

The overall results of this study suggest that *S. sapinea* isolates from *P. radiata* in the Southern Hemisphere did not originate from native *P. radiata*. Rather, it appears that exotic *P. radiata* acquired this endophytic pathogen from other *Pinus* within the exotic environment. Our findings also strongly support the hypothesis that *S. sapinea* has been introduced into South Africa repeatedly. This is in contrast to Australia where the fungus appears to have been introduced rarely. This is particularly interesting because afforestation with *Pinus* commenced across the Southern Hemisphere at about the

same time. (Empire Forestry Association 1929, Legat 1930, Turner 1932, Lavery 1986, Balocchi *et al*. 1998). However, South Africa was colonised *ca* 100 years before Australia and New Zealand and during that time, many exotic pines were cultivated in parks and gardens (Poynton 1957). In addition, Australia and New Zealand are islands far from Europe and the long sea voyage, coupled with quarantine laws, have probably resulted in restricted and reduced introductions of this common pine endophyte.

The results of this study emphasise the danger of poor quarantine. If *S. sapinea* has been introduced so frequently into South Africa, it is likely that other forestry pathogens have also been introduced a number of times. High diversity in an introduced fungal population makes the control of pathogens and the breeding of resistant tree lines more difficult. Thus, even if a pathogen is already present in a country, every effort must be made to avoid the introduction of new genotypes. The emphasis should not be on the pathogen as an entity, but rather on its population biology.

ACKNOWLEDGEMENTS

This study depended on a very large collection of isolates of *S. sapinea* from many parts of the world. It would not have been possible without the assistance of a large number of colleagues, family and friends including Fred Burger and Paul Ten Velde in New Zealand, Tom Gordon in California, Bob Boardman in South Australia, Bev and Peter Burgess in Tasmania and Jolanda Roux in South Africa. Assistance was received in Western Australia from Trevor Butcher and Giles Hardy. We also thank Juanita de Wet for providing data on the Californian population and Bernard Slippers and Albe van der Merwe for critically reviewing the manuscript. 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).

REFERENCES

Anagnostakis, S. L. (1983) Conversion of curative morphology in *Endothia parasitica* and its restriction by vegetative compatibility. *Mycologia* **75**: 777–780.

Anagnostakis, S. L. (1988) *Cryphonectria parasitica*, cause of chestnut blight. *Advances in Plant Pathology* **6**: 123–136.

Anagnostakis, S. L., Hau, B. & Kranz, J. (1986) Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in Connecticut and Europe. *Plant Disease* **70**: 536–538.

Anderson, R. L., Belcher, E. & Miller, T. (1984) Occurrence of seed fungi inside slash pine seeds produced in seed orchards in the United States. *Seed Science and Technology* **12**: 795–799.

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

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.

Bowman, K. O., Hutchenson, K., Odum, E. P. & Shenton, L. R. (1971) Comments on the distribution of indices of diversity. In *Statistical Ecology* (G. P. Patil, E. C. Pielou & W. E. Waters, eds): 315–359. Pennsylvanian State University Press, London.

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

Cortesi, P. & Milgroom, M. G. (1998) Genetics of vegetative incompatibility in *Cryphonectria parasitica*. *Applied and Environmental Microbiology* **64**: 2988–2994.

Cortesi, P., Milgroom, M. G. & Bisiachi, M. (1996) Distribution and diversity of vegetative compatibility types in subpopulations of *Cryphonectria parasitica* in Italy. *Mycological Research* **100**: 1087–1093.

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., 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.

Empire Forestry Association (1929) Empire Forestry Conference, 1928. Summary report, resolutions and reports of committees. *Empire Forestry Journal* **8**: 108–119.

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.

Fulbright, D. W. (1984) Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. *Phytopathology* **74**: 722–724.

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.

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, New Zealand.

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

Leslie, J. F. (1993) Fungal vegetative compatibility. *Annual Review of Phytopathology* **31**: 127–150.

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

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

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

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

Milgroom, M. G., Lipari, S. E. & Powell, W. A. (1992) Comparison of genetic diversity in the chestnut blight fungus, *Cryphonectria (Endothia) parasitica* from China and the US. *Mycological Research* **96**: 1114–1120.

Morelet, P. M. & Chandelier, P. (1993) A case for variability in *Sphaeropsis sapinea*. *European Journal of Forest Pathology* **23**: 317–320.

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.

Poynton, R. J. (1957) *Notes on Exotic Forest Trees in South Africa*. 2nd edn. Vol. 38. Government Printer, Pretoria.

Poynton, R. J. (1977) *Pinus radiata* D. Don. In *Report to the South African Regional Commission for Conservation and Utilization of the Soil (SARCCUS) on Tree Planting in Southern Africa*. Vol. 1. *The Pines* (R. J. Poynton, ed.): 417–441. South African Forestry Research Institute, Pretoria.

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

Smith, D. R. & Stanosz, G. R. (1995) Confirmation of two distinct populations of *Sphaeropsis sapinea* in the Northern Central United States using RAPDs. *Phytopathology* **85**: 669–704.

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.

Smith, H., Wingfield, M. J., de Wet, J. & Coutinho, T. A. (2000) Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. *Plant Disease* **84**: 139–142.

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.

Sutton, B. C. (1980) *The Coelomycetes*. Commonwealth Mycological Institute, Kew.

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.

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

Turner, E. P. (1932) A retrospective of forestry in New Zealand 1894–1931. *Empire Forestry Journal* **11**: 198–212.

van Heerden, S. W. & Wingfield, M. J. (2001) Genetic diversity of *Cryphonectria cubensis* isolates in South Africa. *Mycological Research* **105**: 94–99.

van Heerden, S. W., Wingfield, M. J., Coutinho, T. A., Van Zyl, L. M. & Wright, J. A. (1997). Diversity of *Cryphonectria cubensis* isolates in Venezuela and Indonesia. *Proceedings of the IUFRO Conference on Silviculture and Improvement of Eucalypts, Salvador, Brazil, 24–29 August*: 142–146.

van Zyl, L. M., Wingfield, M. J., Alfenas, A. C. & Crous, P. W. (1998) Population diversity amongst isolates of *Cryphonectria cubensis*. *Forest Ecology and Management* **112**: 41–47.

Wang, C.-G., Blanchette, R. A., Jackson, W. A. & Palmer, M. A. (1985) Differences in conidial morphology among isolates of *Sphaeropsis sapinea*. *Plant Disease* **69**: 838–841.

Wingfield, M. J. (1999) Pathogens in exotic plantation forestry. *International Forestry Review* **1**: 163–168.

Corresponding Editior: *R. F. Park*