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Cryptic species, native populations and biological invasions by a eucalypt forest pathogen

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Abstract

Human-associated introduction of pathogens and consequent invasions is very evident in areas where no related organisms existed before. In areas where related but distinct populations or closely related cryptic species already exist, the invasion process is much harder to unravel. In this study, the population structure of the *Eucalyptus* leaf pathogen *Teratosphaeria nubilosa* was studied within its native range in Australia, including both commercial plantations and native forests. A collection of 521 isolates from across its distribution was characterized using eight microsatellite loci, resulting in 112 multilocus haplotypes (MLHs). Multivariate and Bayesian analyses of the population conducted in STRUCTURE revealed three genetically isolated groups (A, B and C), with no evidence for recombination or hybridization among groups, even when they co-occur in the same plantation. DNA sequence data of the ITS (*n* = 32), β-tubulin (*n* = 32) and 27 anonymous loci (*n* = 16) were consistent with microsatellite data in suggesting that *T. nubilosa* should be considered as a species complex. Patterns of genetic diversity provided evidence of biological invasions by the pathogen within Australia in the states of Western Australia and New South Wales and helped unravel the pattern of invasion beyond Australia into New Zealand, Brazil and Uruguay. No significant genetic differences in pathogen populations collected in native forests and commercial plantations were observed. This emphasizes the importance of sanitation in the acquisition of nursery stock for the establishment of commercial plantations.

Keywords: *Eucalyptus*, forest pathogen, microsatellite, Mycosphaerella leaf disease, population genetics, STRUCTURE, *Teratosphaeria nubilosa*

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Introduction

Commercially propagated plants are generally cultivated outside their areas of endemism, and they have often been accompanied by the accidental introduction of many of their natural pests and diseases (Fry *et al.* 1993; Stukenbrock & McDonald 2008; Mitchell *et al.* 2010). This is also the case for *Eucalyptus* spp., a native to Australia, their introduction worldwide for forestry and amenity plantings has been accompanied by the introduction of many important pests and pathogens (Wingfield *et al.* 2008). *Eucalyptus* spp. are also grown for pulp and timber production in commercial plantations in Australia (Potts *et al.* 2004; Nichols *et al.* 2010). To establish these commercial plantations, certain *Eucalyptus* species, in particular, *E. globulus* and *E. nitens*, have been taken from their endemic areas of distribution and introduced into new, formerly isolated, biogeographical areas within Australia (Potts *et al.* 2004).

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These plantings into new biogeographical areas have also been faced with the appearance of their associated native pathogens (Maxwell *et al.* 2001; Barber *et al.* 2008; Jackson *et al.* 2008). This suggests that the natural geographical distribution of *Eucalyptus* pathogens has been concurrently modified and expanded via human intervention within Australia.

The introduction of pathogens into new areas has the potential to alter the species composition of resident native fungal communities (Desprez-Loustau *et al.* 2007). Likewise, at the population level, the introduction of novel genotypes into new areas has the potential to alter the genetic diversity of native fungal populations (Desprez-Loustau *et al.* 2007). Recombination and hybridization of native and introduced pathogens are considered a threat to both native forest ecosystems and commercial plantations (Gilbert 2002; Brasier 2008). Because *Eucalyptus* commercial plantations are often located close to native stands (Burgess *et al.* 2006; Barber *et al.* 2008), Australia represents an excellent model system to study the possible influence of anthropogenic forestry activities in the native fungal communities and populations (Burgess *et al.* 2006).

Application of molecular markers to assess the genetic diversity of populations has made it possible to ascertain the centres of origin (Burnett 2003; Hayden *et al.* 2003; Zhan *et al.* 2003; Stukenbrock *et al.* 2007; Zhou *et al.* 2007) and the pathways of introduction of several plant pathogens (Fry *et al.* 1993; Brown & Hovmøller 2002). Species in native populations are typically genetically diverse because of the historical accumulation of novel mutations (Burnett 2003; Stukenbrock *et al.* 2007). Biological invasions, including those by plant pathogens, are usually accompanied by a reduction in their population diversity (Dlugosch & Parker 2007; Suarez & Tsutsui 2008). This is explained by the 'founder effect', where a reduced number of individuals carrying a fraction of the diversity of the original population, establish a new population into a new area (Nei *et al.* 1975; Ficetola *et al.* 2008). However, multiple introductions are common in biological invasions (Dlugosch & Parker 2007) including plant pathogens (Smart & Fry 2001; Dutech *et al.* 2010), and when organisms are introduced multiple times in a certain area, introduced populations can be as diverse (or even more diverse) than native populations (Kolbe *et al.* 2004; Estoup & Guillemaud 2010; Mitchell *et al.* 2010).

A very common disease observed in both native and commercial eucalypt plantations is Mycosphaerella leaf disease (MLD) (Crous 1998; Carnegie 2007b). This disease is caused by more than 120 species belonging to the families *Mycosphaerellaceae* and *Teratosphaeriaceae* (Ascomycetes) (Crous 1998; Burgess *et al.* 2007; Carnegie *et al.* 2007; Crous *et al.* 2007). The leaf pathogen *Teratosphaeria nubilosa* is one of the most aggressive species causing MLD (Carnegie 2007b; Hunter *et al.* 2009), resulting in premature leaf abscission and reduction in tree growth in commercial plantations, which can lead to significant economic losses (Lundquist & Purnell 1987; Carnegie & Ades 2003; Milgate *et al.* 2005; Andjic *et al.* 2010). *Teratosphaeria nubilosa* is native to Australia, and it has been accidentally introduced into many countries including Brazil, Ethiopia, Kenya, New Zealand, Portugal, Spain, South Africa, Tanzania, Uruguay and Zambia (Dick 1982; Hunter *et al.* 2008, 2009; Pérez *et al.* 2009a,b).

It has been suspected that *T. nubilosa* might represent a species complex, because substantial intraspecific morphological and molecular variation has been observed in a global sample of isolates (Crous *et al.* 2006; Hunter *et al.* 2009). The presence of complexes of cryptic species is common throughout the tree of life (Bickford *et al.* 2007), and in fungi in particular, because fungal morphology is simpler than that of macro-organisms, making the distinction between recently diverged species or sibling species very difficult (Taylor *et al.* 2006). One way to recognize species within species complexes is to sequence multiple loci across the genome, an approach also known as multilocus sequence typing (MLST) (Taylor & Fisher 2003). MLST also has the advantage that it can be used to test the genealogical concordance phylogenetic species concept (GCPSC) (Taylor *et al.* 2000). Here, the concordance of genealogies across unlinked loci is used to provide an indication of separate evolutionary histories of the individuals (or populations) being compared, indicating species barriers if opportunities for mating exist (Taylor *et al.* 2000). This concept has been widely applied and has allowed the discovery of numerous cryptic species in genera of animal and human pathogens (Taylor *et al.* 2006), as well as plant pathogenic and endophytic fungi (O'Donnell *et al.* 2004; Andjic *et al.* 2007; Grünig *et al.* 2007; Pavlic *et al.* 2009).

The population biology of *T. nubilosa* has been intensely studied in South Africa, where it is an introduced pathogen (Hunter *et al.* 2008; Pérez *et al.* 2010). However, the population biology of the pathogen has not been carefully considered in Australia where it is native (Hunter *et al.* 2008) occurring in most regions of the country (Maxwell *et al.* 2001; Mohammed *et al.* 2003; Carnegie 2007a; Barber *et al.* 2008). In this study, we examined the population structure of *T. nubilosa* in Australia using eight microsatellite markers and DNA sequence data. An initial step was to determine whether *T. nubilosa* represents a single species or a complex of genetically isolated cryptic species. This was achieved by applying two independent analyses: (i) Bayesian and multivariate analyses of microsatellite

derived data and (ii) phylogenetic reconstructions using DNA sequence data from 29 loci. Once isolates had been sorted into population/phylogenetic groups, we (i) determined the most likely centre of origin and the human-introduced area of distribution for each population/phylogenetic group, (ii) tested whether recombination or hybridization has occurred recently among native and human-introduced pathogen populations and (iii) compared the genetic structure of pathogen populations colonizing native forests and adjacent commercial plantations.

Materials and methods

Sampling and isolations

Most sampling was carried out in Eastern Australia, specifically in the states of New South Wales (NSW), Victoria and Tasmania during October and November 2008. This geographical area is considered to be the native range of *T. nubilosa* (Hunter *et al.* 2009). Samples of leaves with typical *T. nubilosa* symptoms were taken from *Eucalyptus dunnii, E. globulus, E. nitens* and *E. nitens* × *E. globulus* hybrids in both plantations and research trials (Table 1, Fig. 1). Additionally, trees in native *E. globulus* forests (including its sub-species) in Victoria and Tasmania were sampled (Table 1, Fig. 1). Furthermore, sampling included *T. nubilosa* populations from Western Australia where the pathogen is known to have been introduced, most probably after 1994 (Maxwell *et al.* 2001). *Teratosphaeria nubilosa* isolates collected in Brazil, New Zealand and Uruguay were also included for comparative purposes and because these populations have not been considered in any previous population study (Pérez *et al.* 2009a,b) (Table 1).

In all cases, sampling was performed by randomly collecting one to five leaves per diseased tree. Fungal isolations were conducted using the method described by Crous (1998). All fungal strains obtained in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Leaf lesions collected in Victoria and Tasmania often contained pseudothecia that did not release ascospores because they were either immature or inordinately old. This hampered the isolation process for some populations. To overcome this limitation, fungal DNA was extracted directly from lesions for those sites where the sample size was considered insufficient for analysis. In this way, populations from Victoria and Tasmania were derived from 111 DNA samples and 59 single-ascospore cultures. Thus, a total of 390 fungal cultures and 111 DNA samples were analysed, making up a total of 521 *T. nubilosa* samples (Table 1).

DNA extraction, loci amplification and allele size determination

Fungal DNA was extracted either directly from lesions or from actively growing mycelium arising from monosporic cultures. DNA was extracted from lesions using the ZR-96 Fungal/Bacterial DNA Kit™ (Zymo Research, USA), following the manufacturer's protocols. DNA extraction from mycelium was conducted as described in Pérez *et al.* (2010).

Eight microsatellite loci (MN-1, MN-2, MN-3, MN-7, MN-9, MN-10, MN-11 and MN-14) were amplified using the fluorescently labelled *T. nubilosa*-specific primers developed by Hunter *et al.* (2006). PCRs using the primer pairs MN-4 and MN-8 were not successful for all isolates in the collection and were therefore not used. PCR cycling conditions were the same as those used by Hunter *et al.*(2006). The allele size of each locus was determined on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). GENESCAN and GENEMAPPER software packages (Applied Biosystems) were used for further analyses of the data.

Isolate grouping

A matrix of data including the allele size for each of the eight loci in all 521 isolates was constructed. To avoid bias in subsequent analyses because of repeated genotypes (Grünwald *et al.* 2003), the matrix of data were clone-corrected using GENCLONE version 2.0 (Arnaud-Haond & Belkhir 2007), retaining only one representative isolate per genotype for the analyses. A total of 112 multilocus haplotypes (MLHs) were obtained from the entire collection of 521 *T. nubilosa* isolates (see results), and therefore, most analyses were then carried out on these 112 MLHs (clone-corrected data sets). An exception was made when comparing genetic diversity among native forests and adjacent commercial plantations because six MLHs were shared among those types of forests. Therefore, these six MLHs were included in each data subset totalling 118 MLHs on those comparisons.

Populations were defined on the basis of their geographical origin. Two different clustering methods were used to explore the genetic relationships among isolates: multivariate analyses and Bayesian algorithms. These methods were chosen because they analyse only the matrix of microsatellite data, therefore avoiding biases in a priori grouping of isolates (Pritchard *et al.* 2000). Multivariate analyses included principal component analysis (PCA), which was performed using XLSTAT (Microsoft Corporation). The Pearson correlation coefficient was used to perform the multidimensional scaling. For Bayesian analysis, clone-corrected data sets were analysed in STRUCTURE version 2.2 (Pritchard *et al.* 2000)

Table 1 *Teratosphaeria nubilosa* isolates and DNA samples used

Country	State	Site no.	Locality	Native forest/ plantation	Number of isolates/ DNA samples	Number of multilocus haplotypes	Host	GPS coordinates/ references
Australia	New South Wales	1	Tabulam	Plantation	32	12	*Eucalyptus globulus* spp. *maidenii*	28° 50′ S, 152° 29′E
		2	Tabulam	Plantation	29	10	*Eucalyptus dunnii* (native)*	28° 50′ S, 152° 29′E
		3	Callaghna creek	Plantation	45	7	*Eucalyptus globulus bicostata*	31° 51′ S, 152° 03′E
		4	Rappville	Plantation	23	12	*E. dunnii* (native)*	
	Tasmania	5	Orford	Native	8	7	*Eucalyptus globulus globulus*	42° 34′ S, 147° 52′E
		6	Three thumbs	Native	10	9	*E. g. globulus*	42° 36′ S, 147° 52′E
		7	Forester peninsula	Native	2	1	*E. g. globulus*	42° 58′ S, 147° 55′E
		8	Gevestone	Native	19	14	*E. g. globulus*	43° 09′ S, 146° 50′E
		9	Meunna	Plantation	1	1	*E. g. globulus*	41° 04′ S, 145° 28′E
		10	Takone	Plantation	8	5	*E. g. globulus*	41° 01′ S, 144° 51′E
			Takone	Plantation	8	7	*E. g. globulus* × *Eucalyptus nitens* hybrids	41° 01′ S, 144° 51′E
		11	Research trial	Plantation	21	5	*E. g. globulus*	
	Victoria	12	Boombala†	Native	11	6	*E. globulus*	36° 55′ S, 149° 45′E
		13	Genoa	Native	11	2	*E. globulus*	37° 32′ S, 149° 23′E
		14	Gori	Native	10	5	*E. globulus*	38° 46′ S, 143° 32′E
		15	Johanna	Native	3	1	*E. globulus*	38° 44′ S, 143° 27′E
		16	Murrongowar	Native	2	2	*E. globulus*	37° 36′ S, 148° 42′E
		17	Cann River	Native	1	1	*E. globulus*	37° 33′ S, 149° 09′E
		18	Pinnak	Native	1	1	*E. globulus*	37° 31′ S, 148° 25′E
		19	Nowa Nowa	Plantation	1	1	*E. globulus*	37° 40′ S, 148° 18′E
		20	Kinglake	Plantation	18	7	*E. g. globulus*	37° 27′ S, 145° 12′E
		21	Gellibrand	Plantation	13	3	*E. g. globulus*	38° 33′ S, 143° 32′E
		22	Briagolong	Plantation	22	8	*E. g. globulus*	37° 48′ S, 147° 03′E
	Western Australia	23	Perup	Plantation	20	2	*E. g. globulus*	34° 11′ S, 116° 28′E
		24	Perup	Plantation	16	1	*E. g. globulus*	34° 10′ S, 116° 30′E
		25	Leighton	Plantation	30	2	*E. g. globulus*	34° 35′ S, 118° 34′E
		26	Geddes	Plantation	5	1	*E. g. globulus*	34° 48′ S, 118° 14′E
		27	Narrikup	Plantation	2	1	*E. g. globulus*	34° 49′ S, 117° 45′E
		28	Rocky Gully	Plantation	1	1	*E. g. globulus*	34° 29′ S, 116° 58′E
		29	Beedelup	Plantation	7	1	*E. g. globulus*	34° 22′ S, 115° 57′E
Brazil	Rio Grande do Sul		Osorio	Plantation	42	1	*E. g. globulus*	Pérez *et al.* (2009b)
New Zealand	North Island		Rotorua	Plantation	53	1	*E. g. globulus*	
Uruguay			Multiple plantations	Plantation	46	1	*E. g. globulus*	Pérez *et al.* (2009a)
				Total	521			

*_Eucalyptus dunnii_ is native in this area; however, the sample collection was performed on a _E. dunnii_ plantation.

†Actually Boombala is located in New South Wales by the border with Victoria; however, this population was analysed with isolates from Victoria.

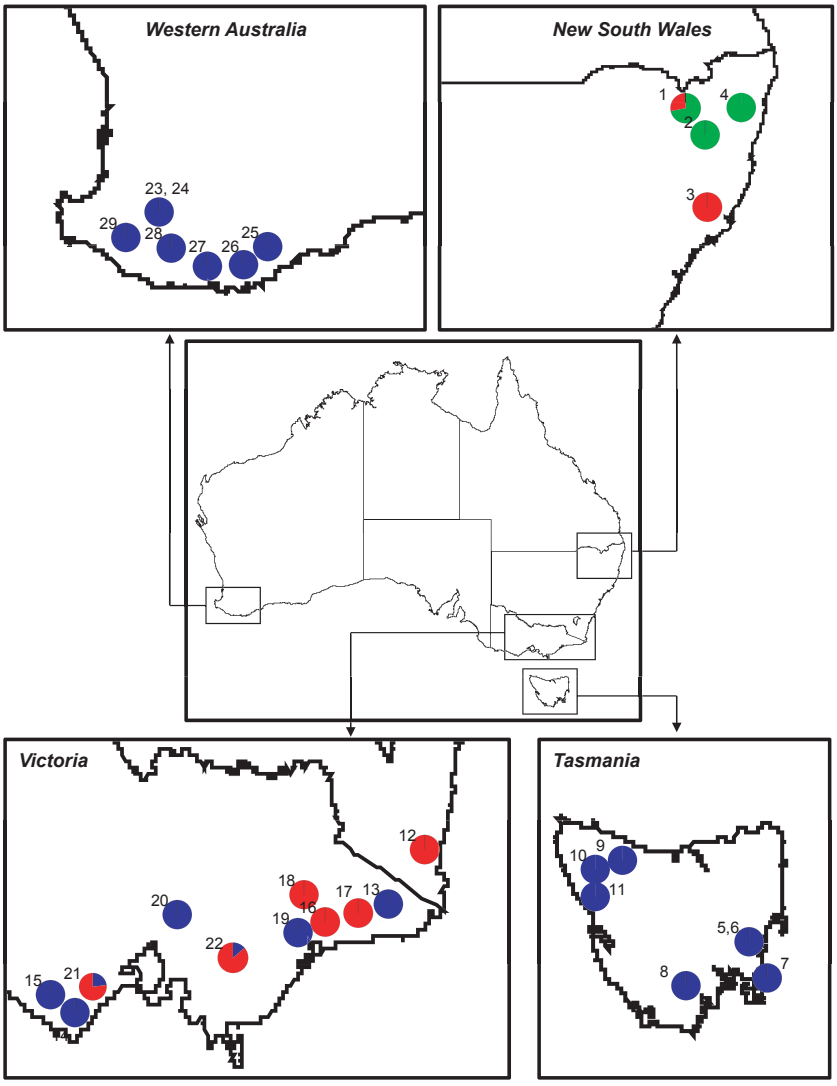


Fig 1. *Teratosphaeria nubilosa* populations collected from native forests and *Eucalyptus* plantations in 29 locations in Australia. Pie charts show the relative proportion of isolates belonging to Group A (green), Group B (blue) and Group C (red) in each location. See Table 1 for reference numbers of locations.

to determine the optimal number of populations (*K*) using a Bayesian Markov chain Monte Carlo (MCMC) clustering algorithm. A total of 200 000 iterations were performed after a burn-in period of 20 000, where *K* values ranged from 1 to 10. The 'nonadmixture model' was chosen as the ancestry model and the independent allele frequency model for setting the parameter λ (Pritchard *et al.* 2000). For the best choice of *K*, the log likelihood values of *K*, *L* (*K*) (Pritchard *et al.* 2000) and *ΔK* values (Evanno *et al.* 2005) were plotted against the ranging *K* values. Once the best value of *K* had been determined, isolates were assigned to populations using STRUCTURE version 2.2 (Pritchard *et al.* 2000). To further investigate potential population substructuring within each *K* group, new sets of MCMC iterations were performed in STRUCTURE version 2.2 (Pritchard *et al.* 2000). The procedure was essentially the same as described earlier with the only exception that the 'admixture model' was chosen as the ancestry model because recombination (admixture) is expected to take place within each *K* group.

DNA sequencing and phylogenetic reconstructions

To test hypotheses regarding the existence of cryptic species, 32 or 16 isolates for respective analyses were chosen to represent groups identified in STRUCTURE and PCA. These isolates also included the ex-epitype culture of *T. nubilosa* (CMW 3282 = CBS 116005), which was collected in Victoria, Australia (Crous *et al.* 2004). Twenty-nine loci were targeted, including the internal transcribed spacers (ITS1 and ITS2) ($n = 32$) [primers; ITS-1 and ITS-4 (White *et al.* 1990)], portions of the β-tubulin-1 and β-tubulin-2 gene regions ($n = 32$) [primers; Bt-1a, Bt-1b, Bt-2a and Bt-2b; (Glass & Donaldson 1995)] and 27 polymorphic anonymous loci from the

genome of *T. nubilosa* ($n = 16$) [using primers developed by (Pérez 2010)]. PCR conditions and sequencing reactions were the same as those used by Pérez *et al.* (2009a) and Pérez (2010). Amplicons were sequenced in both directions using the same primers as those used for the initial amplification.

DNA sequences were analysed, edited and aligned in MEGA version 3.1 (Kumar *et al.* 2004). All sequences were deposited in GenBank (HQ130795-HQ131328). For parsimony analysis, heuristic searches were conducted in PAUP version 4.0b10 (Swofford 2002) using tree-bisection–reconnection (TBR) as the branch swapping algorithm and the following parameters: MulTrees were 'on', starting trees were obtained via stepwise addition of 100 random replicates, remaining trees were added in single sequence fashion, and MaxTrees were set at 100. Indels were treated as a single evolutionary event and coded as 0 and 1 according to absence–presence, respectively. Finally, 1000 bootstrap replicates were conducted to determine confidence levels of branching points in the phylograms. Phylograms produced by PAUP were visualized in MEGA version 3.1 (Kumar *et al.* 2004).

Phylogenetic reconstructions were based on 29 loci, where each locus was initially analysed separately. Afterwards, we applied the GCPSC (Taylor *et al.* 2000) to recognize independent evolutionary lineages. Phylogenies were considered concordant where identical nodes were consistently observed and supported by significant bootstrap values. A clade was considered as an independent evolutionary lineage when satisfied both of the following criteria: (i) it was present in the majority of the single-locus genealogies and (ii) it was not contradicted in any other single-locus genealogy at the same level of support (Dettman *et al.* 2003). Additionally, haplotype networks were constructed by pooling DNA sequence data from all 29 loci. Haplotype networks were constructed using the software TCS version 1.21 (Clement *et al.* 2000), which uses parsimony to infer unrooted cladograms. The connection limit was set at 100 steps, and indels were treated as a 5th state.

Gene and genotypic diversity

Isolates were assigned to groups using STRUCTURE (Pritchard *et al.* 2000). Subsequently, populations were defined according to the STRUCTURE results and the geographical origin of the isolates. The number of MLHs per population was calculated using GENCLONE version 2.0 (Arnaud-Haond & Belkhir 2007). The gene diversity (*H*) (Nei 1973), number of alleles, number of private alleles and the number of polymorphic loci were calculated in POPGENE version 1.31 for each population (Yeh *et al.* 1999). In all cases, clone-corrected data sets were used. To compare the genetic diversity among geographical areas, isolates collected from native forests and plantations within the same geographical area were pooled.

To compare the genetic structure of *T. nubilosa* populations collected from native forests and commercial plantations in the same geographical area, the gene diversity (*H*) (Nei 1973), number of alleles, the number of polymorphic loci and the number of MLHs were calculated in POPGENE version 1.31 (Yeh *et al.* 1999). This was possible only for isolates collected in the states of Tasmania and Victoria. Chi-square tests for differences in allele frequencies were calculated for each locus using POPGENE version 1.31 (Yeh *et al.* 1999).

Genotypic diversity ($\hat{G}$) was calculated for every population 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 dependent 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).

Each observed MLH in the *T. nubilosa* population is most likely the result of sexual reproduction events including both intrahaploid mating (yielding identical MLHs as the parental strain) and outcrossing (yielding novel MLHs through recombination) (Giraud *et al.* 2008; Pérez *et al.* 2010). The contribution of each mechanism to the general genotypic diversity was investigated by calculating the P_{sex} index in GENCLONE version 2.0 (Arnaud-Haond & Belkhir 2007; Arnaud-Haond *et al.* 2007). P_{sex} estimates the probability of a given MLH encountered more than once in a sample of N units originating from distinct outcrossing events (Arnaud-Haond *et al.* 2007). In this way, P_{sex} was calculated for each STRUCTURE group separately.

AMOVA and isolation by distance

Two independent AMOVA tests were performed in ARLEQUIN version 3.11 (Excoffier *et al.* 2005). An AMOVA test was run for the entire data set to determine the hierarchical partitioning of the molecular variation among groups. Groups were defined based on the STRUCTURE outputs and geographical origin. All isolates belonging to the same STRUCTURE group (A, B or C) and collected in the same geographical area (NSW,

Tasmania or Victoria) were pooled to perform this AMOVA test regardless of whether they were collected on different sites in native forests or plantations.

A second AMOVA test aimed to compare *T. nubilosa* populations collected in native forests or commercial plantations. This was possible only for populations from Tasmania and Victoria. In this way, clone-corrected *T. nubilosa* populations collected from native forests (collecting sites 5, 6, 8, 12 and 14; Table 1) were compared against *T. nubilosa* populations collected on commercial plantations (collecting sites 10, 11, 20 and 22; Table 1). Sites with a sample size smaller than five MLHs were excluded from this analysis.

To analyse the genetic differentiation as a function of the geographical distance, Mantel tests were conducted in Alleles in Space (AIS) software (Miller 2005). The significance of the correlation was estimated by 1000 random permutations using AIS. Introduced populations from WA, New Zealand, Brazil and Uruguay were not included in the Mantel tests.

Results

Isolate grouping

A total of 112 MLHs were obtained from the entire collection of 521 *T. nubilosa* isolates (Table 2). Further analyses were then carried out on these 112 MLHs (clone-corrected data set). MCMC performed in STRUCTURE showed that the average log Likelihood values for increasing the number of populations (K) reached a plateau where K was set between 3 and 6 (Fig. S1a, Supporting information). For the best choice of K, ΔK was calculated and plotted against K, where a clear peak was observed when $K = 3$ (Fig. S1b, Supporting information). In addition, every MLH was assigned to a particular group with associated probabilities close to the maximum and no admixture for $K = 3$ (boxed in Fig. 2). MLHs assignments were also conducted for $K = 2$, 4 and 5 (Fig. 2). In all cases, the associated probabilities (Q) were lower than those observed for $K = 3$ (Fig. 2). The clear peak observed on the ΔK vs. K plot and the highest associated probabilities for $K = 3$ made it possible to conclude with confidence that $K = 3$ is the best estimate for the real number of distinct genetic groups.

A total of 29, 60 and 23 MLHs were assigned by STRUCTURE to each of the three groups, respectively, and these are hereafter referred to as Groups A, B and C (Table 2, Fig. 2). We further investigated potential population substructuring within each group performing new sets of MCMC iterations in STRUCTURE. These analyses showed that the distribution of the maximum likelihood was the highest for $K = 1$ (−273, −496 and −215 for Groups A, B and C, respectively), indicating a high degree of admixture within each group (Fig. 2).

Three main clusters were observed in the PCA, and these were consistent with the three groups arising in STRUCTURE. The two major components explained 66.5% of the variability (Fig. 3). The three groups observed by both clustering analyses (multivariate and Bayesian) were not perfectly correlated with the geographical origin of the isolates. While isolates from Tasmania (TAS), Western Australia (WA), New Zealand, Brazil and Uruguay were assigned to single groups, those from New South Wales (NSW) and Victoria (VIC) were split

Table 2 Number of isolates and MLHs assigned to different groups by STRUCTURE

Country	State	Number of isolates	Number of MLHs	Group A		Group B		Group C	
				Isolates	MLHs	Isolates	MLHs	Isolates	MLHs
Australia	NSW	129	37	75	29	—	—	54	8
	Tasmania	77	40	—	—	77	40	—	—
	(native)*	(39)	(26)	(—)	(—)	(39)	(26)	(—)	(—)
	(plantations)	(38)	(18)	(—)	(—)	(38)	(18)	(—)	(—)
	Victoria	93	30	—	—	49	16	44	14
	(native)	(39)	(17)	(—)	(—)	(24)	(7)	(15)	(10)
	(plantations)	(54)	(14)	(—)	(—)	(25)	(9)	(29)	(5)
	WA	81	3	—	—	81	3	—	—
Brazil		42	1	—	—	—	—	42	1
New Zealand		53	2	—	—	53	2	—	—
Uruguay		46	1	—	—	—	—	46	1
	Total	521	112						

NSW, New South Wales; WA, Western Australia; MLH, multilocus haplotype.
*Populations collected on native forests and commercial plantations are provided separately in brackets for the states of Tasmania and Victoria were both types of forests were sampled.

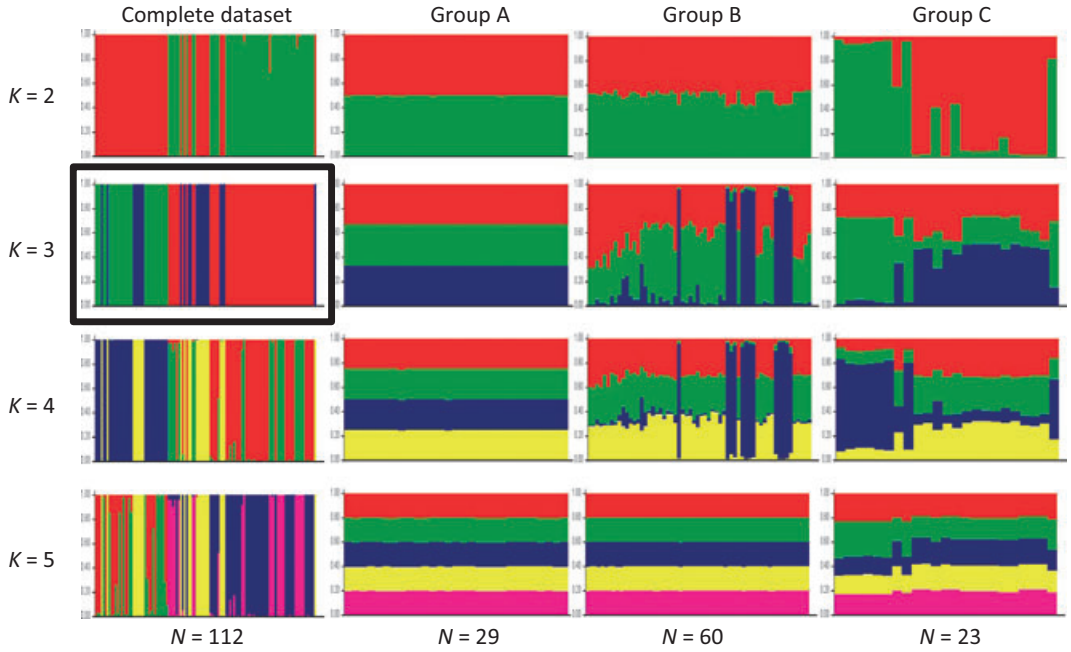


Fig. 2 Assignment of *Teratosphaeria nubilosa* multilocus haplotypes into growing *K* populations using STRUCTURE for the complete data set and for each group separately. The best estimate for the real number of distinct genetic groups *K* = 3 is boxed.

among two groups without any noticeable correlation with hosts or collecting sites (Table 2, Figs 1 and 3a). Consistently with STRUCTURE grouping algorithms, isolates assigned to Groups A, B and C also grouped apart in PCA (Fig. 3b).

Phylogenetic reconstructions

To interrogate the possible recognition of the three groups identified by STRUCTURE and PCA as species, DNA sequence data were considered in the analyses. Five and seven polymorphisms were observed in the ITS and the β-tubulin gene regions, respectively (Table 3). DNA sequencing of the 27 anonymous loci yielded 4020 characters of which 50 were variable and 47 were parsimony informative (Table 3).

Phylogenetic analyses based on the ITS gene region revealed two well-supported clusters that were consistent with STRUCTURE and multivariate grouping results (Fig. 4), except that isolates from Group A and Group C clustered together, while those from Group B clustered apart (Fig. 4). Identical results were observed from each of 20 anonymous loci (TN-1, TN-2, TN-3, TN-5, TN-6, TN-7, TN-9, TN-10, TN-12, TN-16, TN-18, TN-19, TN-20, TN-22, TN-24, TN-25, TN-26, TN-27, TN-28, TN-30) namely that isolates from Group A and Group C clustered together and isolates in Group B clustered separately (TreeBase S12689). To the contrary, in the β-tubulin phylogram, isolates from Group A and Group B clustered together and isolates in Group C clustered apart (TreeBase S12689). Similar results were observed on two anonymous loci (TN-14 and TN-29) (TreeBase S12689). Finally, five anonymous loci (TN-4, TN-13, TN-17, TN-21 and TN-23) showed clusters that were not consistent with either ITS, β-tubulin, STRUCTURE or multivariate grouping results (TreeBase S12689).

Two well-supported clusters were also observed in the haplotype network constructed with the pooled DNA sequence data arising from the 29 loci (Fig. 5). One cluster accommodated all haplotypes from Groups A and C, and another cluster accommodated all haplotypes from Group B (Fig. 5). Haplotypes from Group A were separated by seven missing intermediates from those haplotypes of Group C. Haplotypes from Group B were separated by 42 missing intermediates from those of Groups A and C (Fig. 5).

Gene and genotypic diversity

A total of 92 alleles were recovered from eight polymorphic loci across the entire collection of isolates (Table 4). Only seven alleles were shared among the Groups A, B and C. This implies that, on average, <1 allele per locus was shared among all groups. Ten alleles were shared among Groups A and B, four alleles were shared among Groups A and C, and five alleles were shared among Groups B and C. In contrast,

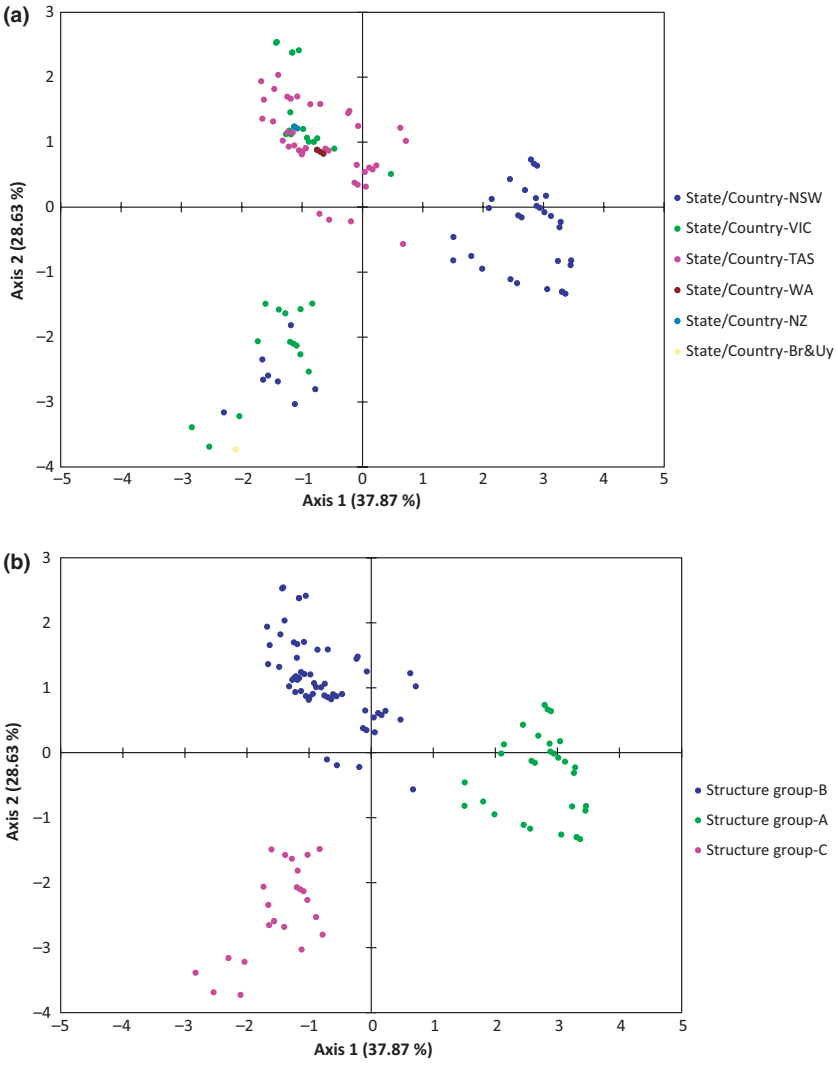


Fig. 3 Principal component analysis (PCA) performed on the 112 multilocus haplotypes (MLHs) of *Teratosphaeria nubilosa* based on eight microsatellite markers. Each point represents a MLH. (a) Point colours representing the geographical origin of isolates. (b) Same PCA plot where point colours represent the STRUCTURE grouping of isolates.

Groups A, B and C had 24, 22 and 20 private alleles, respectively (Table 4). Gene diversity (*H*) was equivalent in all groups ranging from 0.459 to 0.537 (Table 4). Most loci were polymorphic, but locus MN-1 was monomorphic in Group A, locus MN-10 was monomorphic in Group B, and locus MN-11 was monomorphic in both Groups A and B (Table 4). Likewise, microsatellite primer pairs successfully amplified loci MN-4 and MN-8 of isolates from Groups A and C, but were unable to amplify those isolates residing in Group B. These two microsatellite loci were not used for population analyses in this study.

The number of MLHs, alleles, private alleles, polymorphic loci and gene diversity values (*H*) were considerably lower in all introduced populations from WA, New Zealand, Brazil and Uruguay, when compared against native populations from Victoria and Tasmania (Table 4). Similar results were observed in the NSW population of Group C with relatively low values for the aforementioned parameters, despite a relatively large sample size (N = 54) (Table 4).

Equivalent values of gene diversity (*H*), number of alleles, number of polymorphic loci and number of MLHs were observed on populations collected from native forests and plantations in Victoria and Tasmania in both Groups B and C (Table 5). Most alleles were present on both native and commercial plantations, and six MLHs were found colonizing native and commercial trees. Chi-square tests showed that in most cases, differences in allele frequencies among populations were not significant (Table 5).

Genotypic diversity ($\hat{G}$) and percentage of the maximum theoretical value of $\hat{G}$ ($\hat{G}_{max}$) were extremely low to moderate (Table 4). Both values were particularly low in all introduced populations from WA, New Zealand, Brazil and Uruguay ($\hat{G}_{max}$ = 2.27–2.74%) where pop-

ulations were represented by one to three MLHs (Table 4). Native Australian populations from NSW, Victoria and Tasmania showed moderate values ($\hat{G}_{max}$ = 13.01–42.8%). Genotypic diversity of the NSW Group C population was very low ($\hat{G}_{max}$ = 3.11%) resembling those frequencies observed in introduced populations (Table 4).

The number and evenness of MLHs was highly variable in the entire collection, among groups and among populations within groups (Fig. 6, Table 4). Most MLHs (75%) occurred once or twice and corresponded to those MLHs collected in native populations of NSW, Victoria and Tasmania including both native forests and commercial plantations. Conversely, four MLHs occurred more than 40 times and corresponded to those MLHs originating from introduced populations from WA, New Zealand, Brazil, Uruguay and the NSW Group C population (Fig. 6). P_{sex} values were significant ($P < 0.05$) for all introduced populations from WA, New Zealand, Brazil, Uruguay and the NSW Group C population (Fig. 6) and for 33 (79%) of 42 MLHs, which occurred more than once in the NSW, Tasmanian and Victorian populations (Fig. 6). Conversely, only 9 (21%) of 42 MLHs that occurred more than once in Australia showed nonsignificant P_{sex} values ($P > 0.05$) and therefore potentially could have arisen in the population from sexual reproduction through outcrossing (Fig. 6).

AMOVA and isolation by distance

The AMOVA analysis including the entire data set showed that a relatively high percentage (29.3%) of the allelic variation was distributed among Groups A, B and C (Table 6). However, most variation was distributed within these groups (55.5%), and a relatively low level of variation (15.2%) was distributed among populations from distinct geographical areas within the same group (Table 6). The second AMOVA analysis including only those isolates collected on native forests, and commercial plantations showed that a negligible percentage (0.64%) of the allelic variation was distributed among groups collected on those types of forests (Table 7). Most variation was distributed within populations (68.7%). Consistent with the previous AMOVA, a relatively high percentage of variation (30.7%) was distributed between Groups B and C (Table 7). The correlation between genetic and geographical distance (Mantel test) was significant (r = 0.559, $P < 0.001$).

Discussion

In this study, three genetically isolated groups were identified among an extensive sample of *T. nubilosa* within its native range in Australia. While human activities have modified the geographical distribution of the pathogen introducing representatives of novel groups into different biogeographical areas in Australia, no evidence of hybridization was found. These data provide robust evidence to suggest that *T. nubilosa* represents a species complex, which is also supported by DNA sequence data and phylogenetic reconstructions. Furthermore, where appropriate comparisons were possible, it was clear that there are no genetic differences between pathogen populations colonizing native forests and those that have moved to adjacent commercial plantations.

Analyses of microsatellite data revealed the presence of three discrete groups among isolates treated as the single species, *T. nubilosa*. Very few alleles were shared among groups (on average <1 per locus), and approximately half of the alleles observed in each group were identified as 'private'. All isolates were assigned to a particular group with high associated probabilities (*Q*) in STRUCTURE, and no potential hybrids or recombinants were identified in individual assignments in STRUCTURE (Pritchard *et al.* 2000). AMOVA tests showed that ~30% of the molecular variation was distributed among those three discrete groups. DNA sequence data provided results consistent with those arising from microsatellite analyses and also supported the existence of discrete groups in *T. nubilosa*. Four fixed polymorphisms were observed in the ITS gene region of Group B isolates. This is higher than for other *Mycosphaerella* spp. and *Teratosphaeria* spp., where commonly one to three fixed polymorphisms in this gene region reflect discrete species (Goodwin *et al.* 2001; Crous *et al.* 2006). Group C contains the ex-epitype specimen of *T. nubilosa* (isolate CMW 3282) (Crous *et al.* 2006) and should be treated as *T. nubilosa sensu stricto*. Isolates representing Group B were consistently separated from the *T. nubilosa sensu stricto* group (Group C) in 24 (ITS, β-tubulin and 22 anonymous loci) of 29 phylograms analysed and also grouped apart in a well-supported clade in the haplotype network. No contradictions to this grouping were found in any other single-locus genealogy (Dettman *et al.* 2003). Applying the GCPSC proposed by Taylor *et al.* (2000), our results show that 24 genealogies are concordant suggesting that Groups B and C are genetically isolated species. Therefore, *T. nubilosa* should be considered as a species complex as previously proposed (Crous *et al.* 2006; Hunter *et al.* 2009). This discovery of well-known species representing more than one cryptic species is similar to many ascomycete fungi via the application of contemporary molecular genetic tools and the GCPSC (O'Donnell *et al.* 2004; Taylor *et al.* 2006; Pavlic *et al.* 2009). It is important to take note of the common occurrence of these cryptic species, and

Table 3 DNA sequence polymorphisms contained in the ITS, β-tubulin and 27 anonymous loci from representative isolates. Fixed apomorphies within groups are highlighted and indels coded as presence-absence

Group	State/ Country	Isolate (CMW)	ITS 45*	ITS 89	ITS 113	ITS 331	ITS 410	BT1 210	BT1 414	BT2 82	BT2 86	BT2 202 ACA	BT2 218	BT2 220	T1 35	T1 171	T2 19	T2 82 TT	T3 35	T3 80 CCG	T3 116	T4 81	T4 86	T5 36	T5 59	T5 103	T6 59	T6 105	T6 125
C	Victoria	CMW 3282†	A	C	C	C	T	T	C	G	A	0	G	A	A	A	T	0	C	1	C	C	G	T	A	G	A	G	G
		CMW 30752	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		CMW 30751	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		CMW 30746	T	.	.	.	.	.	T	.	.	.	.	.															
		CMW 30748	T	.	.	.	.	.	T	.	.	.	.	.															
		CMW 30753	T	.	.	.	.	.	T	.	.	.	.	.															
		CMW 30973	T	.	.	.	.	.	T	.	.	.	.	.															
	NSW	CMW 30715	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
		CMW 30947	.	.	.	.	.	.	T	.	.	.	.	.															
		CMW 30956	.	.	.	.	.	.	T	.	.	.	.	.															
	Brazil	CMW 30900	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	Uruguay	CMW 30218	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
A	NSW	CMW 30707	.	.	.	.	.	C	T	.	.	1	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		CMW 30709	.	.	.	.	.	C	T	.	.	1	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		CMW 30717	.	.	.	.	.	C	T	.	.	1	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
		CMW 30711	.	.	.	.	.	C	T	.	.	1	A	.															
		CMW 30718	.	.	.	.	.	C	T	.	.	1	A	.															
		CMW 30720	.	.	.	.	.	C	T	.	.	1	A	.															
		CMW 30952	.	.	.	.	.	C	T	.	.	1	A	.															
B	Victoria	CMW 30745	.	A	G	T	C	C	T	C	.	1	A	.	G	G	C	1	A	0	G	.	.	C	T	A	G	A	A
		CMW 30750	.	A	G	T	C	C	T	C	.	1	A	G	G	G	C	1	A	0	G	.	.	C	T	A	G	A	A
		CMW 30747	.	A	G	T	C	C	T	C	.	1	A	.															
		CMW 30749	.	A	G	T	C	C	T	C	.	1	A	G															
		CMW 30970	.	A	G	T	C	C	T	C	.	1	A	.															
		CMW 30971	.	A	G	T	C	C	T	C	.	1	A	G															
		CMW 30972	.	A	G	T	C	C	T	C	.	1	A	.															
	Tasmania	CMW 30735	.	A	G	T	C	C	T	.	.	1	A	.	G	G	C	1	A	0	G	.	.	C	T	A	G	A	A
		CMW 30730	.	A	G	T	C	C	T	.	.	1	A	.															
		CMW 30737	.	A	G	T	C	C	T	.	.	1	A	.															
		CMW 30740	.	A	G	T	C	C	T	.	.	1	A	.															
	WA	CMW 30723	.	A	G	T	C	C	T	.	.	1	A	.	G	G	C	1	A	0	G	.	.	C	T	A	G	A	A
	New Zealand	CMW 31008	.	A	G	T	C	C	T	C	.	1	A	.	G	G	C	1	A	0	G	.	.	C	T	A	G	A	A

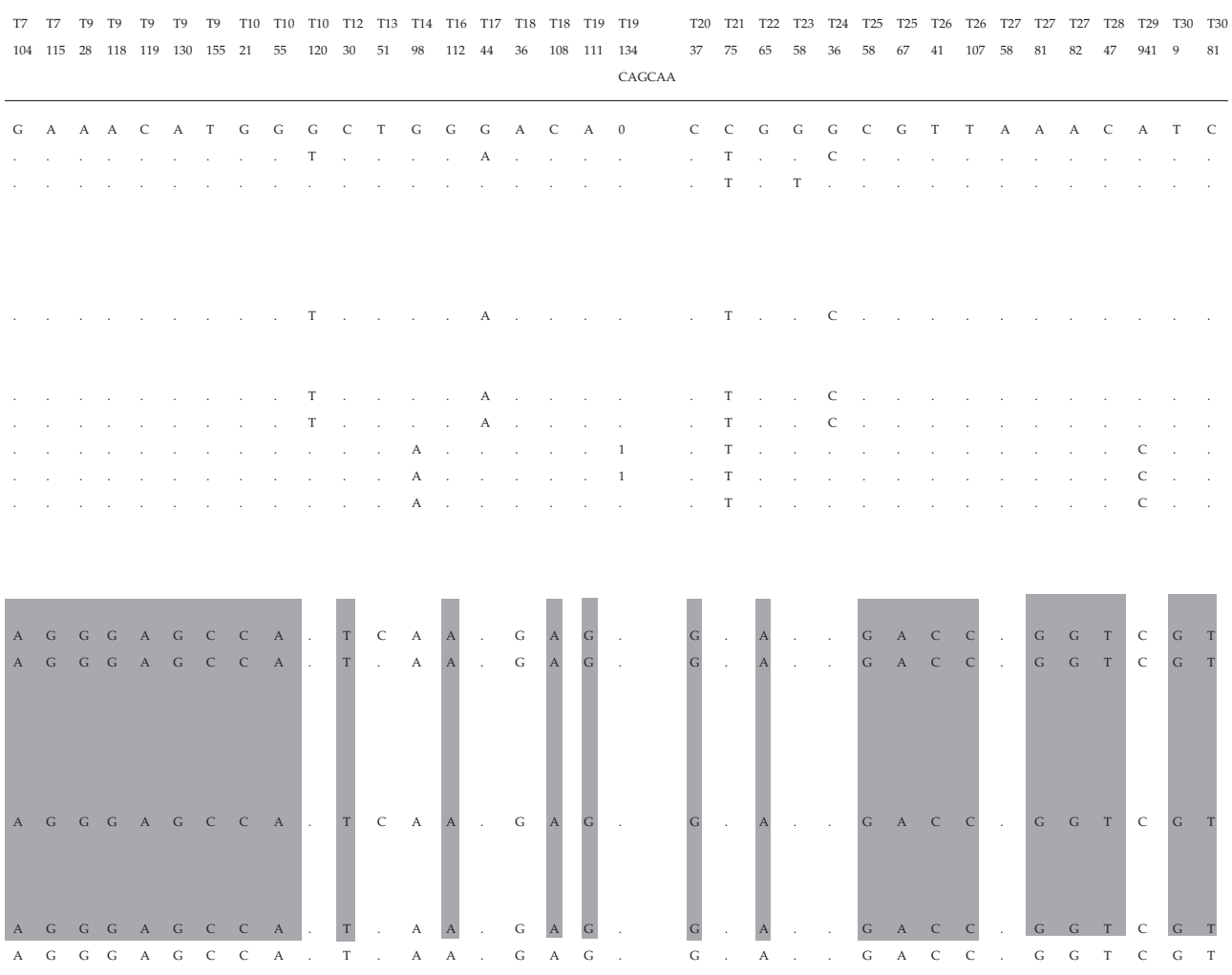
*Position of the substitutions in the DNA strand.

†*Teratosphaeria nubilosa* ex-epitype culture.

the extent of sampling (both in terms of numbers of isolates and in terms of markers analysed) that is needed to distinguish them, especially if they are recently diverged. This can have critical implications for pathogen management in agriculture and forestry (Andjic *et al*. 2007; O'Donnell *et al*. 2009).

Whereas a high number of private microsatellite alleles (24 in 45) were observed in Group A, no 'private' fixed polymorphisms were observed in the DNA sequence data examined within this group. In 22 of 29 phylogenies analysed, Group A isolates grouped together with the *T. nubilosa sensu stricto* group (Group C). DNA sequence polymorphisms show lower level of variation and evolve at a slower rate than microsatellite loci (Brumfield *et al.,* 2003). Our results suggest that Group A is in the process of speciation and has been genetically isolated from Groups B and C, allowing sufficient time for microsatellite alleles to be fixed in the population but not sufficient time for DNA polymorphisms fixation (Taylor *et al*. 2000).

Contrasting values of gene and genotypic diversity were observed among different populations of *T. nubilosa*. The non-native populations from New Zealand, Brazil and Uruguay included in this study all showed very low levels of both gene and genotypic diversity, usually represented by one to three MLHs. These results are similar to those reported in other *T. nubilosa* populations in Spain, Portugal and Tanzania



(Hunter *et al.* 2008) and typical examples of biological invasions in other organisms (Ficetola *et al.* 2008). The low level of gene and genotypic diversities can be explained by the 'founder effect', where a reduced number of individuals carrying a fraction of the diversity of the original population, establish a new population into a new area (Ficetola *et al.* 2008; Stukenbrock & McDonald 2008) or because of the stepwise invasion events, where introduced populations originate from other non-native populations with lower levels of diversity (Clegg *et al.* 2002; Lombaert *et al.* 2010). While introduced populations can be highly diverse (Kolbe *et al.* 2004; Estoup & Guillemaud 2010; Mitchell *et al.* 2010) because of multiple introductions and admixture (Smart & Fry 2001; Dlugosch & Parker 2007; Dutech *et al.* 2010), our results suggest that this is not the case for *T. nubilosa* where very low diversity was observed in all non-native invading populations studied.

Results of this study show that *T. nubilosa* in New Zealand populations correspond to Group B and Brazilian and Uruguayan populations correspond to Group C (=*T. nubilosa sensu stricto*). Using DNA sequence data available in GenBank, it was also possible to determine that *T. nubilosa* populations from Ethiopia, Kenya, Portugal, Spain, South Africa and Tanzania, (from Hunter *et al.* 2009) correspond to isolates in Group C (=*T. nubilosa sensu stricto*). This implies that there have been at

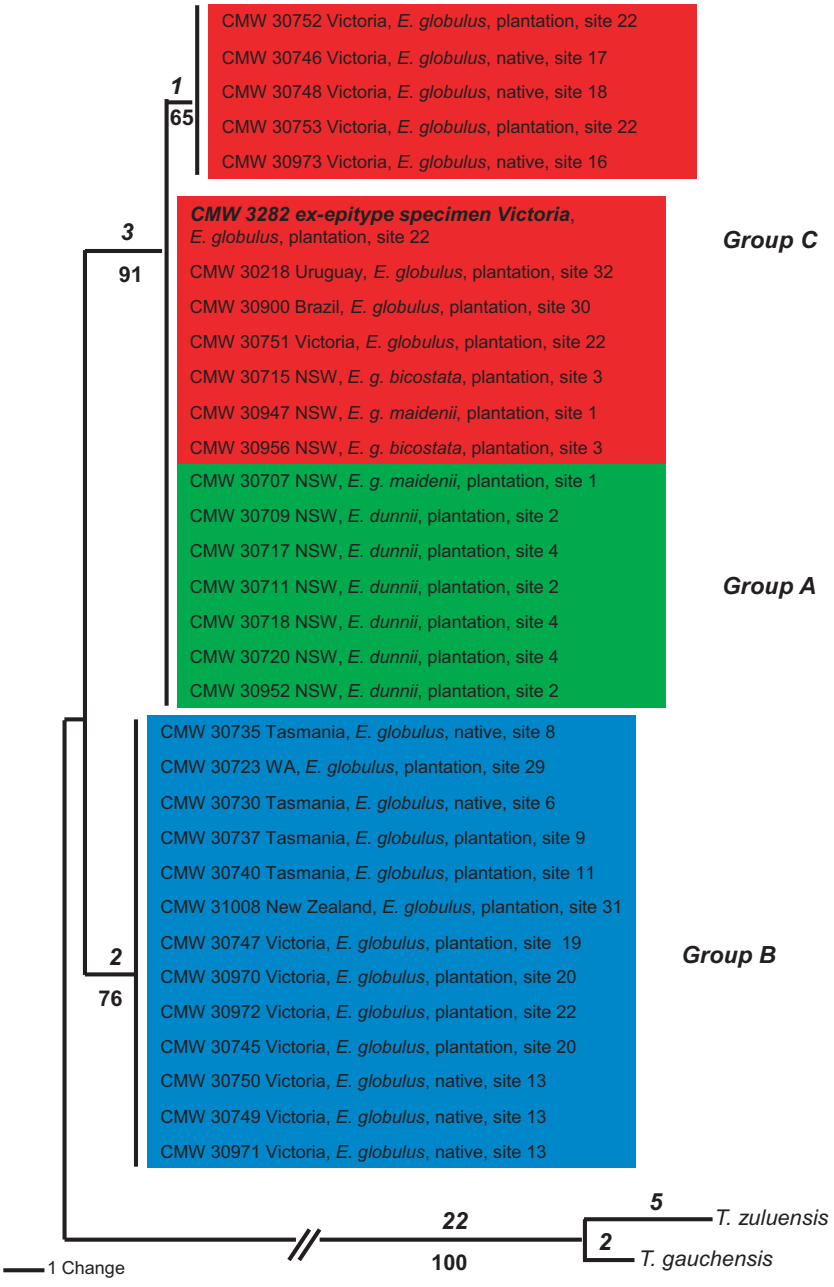


Fig. 4 Consensus ITS phylogram obtained using parsimony and heuristic search. Branch length and bootstrap support values after 1000 randomizations are shown above and below the branching points, respectively. The geographical origin, type of forest (native or commercial plantation), collecting site (see Table 1) and STRUCTURE grouping of isolates are also provided; Group A (green), Group B (blue) and Group C (red).

least two discrete introductions of the pathogen into different regions of the world and from different source populations, consistent with the view presented by Hunter *et al.* (2009). Breeding for resistance is the only economically viable management strategy for MLD (Tibbits *et al.* 1997; Wingfield 2003). Because more diverse pathogen populations present high evolutionary potential to break host resistance (McDonald & Linde 2002), the reinforcement of quarantine measures is strongly advised to avoid novel introductions of new MLHs or new groups of *T. nubilosa* in those countries that populations of the pathogen having low genetic diversity.

Information generated from non-native invading *T. nubilosa* populations studied in Brazil, New Zealand and Uruguay (where populations were not at all diverse and represented by few genotypes) was used to find evidence of potential biological invasions within Australia. Two clearly introduced populations and consequent biological invasions were identified within Australia, one in WA and one in NSW. The WA population belonging to Group B was represented by a single MLH, and the NSW population of Group C was represented by a predominant MLH and seven other single MLHs. Historical reports suggest that *T. nubilosa* was introduced into WA

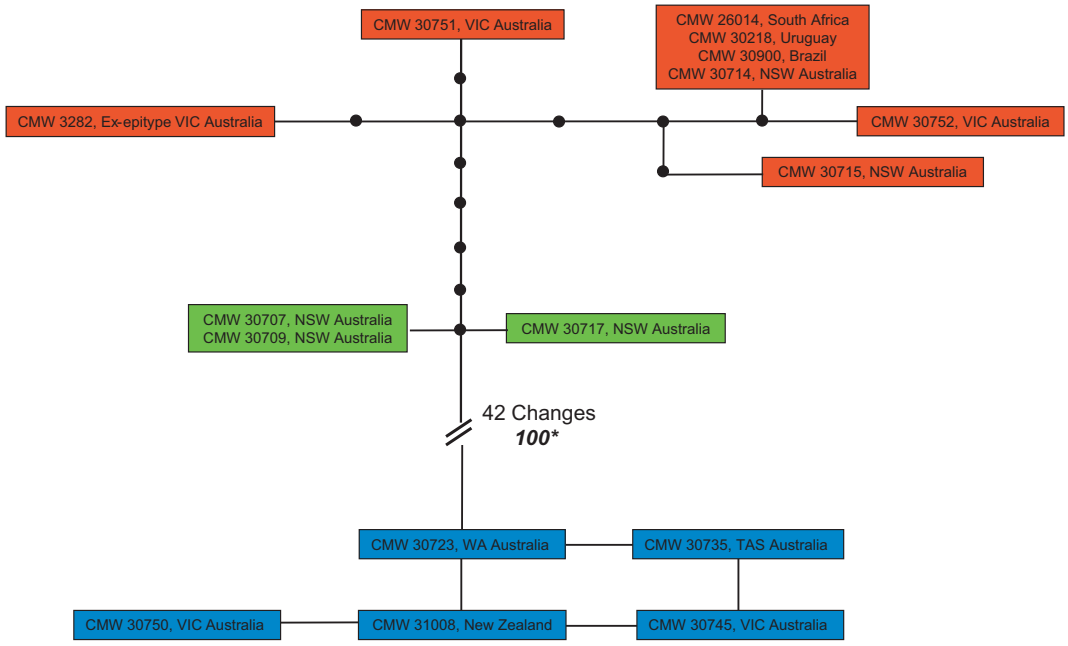


Fig. 5 Parsimony haplotype network from sequence data of 29 loci. Dots indicate hypothetical missing intermediate haplotypes. (*) Bootstrap support value after 1000 randomizations. The geographical origin and STRUCTURE grouping of isolates are also provided; Group A (green), Group B (blue) and Group C (red).

Table 4 Gene and genotypic diversity parameters observed in populations belonging to discrete groups

Group	State/Country	N	No multilocus haplotypes	N_a	N_p	H	PL	Monomorphic loci	$\hat{G}$	$\hat{G}_{max}$
A	NSW	75	29	45	24	0.471	6	1, 11	6.29	14.3
	Total group A	75	29	45	24	0.471	6	1, 11		
B	Tasmania	77	40	33	7	0.437	6	10, 11	18.8	42.8
	(native)*	(39)	(26)	(27)	(3)	(0.385)	(6)	(10, 11)		
	(plantations)	(38)	(18)	(24)	(1)	(0.432)	(6)	(10, 11)		
	Victoria	49	16	26	5	0.409	5	1, 10, 11	7.67	17.4
	(native)*	(24)	(7)	(18)	(2)	(0.337)	(5)	(1, 10, 11)		
	(plantations)	(25)	(9)	(17)	(3)	(0.349)	(5)	(1, 10, 11)		
	WA	81	3	10	0	0.083	1	All but 9	1.05	2.38
	New Zealand	53	2	9	1	0.063	1	All but 9	1.21	2.74
	Total group B	260	60	44	22	0.459	6	10, 11		
C	NSW	54	8	16	4	0.266	6	3, 7	1.37	3.11
	Victoria	44	14	30	10	0.472	8	None	5.72	13.01
	(native)*	(15)	(10)	(23)	(3)	(0.398)	(6)	(10, 1)		
	(plantations)	(29)	(5)	(22)	(2)	(0.470)	(7)	(7)		
	Brazil	42	1	8	1	0.000	0	All	1.00	2.27
	Uruguay	46	1	8	1	0.000	0	All	1.00	2.27
	Total group C	186	23	36	20	0.537	8	None		
	Total	521	112	92						

N, sample size; N_a, number of alleles; N_p, number of private alleles; H, gene diversity (Nei 1973); PL, number of polymorphic loci; $\hat{G}$, genotypic diversity (Stoddart & Taylor 1988); $\hat{G}_{max}$, percentage of the maximum theoretical value of $\hat{G}$.
*Populations collected on native forests and commercial plantations are provided separately in brackets for the states of Tasmania and Victoria were both types of forests were sampled.

after 1994 (Maxwell *et al.* 2001) as it was not reported in previous surveys conducted in this area (Carnegie *et al.* 1997). Commercial *Eucalyptus* plantation establishment has recently accelerated in NSW (Nichols *et al.* 2010), it is thus likely that the introduction of Group C isolates into NSW has been associated with the movement of germplasm to establish these new plantations in this area. *Teratosphaeria nubilosa* was not commonly observed in early

Table 5 Gene diversity (*H*) for the eight microsatellite loci across *Teratosphaeria nubilosa* populations collected on native forests and commercial plantations

	Group B										Group C				
	Tasmania					Victoria					Victoria				
	Native	Plantation				Native	Plantation				Native	Plantation			
	Gene diversity (*H*)		χ^2	d.f.	*P*	Gene diversity (*H*)		χ^2	d.f.	*P*	Gene diversity (*H*)		χ^2	d.f.	*P*
MN-1	0.571	0.593	4.95	2	0.08	0	0	—	—	—	0	0.320	2.14	1	0.14
MN-2	0.382	0.617	**20.93***	**4**	**<0.01**	0.571	0.765	12.75	6	0.05	0.580	0.640	9.00	4	0.06
MN-3	0.686	0.568	**20.56**	**6**	**<0.01**	0.449	0.494	5.84	3	0.12	0.480	0.320	2.14	1	0.14
MN-7	0.639	0.568	4.07	3	0.25	0.694	0.444	3.00	3	0.39	0.180	—	0.54	1	0.46
MN-9	0.544	0.568	10.31	7	0.17	0.571	0.494	**16.00**	**4**	**<0.01**	0.760	0.800	7.13	6	0.31
MN-10	0	0	—	—	—	0	0	—	—	—	0	0.320	2.14	1	0.14
MN-11	0	0	—	—	—	0	0	—	—	—	0.480	0.720	3.50	4	0.48
MN-14	0.260	0.543	4.47	3	0.21	0.408	0.593	4.26	2	0.12	0.700	0.640	6.60	4	0.16
N_a	27	24				18	17				23	22			
PL	6	6				5	5				6	7			
N	39	38				24	25				15	29			
No MLHs	26	18				7	9				10	5			

H, gene diversity (Nei 1973); d.f., degrees of freedom; N_a, number of alleles; PL, number of polymorphic loci; *N*, sample size; No MLHs, number of multilocus haplotypes.
*Significant *P* values ($P < 0.05$) are shown in bold.

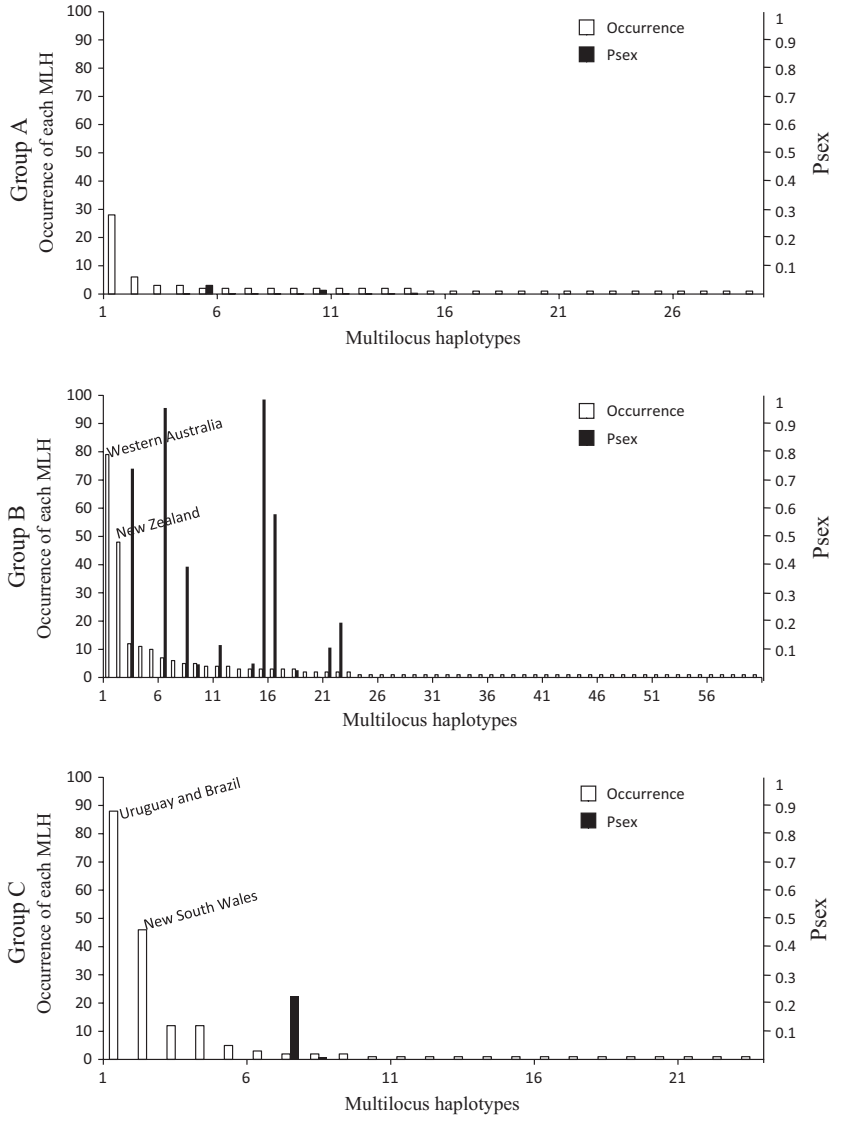


Fig. 6 Occurrence of the 112 *Teratosphaeria nubilosa* multilocus haplotypes (MLHs) (white) assigned to Group A (n = 29), Group B (n = 60) and Group C (n = 23), and P_{sex} probabilities associated to each MLH (black).

Table 6 AMOVA showing the hierarchical partitioning of the molecular variation for the entire data set

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups A B and C	2	88.5	0.94 Va	29.30
Among populations within groups	5	29.2	0.49 Vb	15.16
Within populations	104	184.8	1.78 Vc	55.53
Total	111	339.4	3.20	

d.f., degrees of freedom.

surveys conducted during 1996–2005, but was regularly observed on *E. dunnii* and *E. globulus* subsp. *maidenii* (in trials) in later surveys on *E. nitens* (Carnegie 2007a,b). It is probable that the introduction of isolates representing Group C in NSW accounts for the increased severity of disease in commercial plantations and research trials in this area. The results of this study also show that while the introduced population belonging to Group C coexists with isolates of native Group A in NSW, even in the same plantation (Tabulam), there is no evidence of recombination between them. Our results show that human intervention has altered the species composition of resident native fungal communities through the introduction of novel genotypes and new pathogenic species consistent with the view of Desprez-Loustau *et al.*(2007).

Table 7 AMOVA showing the hierarchical partitioning of the molecular variation for *Teratosphaeria nubilosa* populations collected on native forests and commercial plantations

Source of variation	d.f.*	Sum of squares	Variance components	Percentage of variation
Among groups collected from native and commercial plantations	1	8.1	0.01 Va	0.64
Among populations within groups	7	47.5	0.68 Vb	30.70
Within populations	64	96.6	1.51 Vc	68.66
Total	72	152.2	2.20	

d.f., degrees of freedom.

Highly diverse *T. nubilosa* populations were also observed in Australia where the pathogen is considered to be native (Hunter *et al.* 2009). Native populations are usually genetically diverse because of the accumulation of alleles over time (Stukenbrock & McDonald 2008). The highest levels of gene diversity for isolates in Groups A and C were observed in NSW and Victoria, respectively. We therefore hypothesize that (i) Group A is native in NSW colonizing native *Eucalyptus dunnii* and introduced *E. globulus* and (ii) Group C is native in Victoria colonizing both native and commercial *E. globulus* forests and potentially a small number of other native *Eucalyptus* hosts not sampled in this study. Conversely, isolates in Group B occurred in both Tasmania and Victoria, coexisting with those in Group C only in Victoria. Gene and genotypic diversities of isolates in Group B were equivalent in Tasmania and Victoria, occurring in both native forests and commercial plantations. It is therefore not possible to hypothesize whether Group B isolates are native to Tasmania and introduced (and naturalized) in Victoria or whether they are native to both regions. Interestingly, the distribution of Group B isolates aligns with the native distribution of *E. globulus* in Tasmania and Victoria (Potts *et al.* 2004).

Invasive populations were dominated by a few commonly occurring MLHs, while native Australian populations were characterized by a large number of MLHs that occurred only once or twice (75%). Apart from reflecting the invasion history of the fungus (as discussed earlier), the results are also consistent with the reproductive strategy of this species (see Pérez *et al.* 2010). The anamorphic state of *T. nubilosa* has never been observed in nature despite intensive efforts to find it, and it has been proposed that this pathogen has an exclusively sexual mode of reproduction (Pérez *et al.* 2010). It has, however, been shown that while sexual reproduction through selfing [intrahaploid mating sensu Giraud *et al.* (2008)] is the main reproductive strategy of *T. nubilosa*, outcrossing also occurs (at low levels) in populations (Pérez *et al.* 2010). Results from the present study also suggest that sexual reproduction through intrahaploid mating is common in this species [e.g. P_{sex} values were significant for all introduced populations ($P < 0.001$)]. This pattern of reproduction has also been observed in other plant pathogens (Giraud *et al.* 2008; Billiard *et al.* 2011). The low frequency MLHs that occurred once or twice, typical of Tasmanian and Victorian populations, could have potentially arisen from distinct outcrossing events at the population level. A mixed mating system combining intrahaploid mating and outcrossing is the most likely explanation for differences in the genotypic diversity and number of MLHs among native and non-native *T. nubilosa* populations as reported before for this species (Pérez *et al.* 2010) and other plant pathogens such as *Sclerotinia sclerotiorum*, *Armillaria gallica* and *Phytophtora infestans* (Milgroom 1996; Taylor *et al.* 1999).

Equivalent values of gene diversity (*H*), number of alleles, number of polymorphic loci and number of MLHs were observed in populations colonizing commercial plantations and nearby native forests in both Tasmania and Victoria. Negligible percentage of molecular variation was attributed to among groups collected from native forests and commercial plantations in AMOVA tests. Six MLHs were found to colonize indistinctly native plants and trees in commercial plantations. These results reflect continuous host jumping from native and planted trees and *vice versa*. It is likely that the initial colonization of commercial plantations originated from pre-existing native pathogen populations colonizing nearby native eucalypt forests. Because all three groups do not occur in all geographical regions of Australia, further introductions within the country should be avoided. This is particularly because our results suggest that the pathogen has the potential to colonize the nearby native forests. This situation should be evaluated as it has been shown in the past that the introduction of new species or genotypes can be a threat to both native ecosystems and plantations (Brasier 2008).

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Data accessibility

DNA sequences: GenBank accessions HQ130795–HQ131328.

Phylogenetic data: TreeBASE S12689.

Individual-by-individual sampling locations, microsatellite genotypes and clone-corrected data set: DRYAD entry DOI: 10.5061/dryad.541v5.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Optimal number of populations ($K = 3$) as determined by (a) the LnK and (b) Delta K with increasing K values obtained from STRUCTURE analyses.

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