Global movement and population biology of *Mycosphaerella nubilosa* infecting leaves of cold-tolerant *Eucalyptus globulus* and *E. nitens*

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Using 10 polymorphic DNA-based microsatellite markers, the genetic diversity of eight *Mycosphaerella nubilosa* populations from *Eucalyptus*, comprising 497 isolates from five different countries, was studies using a hierarchical sampling regime. *Mycosphaerella nubilosa* from eastern Australia (New South Wales) had higher gene (0·506) and genotypic (76%) diversity than other populations, supporting the view that this represents the origin of the pathogen. It was also evident that *M. nubilosa* populations from Europe and Tanzania were clonal, with the same multilocus haplotypes occurring in South Africa, but being absent in Australia. This suggests that *M. nubilosa* may have been introduced into Europe via Africa, with a pathway of gene flow from Australia to South Africa, further into Africa and finally to Europe.

Keywords: cold-tolerant *Eucalyptus* spp., gene diversity, gene flow, genotypic diversity, microsatellite markers, mycosphaerella leaf blotch

Introduction

The ascomycete genus *Mycosphaerella* includes more than 80 species of fungi that have been associated with leaf diseases of *Eucalyptus* spp. (Crous, 1998; Crous *et al.*, 2004, 2006). Infection by *Mycosphaerella* spp. can affect photosynthesis in young *Eucalyptus* leaves, resulting in premature defoliation and leading to reduced growth of trees (Park & Keane, 1982b; Carnegie & Ades, 2003; Pinkard & Mohammed, 2006).

Mycosphaerella nubilosa* is one of the most important species on *Eucalyptus*, causing mycosphaerella leaf blotch (MLB), particularly on cold-tolerant species such as *E. globulus* and *E. nitens* (Park & Keane, 1987; Carnegie *et al.*, 1998; Carnegie & Ades, 2002; Hunter *et al.*,

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2004a). This fungus typically infects juvenile leaves and susceptible trees can be severely defoliated (Park & Keane, 1982b; Lundquist & Purnell, 1987).

Mycosphaerella nubilosa was first described from *Eucalyptus* leaves in Victoria, Australia (Cooke, 1891) and subsequently in Tasmania, New South Wales, South and Western Australia (Hansford, 1956; Park & Keane, 1982a,b; Park, 1988; Maxwell *et al.*, 2001; Milgate *et al.*, 2001; Carnegie, 2007a,b). Elsewhere *M. nubilosa* occurs in the North Island of New Zealand (Dick, 1982) and in Africa, in Ethiopia, Kenya, South Africa, Tanzania and Zambia (Crous *et al.*, 2004, 2006; Hunter *et al.*, 2004a; Alemu *et al.*, 2006), and was accidentally introduced into commercial plantations of *E. globulus*, where it caused serious damage in Spain and Portugal (Crous *et al.*, 2004).

Apart from *E. globulus* and *E. nitens*, *M. nubilosa* infects *E. bridgesiana*, *E. cypellocarpa*, *E. dunnii*, *E. grandis*, *E. quadrangulata*, *E. globulus* ssp. *bicostata* and ssp. *maidenii* (Dick, 1982; Crous, 1998; Crous *et al.*, 2004; Hunter *et al.*, 2004a,b; Milgate *et al.*, 2005; Carnegie, 2007b). Because of their rapid growth, frost tolerance and favourable wood qualities, *E. nitens* and *E. globulus* are

amongst the most popular *Eucalyptus* spp. propagated for commercial forestry in the above countries and are particularly susceptible to *M. nubilosa*; MLB can be a serious impediment to the sustainability of *E. nitens* and *E. globulus* plantations.

Mycosphaerella nubilosa naturally occurs only in its sexual state, producing many small black pseudothecia that may be amphigenous, but are predominantly hypophyllous on lesions (Crous, 1998; Crous *et al.*, 2004). When leaves are wet, ascospores are released from pseudothecia and act as wind-dispersed primary inoculum and infect via stomata (Park & Keane, 1982b; Park, 1988). Although the anamorph state *Uwebraunia juvenis* was linked to *M. nubilosa* (Crous *et al.*, 2004), this connection has not been reconfirmed and remains doubtful.

Most research conducted on *M. nubilosa* has focussed on its epidemiology, taxonomy, host association and phylogeny, but little is known of its population biology. The aim of this study was, therefore, to investigate the population biology of *M. nubilosa* from several countries by determining the number of alleles at 10 polymorphic microsatellite loci. There were three primary objectives: (i) consideration of the genetic diversity of *M. nubilosa*, (ii) examination of this variation between different countries, and (iii) determination of the likely origin and global movement of *M. nubilosa*.

Materials and methods

Isolates

Diseased *Eucalyptus* leaves infected with *M. nubilosa* were collected from Australia, Portugal, South Africa, Spain and Tanzania (Table 1).

Two populations of *M. nubilosa* were collected in Australia. From eastern Australia, one leaf was randomly collected from each of 40 *E. globulus* trees in one plantation near Bonalbo, north-eastern New South Wales (EA-MT). Similarly, in Western Australia one leaf was randomly

collected from each of 32 *E. globulus* trees in the south of the state (WA-MT). One plantation of *E. globulus* was sampled in northern Spain and one leaf was selected from each of 55 trees (S-MT). Likewise, one plantation of *E. globulus* was sampled in central Portugal, where 42 leaves were taken from an equal number of *E. globulus* trees (P-MT).

In Njombe, Tanzania, 56 *E. globulus* trees were randomly sampled, and one leaf was collected from each tree (T-MT). In South Africa, three plantations of *E. nitens* were sampled in the Mpumalanga Province, namely Issabelladale, Wynton and Rooihoogte. From Rooihoogte, 48 leaves displaying spots were randomly collected from a single tree (R-1T). At this plantation, a single lesion from a single leaf was sampled and 32 isolates were collected from this lesion (R-1L). In addition, a single leaf was randomly collected from each of 56 trees within Roooihoogte (R-MT). From Wynton, 64 leaves exhibiting MLB symptoms were collected from a single tree (W-1T). Furthermore, 64 trees in this plantation were randomly selected and one diseased leaf was taken from each (W-MT). From Issabelladale, 40 leaves showing MLB lesions were randomly collected from one tree (I-1T). In addition, 60 trees within the same plantation were randomly selected and one leaf was collected from each of these trees (I-MT).

Isolation, *in vitro* culture and DNA extraction

Isolation of *M. nubilosa* from diseased *Eucalyptus* leaves followed the protocol of Crous (1998). One ascospore per lesion, exhibiting the type-F ascospore germination pattern (Crous, 1998; Crous *et al.*, 2004), was cut from the agar surface and transferred to fresh 2% malt extract agar and incubated at 25°C for approximately 2–3 months to ensure sufficient mycelial growth. All isolates of *M. nubilosa* used in this study were deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Table 1 Origins of *Mycosphaerella nubilosa* isolates used for this study

Country	Area	Plantation	Hierarchical Level	PC[a]	Host	Collector	Date	No. isolates[b]
South Africa	Mpumalanga	Rooihoogte	Single lesion	R-1L	*E. nitens*	G. C. Hunter	2005	32
			Single tree	R-1T	*E. nitens*	G. C. Hunter	2005	48
			Plantation	R-MT	*E. nitens*	G. C. Hunter	2005	56
		Issabelladale	Single tree	I-1T	*E. nitens*	G. C. Hunter	2005	30
			Plantation	I-MT	*E. nitens*	G. C. Hunter	2005	29
		Wynton	Single tree	W-1T	*E. nitens*	G. C. Hunter	2005	49
			Plantation	W-MT	*E. nitens*	G. C. Hunter	2005	49
Australia	Western Australia	N/A	Plantation	WA-MT	*E. globulus*	A. Maxwell	2002	32
	New South Wales	Bonalbo	Plantation	EA-MT	*E. globulus*	A. J. Carnegie	2004	19
Spain			Plantation	S-MT	*E. globulus*	J. P. M. Vasquez	2002	55
Portugal			Plantation	P-MT	*E. globulus*	M. J. Wingfield	2005	42
Tanzania			Plantation	T-MT	*E. globulus*	J. Roux	2004	56
							Total	497

[a]Population code assigned to each population and subpopulation.
[b]Number of isolates per hierarchical sampling level.

Subsequently, mycelium from actively growing single-spore cultures of *M. nubilosa* was scraped from the surface of cultures, freeze-dried for 24 h and ground to a fine powder using liquid nitrogen. DNA was isolated using the phenol:chloroform (1:1) extraction protocol described by Hunter *et al*. (2004a,b). Isolated DNA was separated by electrophoresis on 1% agarose gels (w/v) (Roche Diagnostics), stained with ethidium bromide and visualized under ultraviolet light.

PCR amplification and allele size determination

DNA from isolates of *M. nubilosa* served as template DNA for polymerase chain reactions (PCR). Fluorescently labelled primers used for PCR reactions were the 10 polymorphic microsatellite primers developed by Hunter *et al*. (2006). The PCR reaction mixture and conditions were the same as those described previously (Hunter *et al*., 2006). Amplified DNA was visualized on 2% agarose gels stained with ethidium bromide and viewed under ultraviolet light. Sizes of PCR amplicons were estimated by comparison against a 100-bp molecular weight marker (O'RangeRuler™ 100-bp DNA ladder) (Fermentas Life Sciences). PCR products were purified using Sephadex G-50 (Sigma Aldrich) in Centri-sep Spin Columns (Princeton Separations) as outlined by the manufacturer.

Allele sizes of amplified PCR products were determined following the method of Hunter *et al*. (2006). The number of alleles per locus and the total number of multilocus haplotypes (MLHs, hereafter referred to as haplotypes) across all the *M. nubilosa* populations were determined.

Gene and genotypic diversity

The frequency of alleles at each locus was calculated for every *M. nubilosa* population. Allele diversity was also determined using the software program POPGENE (Yeh *et al*., 1999) and the gene diversity (*H*) of Nei (1973) was calculated at each locus and also averaged over all loci:

$$H = 1 - \sum x_k^2$$

Chi-squared tests for differences in allele frequencies between the *M. nubilosa* populations were also calculated from clone-corrected datasets (Workman & Niswander, 1970).

Every *M. nubilosa* isolate was assigned a haplotype code according to the observed allele sizes, across the 10 microsatellite loci. Genotypic diversity (*G*) was calculated using the equation of Stoddart & Taylor (1988):

$$G = 1 \bigg/ \sum_{x=0}^{N} [f_X(X/N)^2]$$

To compare genotypic diversities between the various *M. nubilosa* populations, the maximum percentage of genotypic diversity ($\hat{G}$) was calculated:

$$\hat{G} = G/N \times 100$$

(Chen *et al*., 1994). Genotypic diversity values were also compared between *M. nubilosa* populations at the plantation level for significant differences using a *t*-test (Chen *et al*., 1994).

Population differentiation and gene flow

Clone-corrected populations of *M. nubilosa* were used to calculate population differentiation (θ) between the South African and Australian *M. nubilosa* populations using the computer software program MULTILOCUS (Agapow & Burt, 2001), using 1000 randomized datasets. The population differentiation value (θ), Weir's formulation of Wrights' F_{ST}, was calculated (Weir, 1996; Agapow & Burt, 2001). Furthermore, gene flow (*M*) was calculated from theta (θ) using the equation of Cockerham & Weir (1993):

$$M = [(1/\theta) - 1]/2.$$

Results

Isolates

A total of 497 isolates of *M. nubilosa* were collected from diseased *Eucalyptus* leaves (Table 1). These represented a wide range of geographic locations and also different hierarchical levels, and included 349 isolates from Africa, 97 from Portugal and Spain, and 51 from Australia.

PCR amplification and allele size determination

A total of 66 alleles were observed across all 10 loci for all the *M. nubilosa* populations, with an average of 6·6 alleles per locus. The Australian *M. nubilosa* populations WA-MT and EA-MT exhibited the greatest number of alleles across all loci, namely 22 and 44, respectively (Table 2).

South African populations exhibited much lower numbers of alleles across the 10 loci than those from Australia. In total, 25 alleles were observed across all of the South African *M. nubilosa* populations at the various hierarchical levels and the majority of these alleles were commonly shared. South African populations R-MT and I-1T exhibited the most alleles (a total of 18) (Table 2). One population (R-1L) collected from a single lesion on an *E. nitens* leaf from Rooihoogte yielded a total of 13 different alleles for the 10 loci across 32 *M. nubilosa* isolates (Table 2). For the isolates collected in Spain, Portugal and Tanzania all loci were monomorphic.

Across all *M. nubilosa* populations, all of the 10 microsatellite loci were polymorphic. Both the South African and Western Australian populations exhibited 60% polymorphic loci, whilst 80% of loci were polymorphic for the population EA-MT in New South Wales.

Gene and genotypic diversity

The South African *M. nubilosa* populations had a moderate to high gene diversity, ranging from 0·149 to 0·250 for populations R-1L and W-MT, respectively (Table 2).

Table 2 Number of alleles and gene diversity at 10 microsatellite loci (MN-1–MN-14) determined using the program POPGENE for populations of *Mycosphaerella nubilosa* collected in South Africa (Rooihoogte, Issabelladale and Wynton) and Australia (Western Australia and New South Wales)

| | South Africa | | | | | | | Australia | |
| | Rooihoogte | | | Issabelladale | | Wynton | | WA | NSW |
	R-1L	R-1T	R-MT	I-1T	I-MT	W-1T	W-MT	WA-MT	EA-MT
N^a	32	48	56	30	29	49	49	32	19
N^b	4	24	24	16	17	22	23	10	16
Alleles	13	17	18	18	16	16	17	22	44
P	3	6	5	5	5	5	6	6	8
P (%)	30	60	50	50	50	50	60	60	80
H	0·149	0·240	0·248	0·222	0·211	0·231	0·250	0·242	0·506
H^*	0·125	0·258	0·255	0·248	0·242	0·247	0·255	0·246	0·506

N^a = number of isolates used in uncorrected dataset; N^b = number of isolates used in clone-corrected dataset; P = number of polymorphic loci; P (%) = percentage of polymorphic loci; H = gene diversity of population (Nei, 1973); H^* = gene diversity of clone-corrected population.

Table 3 Number of multilocus haplotypes (MLHs), genotypic diversity and maximum percentage of genotypic diversity of *Mycosphaerella nubilosa* isolates compiled from 10 polymorphic microsatellite markers. Haplotypes from Spain (S-MT), Portugal (P-MT) and Tanzania (T-MT) all at the plantation level. Those from three plantations in South Africa at different hierarchical levels: Rooihoogte plantation at the plantation level (R-MT), within one tree (R-1T) and within one lesion (R-1L); Issabelladale plantation at the plantation level (I-MT) and within one tree (I-1T); and Wynton plantation at the plantation level (W-MT) and within one tree (W-1T). Also two *M. nubilosa* populations, both at the plantation level, from Australia: Western Australia (WA-MT) and New South Wales (EA-MT)

				South Africa							Australia	
				Rooihoogte			Issabelladale		Wynton		WA	NSW
	Spain	Portugal	Tanzania									
	S-MT	P-MT	T-MT	R-1L	R-1T	R-MT	I-1T	I-MT	W-1T	W-MT	WA-MT	EA-MT
N^a	55	42	56	32	48	56	30	29	49	49	32	19
$N (g)^b$	1	1	1	4	24	24	16	17	22	23	10	16
G^c	N/A	N/A	N/A	2·26	15·78	16·88	6·63	8·73	9·61	14·82	4·86	14·49
$\hat{G}^d$	N/A	N/A	N/A	7·1	32·9	30·14	22·1	30·1	19·6	30·2	15·2	76·3

[a] Number of *M. nubilosa* isolates.
[b] Number of MLHs.
[c] Genotypic diversity (Stoddart & Taylor, 1988).
[d] Maximum percentage of genotypic diversity.

Gene diversity for the Western Australia population (WA-MT) (0·242) was comparable with that of the South African populations. In contrast, a substantially higher gene diversity value of 0·506 was observed for the New South Wales population (EA-MT).

A total of 68 haplotypes were observed across all *M. nubilosa* populations from the various locations considered (Table 3). Almost half (49%) of the haplotypes were unique and detected only once. The South African populations had a total of 35 haplotypes and the majority of these haplotypes were distributed among the various South African populations. However, seven haplotypes were unique to certain South African populations. None of the haplotypes that were observed for the South African populations occurred in either of the Australian populations. However, the Spanish and Portuguese populations both had only one haplotype, which was also observed in the R-MT, W-1T and W-MT *M. nubilosa* populations of South Africa. The Tanzanian population also had only one haplotype, which was also observed in six of the seven South African populations.

A total of 26 haplotypes were observed across the two Australian populations WA-MT and EA-MT, the former with 10 unique haplotypes, the latter with 16.

Genotypic diversity (G) varied between the various populations of *M. nubilosa* from different locations (Table 3). The lowest maximum percentage of genotypic diversity ($\hat{G}$), 7·1% was observed for the South African *M. nubilosa* isolates obtained from a single lesion (R-1L). However, maximum percentages of genotypic diversity for South African *M. nubilosa* populations at the plantation level were similar to each other, with values of 30·14, 30·1 and 30·2% for R-MT, I-MT and W-MT, respectively (Table 3). Interestingly, the Western Australian population (WA-MT) had the second lowest value of genotypic diversity at 15·2% of the theoretical maximum value, which was lower than for all the South African populations except population R-1L. The highest genotypic diversity

Table 4 Gene diversity (*H*) and contingency χ² tests for differences in allele frequencies for the 10 microsatellite loci across the clone-corrected South African populations of *Mycosphaerella nubilosa* from different hierarchical levels. (A) Within one lesion (R-1L), within one tree (R-1T) and within one plantation (R-MT) of the Rooihoogte plantation. (B) Within one tree (I-1T) and within one plantation (I-MT) of the Issabelladale plantation. (C) Within one tree (W-1T) and within one plantation (W-MT) of the Wynton plantation. (D) Comparison between three South African plantations at the plantation level (R-MT, I-MT and W-MT). Determined χ² values not significant

	Gene diversity (*H*)							A		B		C		D	
	R-1L	R-1T	R-MT	I-1T	I-MT	W-1T	W-MT	χ^2	d.f.	χ^2	d.f.	χ^2	d.f.	χ^2	d.f.
MN-3	0·00	0·49	0·49	0·43	0·46	0·48	0·50	3·0	2	0·06	1	0·22	1	2·2	2
MN-4	0·38	0·50	0·49	0·55	0·50	0·48	0·50	0·9	2	1·22	2	0·22	1	0·7	2
MN-8	0·38	0·50	0·57	0·54	0·48	0·52	0·48	3·3	6	1·10	2	1·07	2	3·6	6
MN-9	0·00	0·47	0·52	0·46	0·46	0·48	0·54	3·6	4	1·37	2	1·07	2	3·9	6
MN-10	0·00	0·08	0·00	0·00	0·00	0·00	0·08	1·2	2	–	–	0·98	1	1·8	2
MN-11	0·50	0·53	0·49	0·49	0·53	0·50	0·45	2·2	4	1·10	2	1·07	1	5·8	4
N[a]	4	24	24	16	17	22	23								
Mean	0·13	0·23	0·25	0·25	0·24	0·25	0·25								

[a]Number of *M. nubilosa* isolates from the clone-corrected dataset.

Table 5 Gene diversity (*H*) and contingency χ² tests for differences in allele frequencies for the 10 polymorphic microsatellite loci across the clone corrected *Mycosphaerella nubilosa* populations from (A) South Africa [RSA = (R-MT + I-MT + W-MT)] and Western Australia (WA-MT), (B) South Africa [RSA = (R-MT + I-MT + W-MT)] and eastern Australia (EA-MT), and (C) Western Australia (WA-MT) and New South Wales (EA-MT)

	Gene diversity (*H*)			A		B		C	
Locus	RSA	WA-MT	EA-MT	χ^2	d.f.	χ^2	d.f.	χ^2	d.f.
MN-1	0·00	0·00	0·00	–	–	51·0*	1	26·0*	1
MN-2	0·00	0·18	0·88	3·6	1	46·5*	9	22·2*	10
MN-3	0·50	0·00	0·30	7·8*	1	15·9*	2	2·1	1
MN-4	0·50	0·00	0·69	9·4*	1	38·2*	4	22·2*	4
MN-7	0·00	0·32	0·43	7·3*	1	51·0*	2	26·0*	3
MN-8	0·54	0·84	0·63	39·6*	9	22·6*	8	20·4*	10
MN-9	0·54	0·32	0·76	5·1	3	46·4*	9	26·0*	9
MN-10	0·06	0·00	0·63	0·3	1	42·2*	5	19·0*	4
MN-11	0·52	0·62	0·00	19·0*	3	15·0*	2	9·9*	2
MN-14	0·00	0·18	0·73	3·6	1	51·0*	4	22·4*	4
N[a]	35	10	16						
Mean	0·27	0·25	0·51						

*Indicates significant χ² values (*P* < 0·05).
[a]Number of *M. nuilosa* isolates from the clone-corrected dataset.

(76% of the theoretical maximum) was observed in the New South Wales population (EA-MT). No significant differences (*P* < 0·05) in genotypic diversities were observed between *M. nubilosa* populations at the plantation level (R-MT, I-MT, W-MT, WA-MT and EA-MT) using a *t*-test (data not shown).

Population differentiation and gene flow

Based on contingency chi-squared tests, there were no significant differences in allele frequencies at any of the loci when the *M. nubilosa* subpopulations from the Rooihoogte (R-1L, R-1T and R-MT), Issabelladale (I-1T and I-MT) and Wynton (W-1T and W-MT) *E. nitens* plantations in South Africa were considered (Table 4). Three subpopulations at the plantation level from South

Africa (R-MT, I-MT and W-MT) were also compared with each other and no significant differences were observed between the allele frequencies at any of the 10 loci (Table 4). These three South African populations were, therefore, combined at the plantation level and this dataset was clone-corrected, in order to compare it to the two Australian populations (WA-MT and EA-MT).

Based on contingency chi-squared tests, significant differences (*P* < 0·05) in allele frequencies were observed at nine of the 10 polymorphic loci when the two Australian *M. nubilosa* populations (WA-MT and EA-MT) were compared against each other (Table 5). Significant differences (*P* < 0·05) in allele frequencies were also observed at five loci when the combined South African *M. nubilosa* population (RSA) was compared with the Western Australian population (WA-MT), and for all 10 loci when population

Table 6 Population differentiation values (θ), for populations of *Mycosphaerella nubilosa* above the diagonal, calculated after 1000 randomizations using the program MULTILOCUS. Gene flow (number of migrants) (*M*) indicated below the diagonal

	R-MT	I-MT	W-MT	WA-MT	EA-MT
R-MT		−0·0054	−0·03034	0·25*	0·55*
I-MT	9·63		−0·0061	0·31*	0·54*
W-MT	16·93	82·47		0·29*	0·55*
WA-MT	1·54	1·13	1·24		0·49*
EA-MT	0·41	0·43	0·41	0·52	

*Indicates significant values (*P* < 0·001).
R-MT, Rooihoogte; I-MT, Issabelladale; W-MT, Wynton, South Africa.
WA-MT, Western Australia; EA-MT, Eastern Australia.

RSA was compared with the population from New South Wales (Table 5).

Population differentiation values (θ) were not significantly different between any of the South African *M. nubilosa* populations at the plantation level (R-MT, I-MT and W-MT) (Table 6). Gene flow (*M*) between the three South African populations was high, with the greatest number of migrants (*M* = 82·47) occurring between the W-MT and I-MT populations and the least (*M* = 9·63) occurring between the I-MT and R-MT populations (Table 6). There were, however, significant differences (*P* < 0·001) at the plantation level between the three South African populations (R-MT, I-MT and W-MT) and the two Australian populations (WA-MT and EA-MT) based on θ values (Table 6). Similarly, θ values indicated a significant population differentiation (*P* < 0·001) between the two Australian populations (Table 6), with very little gene flow (*M* = 0·52) observed between the Western Australian and New South Wales populations. However, there was low gene flow (*M* = 1·13–1·54) between the Western Australian population (WA-MT) and the three South African *M. nubilosa* populations (R-MT, I-MT and W-MT) and extremely limited gene flow (*M* = 0·41–0·43) between the other Australian population (EA-MT) and the three South African populations (Table 6).

Discussion

Mycosphaerella nubilosa is one of the most important leaf pathogens of *E. nitens* in South Africa and of *E. globulus* in Australia. In turn, these trees represent two of the most important sources of wood fibre derived from plantations. South Africa has the oldest plantation forestry programme based on non-native species in the world (Burgess & Wingfield, 2001) and *M. nubilosa* was the first pathogen of *Eucalyptus* to be reported from this country (Doidge, 1950). It has subsequently appeared in many areas of the world where *E. nitens* and *E. globulus* are grown. Although it has been assumed that the pathogen originated in Australia and that it was accidentally introduced into other parts of the world, this hypothesis has never been tested experimentally.

Mycosphaerella nubilosa was originally identified from Victoria in eastern Australia (Cooke, 1891). Since this initial identification, it has been hypothesized that eastern Australia would represent the centre of origin for *M. nubilosa*. Gene diversity and maximum percentage of genotypic diversity for the New South Wales *M. nubilosa* (EA-MT) population was the highest of all populations evaluated during this study. Furthermore, the (EA-MT) population had a greater number of alleles than the other *M. nubilosa* populations. It is expected that older pathogen populations representing centres of origin have higher gene diversity values as a result of the accumulation of mutations over time (McDonald, 1997). Results of this study, therefore, support the view that *M. nubilosa* originated from eastern Australia.

An interesting observation in this study was the presence of shared alleles between Western Australia and South Africa. This finding suggests that *M. nubilosa* could have been introduced into South Africa from Western Australia and not from eastern Australia. However, *M. nubilosa* was not detected in Western Australia until 1999 (Maxwell *et al.*, 2001), despite previous surveys (Carnegie *et al.*, 1997), suggesting that it was introduced relatively recently into that area. There are also no known hosts of *M. nubilosa* that naturally occur in Western Australia. Furthermore, commercial forestry with *E. globulus* (a species native to Tasmania and southern Victoria, Australia) only began in Western Australia during the 1980s. It thus seems unlikely that the fungus was introduced into South Africa from Western Australia. An alternative hypothesis suggests inoculum from eastern Australia, not sampled in this study, served as a source population for both Western Australia and South Africa. The fact that *E. globulus* seed from eastern Australia was the first *Eucalyptus* planting stock to be planted in South Africa would provide additional support, albeit anecdotal, for this view.

Gene diversities derived in this study for the various *M. nubilosa* populations from South Africa were comparable with each other, and lack of significant population differentiation, combined with the high level of gene flow between the South African *M. nubilosa* populations, indicate that these populations are highly homogenous.

In South Africa, the gene diversity of isolates from a single tree was as great as for isolates from a single plantation, and gene flow between plantations was very high. Similar findings of genetic diversity have been found for *M. fijiensis* on banana, where the majority of genetic diversity for that pathogen exists at the plant and plantation levels (Rivas *et al.*, 2004). The results for *M. nubilosa* are also consistent with those for other *Mycosphaerella* pathosystems. For example, the majority of diversity for *M. graminicola* was observed within a single plot of a wheat field (McDonald & Martinez, 1990; Zhan *et al.*, 2003), and likewise with *M. populorum* from north-eastern America, where more than 90% of the genetic diversity was distributed within a single tree (Feau *et al.*, 2005).

Results reported here reveal that some haplotypes of *M. nubilosa* are shared between South Africa, Tanzania

and Europe, indicating gene flow has occurred between these regions, resulting in the establishment and persistence of specific haplotypes in Europe and in Tanzania. Based on gene flow data between Australia and South Africa and the same haplotypes that are shared between South Africa, Tanzania and Europe, it seems likely that *M. nubilosa* was first introduced into South Africa from Australia and that it subsequently spread to other parts of Africa and into Europe. This route of movement is also consistent with the pattern of establishment of *Eucalyptus* plantations in Africa and Europe.

Acknowledgements

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