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Micro- and macrospatial scale analyses illustrates mixed mating strategies and extensive geneflow in populations of an invasive haploid pathogen

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Abstract

Sexual reproduction in fungi involves either a single individual (selfing) or two individuals (outcrossing). To investigate the roles that these two strategies play in the establishment of an invasive alien pathogen, the *Eucalyptus* leaf-infecting fungus, *Teratosphaeria* (*Mycosphaerella*) *nubilosa* was studied. Specifically, the genetic diversity of the pathogen was investigated at micro and macrospatial scales. Interestingly, while data obtained at microspatial scales show clearly that selfing is the main reproductive strategy, at macrospatial scales the population genetic structure was consistent with a genetically outcrossing organism. Additional analyses were performed to explore these apparently discordant results at different spatial scales and to quantify the contribution of selfing vs. outcrossing to the genotypic diversity. The results clearly show that the fungus has a mixed mating strategy. While selfing is the predominant form of mating, outcrosses must have occurred in the pathogen that increased the genotypic diversity of the fungus over time. This mating strategy, coupled with the high levels of geneflow between distant populations of the pathogen, has created an even distribution of maximum diversity from the smallest (leaf) to largest scales (>500 km), which will make breeding for resistance difficult. These data illustrate the evolutionary potential and danger of the introduction of multiple genotypes of a potentially outcrossing pathogen, especially when it has a high dispersal potential.

Keywords: hierarchical sampling, introduced pathogen, microsatellite marker, Mycosphaerella leaf disease, Pareto distributions, population genetics, within lesion diversity

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Introduction

Fungi exhibit several mechanisms of sexual reproduction (Taylor *et al.* 1999). Ascomycetes are typically heterothallic, where sexual reproduction involves two genetically distinct haploid individuals or homothallic, where single individuals are able to undergo sexual reproduction (Milgroom 1996; Taylor *et al.* 1999). Populations of heterothallic fungi are usually diverse, with random association of alleles, since meiotic recombination (outcrossing) generates novel genotypes at each generation. In homothallic species where strains 'self' fertilize (selfing), meiosis does not generate novel genotypes and the sexual spores (ascospores) are typically clonal (Milgroom 1996; Taylor *et al.* 1999). Homothallism, however, does not exclude the possibility of occasional heterothallic outcrossing (Milgroom 1996).

The ascomycete fungus, *Teratosphaeria nubilosa* (=*Mycosphaerella nubilosa*) causes a serious leaf disease of *Eucalyptus* spp., known as Mycosphaerella leaf disease (MLD) (Crous 1998; Hunter *et al.* 2009). The disease results in premature leaf abscission and stunting of tree growth, which can lead to significant economic losses

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for commercial forestry companies (Lundquist & Purnell 1987; Carnegie & Ades 2003; Milgate *et al.* 2005). *Teratosphaeria nubilosa* is native to Australia and has been accidentally introduced into many countries including Brazil, Ethiopia, Kenya, Portugal, Spain, South Africa, Tanzania, Uruguay and Zambia, where it limits growth of *Eucalyptus nitens* and *E. globulus* in plantations (Lundquist & Purnell 1987; Hunter *et al.* 2008, 2009; Pérez *et al.* 2009).

Teratosphaeria nubilosa is believed to be homothallic (Park & Keane 1982), but it is unknown whether outcrossing between genotypes also occurs. It has been proposed that *T. nubilosa* has an exclusively sexual mode of reproduction, because sexual structures (pseudothecia) bearing asci and ascospores are the only reproductive state observed in nature. An asexual state known as *Uwebraunia juvenis* was described for the fungus in culture, when it was known under the name *Mycosphaerella juvenis* (Crous & Wingfield 1996). While *M. juvenis* was later reduced to synonymy with *T. nubilosa* (Crous *et al.* 2004) the occurrence of *U. juvenis* has been observed only once in a culture of *T. nubilosa* (= *M. juvenis*) and this anamorph link is considered doubtful (Wingfield unpublished).

Teratosphaeria nubilosa produces airborne ascospores that are actively discharged from ascomata (Park & Keane 1982). These spores are dispersed by the wind to new leaves and, therefore, serve as the primary source of inoculum (Park 1988). Although wind has the potential to carry ascospores over long distances, they are more likely to be deposited close to their source of origin. Therefore, they have the potential to re-infect the same leaf or leaves on the same tree. Epidemiological studies have shown that *T. nubilosa* lesions of different ages co-occur in the same branch and leaves infected in one infection cycle can be re-infected repeatedly (Park 1988). The result of such re-infections on the distribution of the genetic diversity at small scales has been studied for related pathogens such as *Mycosphaerella musicola* on banana (Hayden *et al.* 2005) and *M. graminicola* on wheat (Linde *et al.* 2002). The role that re-infection plays in the distribution of genetic diversity has, however, not been studied in any species of *Mycosphaerella sensu lato* (including *Teratosphaeria* spp.) on *Eucalyptus* or other trees.

Disease symptoms include round leaf spots less than a centimetre in diameter (Crous 1998; Hunter *et al.* 2009). Typically, when neighbouring lesions meet, they coalesce to form larger irregular blotches over the leaf surface (Park & Keane 1982; Park 1988). Multiple infections on lesions could provide opportunities for genetic interactions among fungal species or fungal genotypes (Linde *et al.* 2002). For example, sexual recombination as well as competition amongst genotypes has been proposed to take place within lesions in the well studied plant pathogenic fungus *Mycosphaerella graminicola* (Linde *et al.* 2002). The analysis of the spatial arrangement of multiple genotypes within lesions could provide valuable information regarding the origin of the infection on the host. For instance, when only one genotype is recovered from a lesion, this lesion is most probably the product of the infection by a single spore. The co-occurrence of multiple genotypes on lesions may be due to the co-infection of the leaf by multiple spores (Linde *et al.* 2002) or by the spermatization of pro-pseudothecia developed on the immature leaf lesion (Maheshwari 1999). In the former situation, a mosaic of neighbouring genotypes that co-exist in close proximity would occur. In the latter case, a predominant genotype spread widely over a lesion would be expected with 'islands' of less frequent genotypes occurring in between.

In this study, the microspatial arrangement of *T. nubilosa* genotypes on lesions and blotches was analysed to test two hypotheses regarding the origin of infections on *E. nitens* leaves and the mating system of the pathogen. The first hypothesis was that lesions originate from single ascospores and that single individuals are able to produce sexual structures and new ascospores via homothallism. Here the presence of multiple genotypes within lesions would reflect the coalescence of lesions developing in close proximity on leaves. The second and alternative hypothesis was that more than one genotype is required on a lesion to enable sexual structures to form, thus reflecting heterothallism. In this case, lesions could initially originate either from multiple ascospores or from single ascospores. As the lesions derived from single ascospores develop, the pathogen would produce pro-pseudothecia fertilized by spermatia from a distant source. Sexual reproduction could also be achieved by somatic contact where mycelia of two genotypes of opposite mating type meet in coalescing lesions.

As *T. nubilosa* occurs widely in South Africa, we used the distribution of the genetic diversity at the population level to test the same two hypotheses regarding the mating system of the pathogen at macrospatial scales. The first hypothesis was that the pathogen is represented by an outcrossing population across the entire country. The second and alternative hypothesis was that the pathogen is represented by a selfing population. A significant gametic disequilibrium would be expected in the latter situation but not in the former. We obtained different and apparently contradictory answers to our hypotheses at micro- and macrospatial scales, suggesting a mixed mode of reproduction as the most likely situation. Therefore, additional analyses were performed with the purpose of quantifying the

contribution of selfing vs. outcrossing to the genotypic diversity of the *T. nubilosa* population.

Materials and methods

Hierarchical microspatial sampling

To understand the distribution of the genetic diversity of *T. nubilosa* over microspatial scales, infections of single lesions, blotches and single leaves were considered (Fig. 1). At the single-lesion level (Fig. 1a), 25 lesions were dissected into 20 square samples of 2 mm each. At the blotch level (Fig. 1b and c), eight blotches were dissected into five to 20 samples of 2–5 mm each. At the single-leaf level, 17 lesions were sampled from a diseased *E. nitens* leaf (Fig. 1d). Only one fungal strain per sample was used in further analyses, except for the blotch level where one to five fungal strains were isolated from each leaf piece. All single lesions and blotches were photographed before isolations were made and the position of every sample on a leaf was noted to map the arrangement of the potential multilocus haplotypes (MLH) within lesions or blotches. Similarly, the single leaves were photographed and the position of every lesion on the leaf lamina was recorded.

Hierarchical macrospatial sampling

A hierarchical sampling strategy was used at three macrospatial scales including those for provinces, plantations and single trees. In 2007, samples were collected in the Mpumalanga, KwaZulu-Natal and Western Cape Provinces of South Africa (Fig. 2, Table 1). Four plantations in Mpumalanga were sampled, three in Lothair (Lothair 1, Lothair 2 and Lothair 3) and one in Sabie (Table 1). In KwaZulu-Natal, samples were collected in Utrecht and Bulwer and in the Western Cape Province in George (Fig. 2, Table 1). For the Lothair, Utrecht and Bulwer populations, sampling was conducted in commercial *E. nitens* plantations and for the George and Sabie populations, sampling was conducted on naturally regenerated *E. globulus* trees less than 1 year old (Table 1). In all cases, one diseased leaf infected with *T. nubilosa* was collected from each of 50 trees extending outwards from a central tree.

At the single-tree level, one severely diseased *E. nitens* tree was chosen at the centre of the plantation and a total of 36 leaves were sampled from this tree. Three whorls, at three heights of approximately 0.2, 1.0 and 1.5 m were chosen. Four branches were chosen per whorl, representing the four compass directions. Three leaves were then sampled at proximal, median and distal positions on the branches with respect to the main stem. At all macrospatial hierarchical levels, only one fungal isolate was analysed per sampled leaf.

Fungal isolations

Fungal strains were isolated using the method described by Crous (1998). Lesions were hydrated for 2 h and attached to the inside of Petri Dish lids, with pseudothecia facing the surface of 2% MEA (Malt Extract Agar). After 24 h, single germinating ascospores exhibiting the Type F ascospore germination pattern, typical of *T. nubilosa* (Crous 1998; Crous *et al.* 2004), were transferred to new Petri dishes to obtain single-ascospore cultures. These cultures were initially incu-

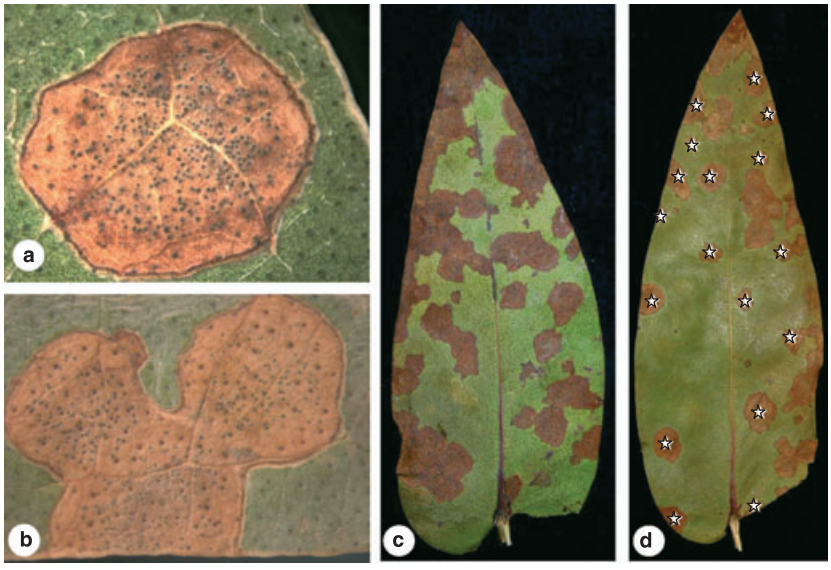

Fig. 1 *Teratosphaeria nubilosa* lesions on *Eucalyptus nitens*. (a) Single round lesion. (b) Small blotch developed from the probable coalescence of three lesions. (c) *E. nitens* leaf showing large blotches developed from the probable coalescence of multiple single lesions. (d). Seventeen lesions studied from a single *E. nitens* leaf indicated with white stars.

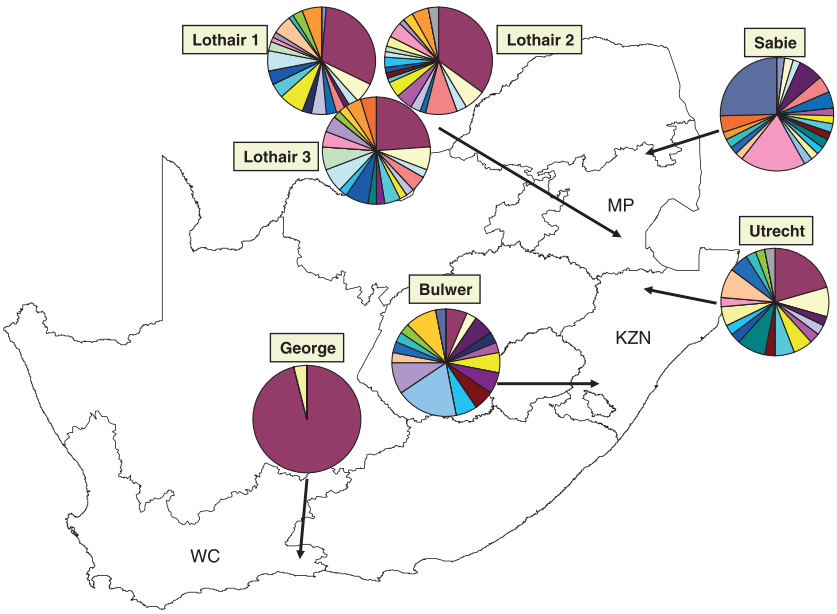

Fig. 2 Pie charts show the relative frequency of the most common 32 MLHs recovered across the seven *T. nubilosa* populations in South Africa. Arrows indicate the approximate location of the populations sampled over the Mpumalanga (MP), KwaZulu-Natal (KZN) and Western Cape (WC) provinces of South Africa.

bated at 15 °C for 2 weeks and then at 20 °C for a further 30 days. A total of 823 fungal isolates were obtained from all the microspatial and macrospatial hierarchical levels of sampling (Table 1). All cultures obtained during this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction, PCR amplification and Genescan analysis

Mycelium was scraped from the surface of actively growing cultures. This was freeze dried and ground to a fine powder using sterile metal beads on a Mixer Mill type MM 301 Retsch® tissue lyser (Retsch, Germany) for 3 min at a frequency of 30 cycles/s. DNA was isolated following the protocol described by Cortinas *et al.* (2004).

Five of the ten species-specific polymorphic microsatellite markers developed for *T. nubilosa* (Hunter *et al.* 2006) have been shown to be polymorphic for South African isolates, while the other five have been shown to be essentially monomorphic (Hunter *et al.* 2008). Thus, the microsatellites MN-3, MN-4, MN-8, MN-9 and MN-11 were selected and amplified using fluorescently labelled primers (Hunter *et al.* 2006). Polymerase Chain Reaction (PCR) mixtures and cycling conditions

Table 1 Number of *Teratosphaeria nubilosa* isolates sampled at different hierarchical levels

Province	Plantation code	Host	Hierarchical level	No. of lesions	Sample size
Mpumalanga	Lothair 1	*E. nitens*	Lesion	25	332
		E. nitens	Blotch	8	125
		E. nitens	Leaf	17	17
		E. nitens	Tree	32	32
		E. nitens	Plantation	42	42
	Lothair 2	*E. nitens*	Tree	22	22
		E. nitens	Plantation	49	49
	Lothair 3	*E. nitens*	Tree	14	14
		E. nitens	Plantation	33	33
	Sabie	*E. globulus*	Plantation	44	44
Kwazulu-Natal	Utrecht	*E. nitens*	Tree	14	14
		E. nitens	Plantation	28	28
	Bulwer	*E. nitens*	Tree	18	18
		E. nitens	Plantation	16	16
Western Cape	George	*E. globulus*	Plantation	37	37
				Total	823

were the same as those described by Hunter *et al.* (2006). Multiplex PCRs were conducted by mixing both forward and reverse primers of the microsatellite MN-3 and MN-11 in the same PCR tube. Similarly, MN-4 and MN-9 were amplified together and MN-8 was amplified in single PCR reactions. GeneScan-500 LIZ Size Standard (Applied Biosystems) was utilized to determine the allele sizes by electrophoresis on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Genescan and Genemapper software packages (Applied Biosystems) were used for further analysis of the data.

Gene and genotypic diversity

Data obtained from single lesions, blotches and single-leaf levels were ignored in further population analyses because the same clone was sampled multiple times. In this way, 474 isolates were not analysed and 349 isolates from the macrospatial hierarchical levels were retained for analysis. Allele frequencies and the number of alleles per locus were calculated for the 349 *T. nubilosa* isolates collected from seven plantations using POPGENE v 1.31 (Yeh *et al.* 1999). To compare allele frequencies among plantations, chi square tests (χ^2) were conducted on clone-corrected data sets using POPGENE v 1.31 (Yeh *et al.* 1999).

The distribution of the genotypic diversity at macrospatial levels was considered by assigning every *T. nubilosa* isolate a multilocus haplotype code, according to the observed allele sizes across the five microsatellite loci. Genotypic diversity ($\hat{G}$) was calculated for every population at the tree and plantation hierarchical levels using the formula of Stoddart & Taylor (1988). The maximum value of $\hat{G}$ is the number of individuals in the sample, which is obtained when all isolates are different (Stoddart & Taylor 1988). Due to the fact that $\hat{G}$ is dependant on the sample size, to compare populations, the percentage of the theoretical maximum of $\hat{G}$ (% $\hat{G}_{max}$) is typically calculated by dividing $\hat{G}$ by the sample size (Stoddart & Taylor 1988; Chen *et al.* 1994). However, when genotypic diversity is low or moderate and the sample size differs; the rarefaction method proposed by Grünwald *et al.* (2003) is more appropriate. Therefore, the maximum value of $\hat{G}$ was calculated by dividing $\hat{G}$ by the smaller sample size (Grünwald *et al.* 2003). As considerable differences in genotypic diversity were observed among populations, the diversity and evenness of MLHs within each population were described using Pareto distributions in GENCLONE v 2.0 (Arnaud-Haond *et al.* 2007; Arnaud-Haond & Belkhir 2007). The Pareto coefficient, β (−1 × regression slope), increases as the diversity and evenness in the sample increase, resulting in a steeper slope in the Pareto plots (Arnaud-Haond *et al.* 2007).

To test whether particular MLHs were over-represented at small scales due to re-infection in the canopy of single trees, the spatial clonal aggregation index (A_c) was calculated using GENCLONE v 2.0 (Arnaud-Haond *et al.* 2007; Arnaud-Haond & Belkhir 2007). Statistical significance of A_c was determined running 10 000 permutations using GENCLONE v 2.0 (Arnaud-Haond & Belkhir 2007). To compare the genotypic composition between single trees and trees at the plantation level, the frequency of each MLH was calculated using GENOTYPE and GENODIVE (Meirmans & van Tienderen 2004). The null hypothesis of random distribution of MLHs was tested conducting 10 000 bootstrap permutations in GENODIVE (Meirmans & van Tienderen 2004). To compare the gene diversity between single trees and trees at the plantation level, allele frequencies were also compared using chi-squared tests (χ^2) on clone-corrected datasets. When differences between the single tree and the plantation hierarchical levels were not significant ($P < 0.05$), the data were pooled to allow comparisons at higher hierarchical levels.

Multilocus haplotypes (MLHs) are likely to arise in the *T. nubilosa* population through sexual reproduction in both homothallic (selfing) and heterothallic fashion (outcrossing). The contribution of each mechanism to the general genotypic diversity was investigated calculating the P_{sex} index in GENCLONE v 2.0 (Arnaud-Haond *et al.* 2007; Arnaud-Haond & Belkhir 2007). P_{sex} estimates the probability of a given MLH encountered more than once in a sample of N units originating from distinct sexual events (outcrossing) (Arnaud-Haond *et al.* 2007). In this way, P_{sex} was calculated for the entire population and for each population separately.

Gametic disequilibrium and population differentiation

Non-random association of alleles (gametic disequilibrium) among the five microsatellite loci was tested by estimating the Index of Association (I_A) and running 1000 permutations in MULTILOCUS v 1.3 (Agapow & Burt 2001). To evaluate the genetic differentiation among *T. nubilosa* collections, each plantation was considered as a single population. Population differentiation was estimated using θ_{st} which is a modification of Wrights' F_{st} (Chen *et al.* 1994). Significant differences in θ_{st} values were tested by running 1000 randomizations in MULTILOCUS (Agapow & Burt 2001). Analyses of the gametic disequilibrium and population differentiation were conducted on clone-corrected data sets (Linde *et al.* 2002). In addition, the genotypic composition of each population was calculated and compared using GENODIVE (Meirmans & van Tienderen 2004) and 10 000 bootstrap permutations.

Results

Microspatial genetic structure

A total of 332 isolates were collected from 25 lesions that had been dissected into 20 small fragments. This represented an average of 13.3 isolates per lesion. All isolates derived from the same lesion showed the same allele for each of the five microsatellite loci. Thus, only one multilocus haplotype (MLH) was recovered from each lesion.

A total of 125 isolates were analysed from eight blotches (coalesced lesions), which represented an average of 15.6 isolates per blotch. Unlike the case for individual lesions, isolates derived from the same blotch showed different alleles across the five microsatellite loci, which allowed for the differentiation of three to seven MLHs per blotch, except in one case. In that case, only one MLH was recovered from a blotch, however, this MLH was the most frequent MLH in the population, occurring 93 times in the entire South African population and five times on a single leaf (see below). When the spatial arrangement of each haplotype was overlaid onto the original image of the blotch, an aggregate pattern of distribution of haplotypes was found, with no intermingling of MLHs across the boundaries of the discrete lesions making up the blotch. In the area where 'original' lesions coalesced to form larger blotches, the same MLHs were found and new MLHs were observed only in two cases. In these cases, two to five additional isolates derived from the same lesion fragment were analysed to search for evidence of potential outcrossing and sib-related recombinants. In all cases, the same MLHs already present in the blotch were recovered and there was no evidence of outcrossing.

Nine MLHs were distinguished from the 17 lesions sampled from a single leaf (Fig 1d). Five MLHs occurred once, two occurred twice, one occurred three times and one occurred five times on the leaf. The MLH that occurred five times on a leaf was also the most common MLH in the entire population (MLH # 1). No pattern of aggregation was observed when the spatial arrangement of the MLHs occurring more than once was overlaid onto the original image of the leaf.

Macrospatial genetic structure

Gene diversity. A total of 28 alleles were observed across the five microsatellite loci for the entire *T. nubilosa* collection (Table 2). All loci except MN-4 and MN-9 in the George population were polymorphic in all populations. The most polymorphic locus was MN-8, which exhibited 12 alleles (Table 2). The majority of alleles (64%) were present at frequencies lower than 2% in the entire data set and between 0% and 12% when subpopulations were analysed separately (Table 2). In contrast, each of the five microsatellite loci exhibited two most common alleles, for which combined frequencies ranged from 95% to 100% for the full data set and from 88% to 100% when individual populations were analysed separately (Table 2). Once the data sets had been clone-corrected and allelic frequencies among the seven populations were compared, differences were not

Table 2 Allele frequencies across the five microsatellite loci for the seven *Teratosphaeria nubilosa* populations

Locus	Allele (bp)	Population						
		Lothair 1	Lothair 2	Lothair 3	Utrecht	Bulwer	George	Sabie
MN-3	309	0.30	0.32	0.38	0.38	0.44	—	0.77
	315	0.69	0.66	0.62	0.62	0.53	0.97	0.23
	306, 312, 321*	0.01	0.01	—	—	0.03	0.03	—
MN-4	155	0.42	0.35	0.49	0.45	0.32	—	0.27
	162	0.58	0.65	0.51	0.55	0.68	1.00	0.73
MN-8	238	0.68	0.75	0.68	0.62	0.56	0.95	0.39
	319	0.30	0.17	0.23	0.33	0.44	0.03	0.61
	185, 211, 220, 230, 247, 256, 266, 304, 312, 328	0.03	0.08	0.09	0.05	—	0.03	—
MN-9	216	0.23	0.27	0.23	0.36	0.68	—	0.45
	218	0.74	0.73	0.77	0.52	0.32	1.00	0.55
	214, 220	0.03	—	—	0.12	—	—	—
MN-11	194	0.30	0.34	0.32	0.31	0.71	—	0.55
	224	0.69	0.65	0.66	0.67	0.26	0.92	0.43
	200, 230, 236	0.01	0.01	0.02	0.02	0.03	0.08	0.02

*Summary of the frequencies of the rare (<5%) alleles.

significant for any of the microsatellite loci (χ^2, $P < 0.05$).

Genotypic diversity

The distribution of the genotypic diversity at the single tree hierarchical level was examined in five plantations. Fourteen to 32 isolates were analysed from single trees which allow differentiating 11–17 MLHs per tree (Table 3). The occurrence of MLHs ranged from one to nine times on single trees. Most MLHs occurred only once on a tree. The position of each isolate in the canopy of the tree was precisely known with respect to its height, branch orientation and distance from the main stem. This allowed of the index of aggregation (A_c) to be calculated for each tree. In all cases, A_c values approached zero and were not significant ($P < 0.05$) (Table 3) indicating that the distribution of the MLHs was not aggregated in the canopy of any tree. Thus, no clear pattern of re-infection could be identified at the single tree hierarchical level.

The frequency of each MLH observed at the single-tree level was not significantly different to its frequency at the plantation level for any of the five plantations assayed ($P < 0.05$) (Table 3). In the same way, when the allelic frequencies among single trees and plantations were compared, differences were not significant for any the of microsatellite loci (χ^2, $P < 0.05$). These results justified pooling isolates obtained from single trees with those derived from the plantation level sampling for posterior analyses of the data.

A total of 60 MLHs were identified across the entire *T. nubilosa* collection. Considering the two most common alleles over the five loci, all ($2^5 = 32$) possible MLHs were recovered in the South African collection of isolates. Twenty-five MLHs (42%) occurred just once and 28 MLHs (47%) occurred between two and 10 times (Fig. 3). Conversely, MLH # 1 occurred 93 times in the entire population and it was by far the most frequent MLH in all but the Sabie and Bulwer populations (Figs 2 and 3). P_{sex} values were not significant in 28 (80%) of 35 MLHs ($P < 0.05$), which

Table 3 Number of *Teratosphaeria nubilosa* isolates and MLHs recovered at the tree and plantation hierarchical levels. Spatial clonal aggregation index (A_c), statistical significance of A_c P (A_c) after 10 000 permutations using GENECLONE v 2.0 (Arnaud-Haond & Belkhir 2007), genotypic diversity ($\hat{G}$) (Stoddard & Taylor 1988), % of the maximum theoretical value of $\hat{G}$ (% $\hat{G}_{max}$) and probability of random distribution of MLHs between the tree and plantation hierarchical levels after 10 000 bootstrap permutations using GENODIVE (Meirmans & van Tienderen 2004) are indicated at the end of each row

Plantation code	Hierarchical level	No. of isolates	No. of MLHs	A_c	P (A_c)	$\hat{G}$	% $\hat{G}_{max}$	P
Lothair 1	Tree	32	17	0.074	0.143	8.53	26.7	0.86
	Plantation	42	21			8.82	27.6	
Lothair 2	Tree	22	12	−0.060	0.814	7.12	34.4	
	Plantation	49	23			7.57	32.3	0.29
Lothair 3	Tree	14	11	0.040	0.242	9.8	70.0	
	Plantation	33	19			10.18	72.6	0.30
Utrecht	Tree	14	11	0.029	0.364	8.91	63.6	
	Plantation	28	20			13.52	96.6	0.93
Bulwer	Tree	18	15	0.030	0.301	11.56	36.1	
	Plantation	16	12			9.84	77.9	0.98
George	Plantation	37	6			—	—	
Sabie	Plantation	44	22			—	—	

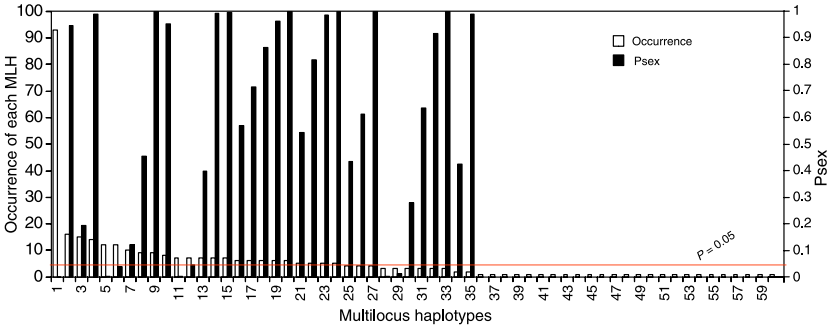

Fig. 3 Occurrence of the sixty MLHs in the entire South African population (white) and P_{sex} probabilities associated to each MLH (black).

Table 4 Genotypic diversity ($\hat{G}$) (Stoddart & Taylor 1988), % of the maximum theoretical value of $\hat{G}$ (% $\hat{G}_{max}$) per population and test of pairwise population differentiation in MLHs composition. Probabilities were obtained after running 10 000 bootstrap permutations in GENODIVE (Miermans & van Tienderen 2004)

Population	$\hat{G}$	$\hat{G}_{max}$	Lothair 1	Lothair 2	Lothair 3	Utrecht	Bulwer	George
Lothair 1	9.25	27.2						
Lothair 2	8.25	24.3	0.16					
Lothair 3	12.77	37.6	0.57	0.53				
Utrecht	15.21	44.7	**0.03**	**0.04**	0.06			
Bulwer	13.14	38.6	**<0.001**	**<0.001**	**<0.001**	**0.02**		
George	1.41	4.2	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	
Sabie	8.72	25.6	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Values in bold: significant differences ($P < 0.05$).

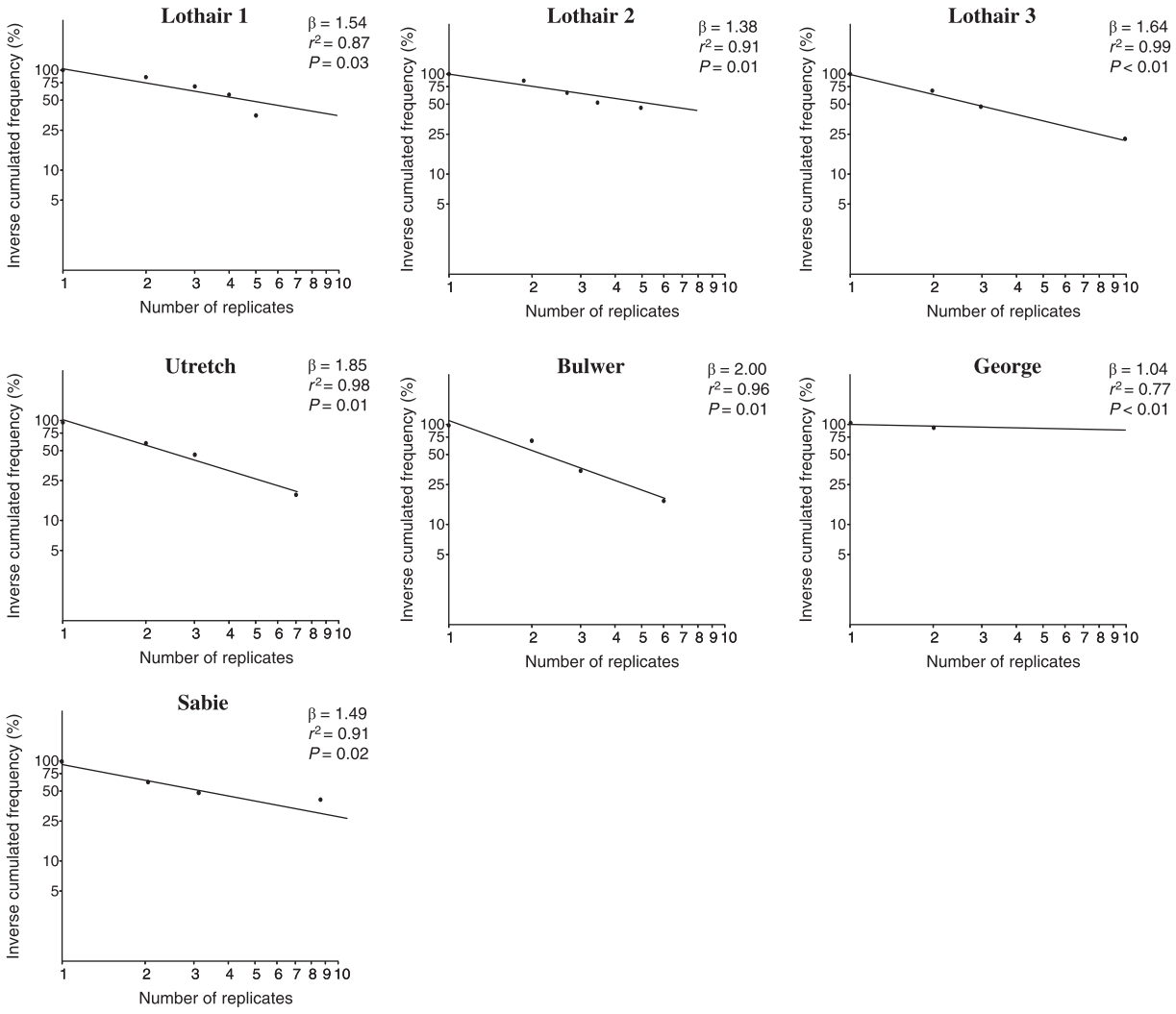

Fig. 4 Pareto plots showing the clonal distribution due to selfing across the seven *T. nubilosa* populations. The Pareto coefficient (β), the correlation coefficient (r^2) and significance of the regression (P) are shown for each population.

occurred more than once in the entire population (Fig. 3). These results were consistent with the values obtained when P_{sex} was calculated for single populations separately (data not shown). Therefore, most MLHs could have arisen from different random outcrossing events.

Table 5 Pair-wise comparisons of population differentiation (θ_{st}) (above diagonal) and *P* values (below diagonal)

	Lothair 1	Lothair 2	Lothair 3	Utrecht	Bulwer	George	Sabie
Lothair 1		−0.011	−0.028	−0.037	−0.011	0.180	−0.024
Lothair 2	0.69		−0.024	−0.015	−0.004	0.115	0.013
Lothair 3	0.67	0.50		−0.028	0.005	0.138	−0.005
Utrecht	0.99	0.16	0.92		−0.014	0.177	−0.025
Bulwer	0.66	0.42	0.41	0.83		**0.213**	−0.025
George	0.15	0.87	0.79	0.34	**<0.01**		**0.221**
Sabie	0.96	0.14	0.37	0.94	0.934	**<0.01**	

Values in bold: significant values of θ_{st} after 1000 randomizations on multilocus.

Between six and 23 MLHs were recovered from single plantations (Table 3). Genotypic diversity ($\hat{G}$) ranged from 4.2% of the theoretical maximum in the George population to 44.7% in the Bulwer population (Table 4). The distribution of the diversity and evenness of MLHs in each population were quite different among populations (Fig. 4). While a skewed distribution of MLHs was observed in the George population ($\beta = 1.04$), where one MLH occurred in 86% of the cases, a more even distribution was observed in the Bulwer and Utrecht populations ($\beta = 2.00$ and $\beta = 1.85$, respectively), where most of the MLHs occurred once or twice (Figs 2 and 4). Intermediate distributions were observed in Lothair and Sabie populations ($\beta = 1.38$ to 1.64), where most MLHs showed comparable frequencies except one or two more frequent MLHs (Figs 2 and 4).

Gametic disequilibrium and population differentiation

The I_A value of the clone-corrected dataset was −0.028 for the entire *T. nubilosa* population and the null hypothesis of random association of alleles could not be rejected ($P < 0.05$). Similarly, when the I_A was estimated for each plantation separately, values approached zero and were not statistically significant, suggesting random association of alleles.

Pairwise comparisons among populations were not significant with values of θ_{st} approaching zero (θ_{st} = −0.037–0.013) except for the George population (Table 5). These results suggest that there is no genetic differentiation among *T. nubilosa* populations on these plantations. Moderate population differentiation (θ_{st} = 0.115–0.223) was observed when the George population was compared with the other populations. This θ_{st} value was significant in only two cases, i.e. when the George population was compared with the Bulwer and the Sabie populations (Table 5). Pairwise comparisons among populations, using the frequencies of the MLHs, were significant for all pairs, except the three Lothair populations (Table 4). Thus, while allelic frequencies (gene pool) are comparable for all populations in South Africa, excluding the one from George, the combination of alleles into MLHs is not even.

Discussion

The results of this study appeared at the outset to be contradictory. Thus, apparently at the microspatial level the pathogen reproduces only by selfing, but at the macrospatial level it is clearly outcrossing. This could have been explained by introduced genetic diversity and crossing events that predate the introduction of this pathogen into South Africa. However, on closer examination, it is clear that each locus has two predominant alleles and every possible combination of these alleles into MLHs is represented in the South African population. It is highly unlikely that this situation would have arisen from a founding population and is more probably due to outcrossing in the South African population from a limited number of introduced genotypes. This study exemplifies the fact, in order to fully understand patterns of establishment of introduced haploid pathogens, it is essential to have knowledge of their life cycle and mating system in order to appropriately interpret data relating to their allelic and genotypic diversity.

At the microspatial scale, one of the most important considerations in this study was to determine whether single or multiple genotypes might occur in discrete lesions and blotches. In all 25 discrete lesions assayed, only one MLH was recovered per lesion. These results show that individual discrete *T. nubilosa* lesions on *E. nitens* leaves result from single infection events. These single lesions are most likely the product of infection by single ascospores, which are the only infective propagules known for the fungus in nature (Park & Keane 1982; Park 1988). Because fungal strains were isolated from mature ascomata that developed on single lesions, our results also confirm previous findings (Park & Keane 1982) that single *T. nubilosa* individuals can produce sexual structures and ascospores in a homothallic fashion (selfing). Because only one MLH was

recovered per lesion in a relatively large sample, the results also suggest that fertilization of proto-pseudothecia by distant sourced spermatia is not a frequent phenomenon in this pathogen.

Between three and seven MLHs were recovered from blotches in most cases. As suggested previously by Park (1988), these results show that blotches originate from the coalescence of single lesions. The fact that we found only one MLH per lesion was unexpected, because a previous study conducted on the same host (Hunter *et al.* 2008) showed the presence of four MLHs in a single lesion. In that study, only a single large lesion was utilized to sample multiple pseudothecia and that lesion was not described in detail. These contradictory results most likely arise from the fact that the lesion analysed by Hunter *et al.* (2008) was most likely a blotch arising from multiple small lesions and not a single discrete lesion. Clearly, great care must be taken in characterizing lesions caused by *T. nubilosa*. Often times, lesions (Fig. 1a) can incorrectly reflect blotches (Fig. 1b and c) that might have arisen from more than one infection event. The presence of multiple individuals within lesions has been reported for several plant pathogens (Müller *et al.* 1997; Linde *et al.* 2002) and it is unclear whether these large lesions constitute blotches of coalesced single lesions or discrete lesions. It is evident that failure to understand whether leaf lesions constitute one or more infection events, could significantly alter the interpretation of data, as clearly appears to have occurred in the study of Hunter *et al.* (2008).

An aggregate pattern of distribution of haplotypes was found in all blotches examined and there was no evidence of intermingling of MLHs across the boundaries of the discrete lesions making up the blotches. Although multiple MLHs were found to co-exist in very close proximity, there was no evidence of outcrossing. Our results contrast with those observed for the heterothallic fungus *M. graminicola*, where the one or two genotypes occupying the majority of the lesion were speckled by several other genotypes, typically carrying complementary mating type genes (Linde *et al.* 2002). Therefore, the results obtained in this study allow us to discard the hypothesis that more than one *T. nubilosa* genotype is required for sexual reproduction to occur in a heterothallic fashion by either somatic contact or fertilization.

The occurrence of multiple MLHs on the same leaf suggests that each lesion is the product of infection by a different ascospore and that re-infection on the same leaf is not a significant factor. It has been shown for different *Mycosphaerella* pathosystems that propagules (mostly asexual conidia) produced in old lesions are able to re-infect the same leaf or neighbouring leaves on the same plant (Linde *et al.* 2002; Feau *et al.* 2005; Hayden *et al.* 2005). In contrast, we found a high number of MLHs on individual trees (even on individual leaves and blotches) and the index of aggregation (A_c) approached zero and it was not significant in all cases. We conclude that if re-infection does occur at small scales in nature, this most probably does not play a significant role in structuring the genetic diversity at the single-tree level.

At macrospatial scales, moderate $\hat{G}_{max}$ values were observed in all South African populations of *T. nubilosa* (Stoddart & Taylor 1988; Milgroom 1996). The index of association observed for the entire population suggests outcrossing and sexual reproduction in a heterothallic fashion (Milgroom 1996; Taylor *et al.* 1999). In the same way, the detection of all possible combinations of the two most common alleles into $2^5 = 32$ MLHs and non-significant P_{sex} values in most cases provide evidence of regular meiotic recombination (Milgroom 1996). Therefore, despite our efforts to find evidence of outcrossing in nature (and delivering no direct evidence for it), we conclude that *T. nubilosa* has undergone outcrossing since its introduction into South Africa. In this way, a mixed model of outcrossing and selfing is the most likely situation. This would be similar to the case for other plant pathogens such as *Sclerotinia sclerotiorum*, *Armillaria gallica* and *Phytophtora infestans* (Milgroom 1996; Taylor *et al.* 1999).

Studying the contribution of outcrossing vs. selfing to genotypic diversity in a haploid homothallic fungus such as *T. nubilosa* is more complex than it is for heterothallic haploid fungi or generally in diploid populations (Milgroom 1996; Halkett *et al.* 2005; Arnaud-Haond *et al.* 2007). In contrast to the vascular plants, Ascomycetes fungi have ephemeral diploid stages and zygotic stages from which to estimate gene associations generated by outcrossing, are consequently not available (Gregorius 2005). Thus, while our data clearly shows that selfing takes place at microspatial scales – as discussed above – P_{sex} values suggest that most MLHs could have potentially arisen from distinct outcrossing events at the population level. The Pareto plots showed that one or two MLHs were dominant in most populations suggesting that selfing is common in nature. Additionally genotypic frequencies were significantly different between populations, indicating that particular MLHs are more dominant in certain populations and that this dominance is maintained through selfing.

No differentiation in the gene frequencies was observed between populations located in Mpumalanga and KwaZulu-Natal Provinces of South Africa. Although these plantations are approximately 570 km apart, *Eucalyptus* plantations are relatively continuous across this area. The presence of shared alleles at even frequencies among plantations suggests that gene flow

via natural wind-dispersal of airborne ascospores (Park 1988) occurs between these *Eucalyptus* growing areas. This suggests that both the continuity in the geographical distribution of the host as well as wind dispersal of ascospores have been important in maintaining these populations undifferentiated as suggested for other *Mycosphaerella* spp. (Linde *et al.* 2002; Rivas *et al.* 2004; Hayden *et al*. 2005).

Interestingly, the George population was genetically differentiated. The George collection was obtained from *E. globulus* as opposed to most other populations originating from *E. nitens*. The George population is geographically separated by more than 800 km from the areas where the other populations were collected and *Eucalyptus* plantations in the area between these sites are not common (Fig. 2). Therefore, our results could equally be explained by host preferences or geographical isolation (Hayden *et al.* 2005). The Sabie population was also collected from *E. globulus*, however, these two populations (Sabie and George) were significantly different (θ_{st} = 0.221, $P < 0.01$), suggesting a dominant influence of geographic isolation. Moreover, most of the MLHs collected in Sabie from *E. globulus* were shared with the populations obtained from *E. nitens* in the same Province (suggesting no host preferences). We therefore hypothesize that the George population may have differentiated from the restricted gene flow between this most distant population and those located in the main *E. nitens* growing areas, rather than by the presence of host preferences among individuals in the *T. nubilosa* population.

A high number of alleles (28) were found for the *T. nubilosa* population in the present study and this was consistent with the results of a previous study of the pathogen in South Africa (Hunter *et al.* 2008). Interestingly, 64% of these alleles were present at frequencies lower than 2%. These low-frequency alleles could have originally been introduced at a very low frequency or they could have been introduced more recently. Alternatively, because plant pathogen populations have the potential to evolve over time (McDonald & Linde 2002; Zhan & McDonald 2004), new alleles are expected to originate from mutation in the new environment and over time they can accumulate in the population, albeit at low frequencies (Zhan & McDonald 2004). Surveys in the native geographical range of *T. nubilosa* will be required in the future to ascertain whether these low-frequency alleles are 'private alleles' found only in South Africa and thus have emerged there or whether they are present in Australia (native geographic range) and were introduced into South Africa.

Unlike the situation in Brazil, Portugal, Spain, Tanzania and Uruguay, where non-native *T. nubilosa* populations are represented by single clones (Hunter *et al.* 2009; Pérez *et al.* 2009), a total of 60 MLHs were observed in South Africa. Genetic diversity of introduced populations is known to be affected by 'propagule pressure', which includes both the number of individuals introduced and the number of introductions (Ficetola *et al.* 2008). Although, the high number of MLHs in South Africa could have originated from either a massive introduction of the pathogen in the early 1920s, when commercial plantations of Eucalypts were initiated in South Africa (Lundquist & Purnell 1987) or through multiple introductions during the course of the last 80 years, most MLHs were a combination of the two predominant alleles. Therefore meiotic recombination of limited introduced genotypes through outcrossing suggested by our data better explains the large numbers of observed MLHs currently in South Africa.

The results of this study show that *T. nubilosa* has a mixed mating system and therefore have a high evolutionary potential *sensu* McDonald & Linde (2002) in South Africa. This will make breeding for resistance more difficult in South Africa which is the only economically viable management strategy for MLD (Wingfield 2003; Carnegie 2007). The high levels of gene flow via wind dispersal of ascospores among plantations renders the implementation of quarantine measures between regions within South Africa futile. However, the reinforcement of quarantine measures with respect to other countries is required to avoid the introduction of new genotypes of *T. nubilosa* or potentially more damaging species of *Teratosphaeria* such as *T. cryptica* (Park 1988; Carnegie 2007).

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