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

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Abstract

Human associated introduction of pests and consequent invasions are very evident in areas where no related organisms existed before. In areas where the geographic distribution is made up of distinct populations or cryptic species closely related to those being introduced, this 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. An extensive sampling was carried out and 521 isolates were characterized using eight microsatellite loci. Multivariate and Bayesian analyses of the population conducted in STRUCTURE revealed three genetically isolated groups, where recombination or hybridization among groups does not occur, even when they co-occur in the same plantation. DNA sequence data of the ITS (n=32), β-tubulin (n=32) and 27 anonymous gene regions (n=16) were consistent with microsatellite data suggesting that *T. nubilosa* should be considered as a species complex as is being discovered for many ascomycete fungi. Clear evidence of biological invasions by the pathogen was identified within Australia in the states of Western Australia and New South Wales and beyond Australia in New Zealand, Brazil and Uruguay. Introduced populations were extremely low diverse showing in most cases one to three MLHs. Non significant genetic differences were observed among pathogen populations collected in native forests and commercial plantations and some MLHs were shared between those types of forests. This raises concerns regarding anthropogenic impact of introduced pathogens arising from the establishment of commercial plantations in Australia, where *T. nubilosa* can easily colonize trees in adjacent native forests.

Introduction

Commercially propagated plants are generally cultivated outside their areas of native occurrence and they have often been accompanied by the accidental introduction of many of their natural pests and diseases (Fry *et al.*, 1993; Mitchell *et al.*, 2010; Stukenbrock & McDonald, 2008). This is also the case of the introduction of *Eucalyptus* spp. worldwide, where important pests and pathogens have been recorded soon after the trees have been established (Wingfield *et al.*, 2008). Eucalypts are native to Australasia with more than 700 known species (Booker & Kleinig, 1994; Potts & Pederick, 2000). *Eucalyptus* spp. are also grown for pulp and timber production in commercial plantations in Australia (Nichols *et al.*, 2010; Potts *et al.*, 2004). 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). Eucalypt introductions into new biogeographical areas of Australia have also been faced with the appearance of their associated native pathogens (Barber *et al.*, 2008; Jackson *et al.*, 2008; Maxwell *et al.*, 2001). 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 is considered a threat to both native forest ecosystems and commercial plantations (Brasier,

2008; Gilbert, 2002). Because *Eucalyptus* commercial plantations are often located close to native stands (Barber *et al.*, 2008; Burgess *et al.*, 2006), 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 centers of origin (Burnett, 2003; Hayden *et al.*, 2003; Stukenbrock *et al.*, 2007; Zhan *et al.*, 2003; Zhou *et al.*, 2007) and the pathways of introduction of several plant pathogens (Brown & Hovmøller, 2002; Fry *et al.*, 1993). Species in native populations are typically genetically diverse due to the successive accumulation of novel alleles arising from mutation over time (Burnett, 2003; Stukenbrock *et al.*, 2007). The introduction of a plant pathogen into a new area is a form of biological invasion (Desprez-Loustau *et al.*, 2007; Mitchell *et al.*, 2010; Philibert *et al.*, 2011) and follows the same stages; transport, arrival (release), establishment and population expansion (Kolar & Lodge, 2001; Lockwood *et al.*, 2005). Biological invasions 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 (Ficetola *et al.*, 2008; Nei *et al.*, 1975). However, genetic diversity of introduced populations is also known to be affected by the "propagule pressure", which includes both the number of individuals introduced and the number of introductions (Colautti *et al.*, 2006; Ficetola *et al.*, 2008; Lockwood *et al.*, 2005). Multiple introductions are common in biological invasions (Dlugosch, Parker, 2007) including plant pathogens (Dutech *et al.*, 2010; Smart & Fry, 2001), and when

organisms are introduced multiple times in a certain area, introduced populations can be as diverse (or even more diverse) as native populations (Estoup & Guillemaud, 2010; Kolbe *et al.*, 2004; Mitchell *et al.*, 2010).

A very common disease observed in both native and commercial eucalypt plantations is Mycosphaerella Leaf Disease (MLD) (Carnegie, 2007b; Crous, 1998). This disease is caused by more than 120 species belonging to the families *Mycosphaerellaceae* and *Teratosphaeriaceae* (Ascomycetes) (Burgess *et al.*, 2007; Carnegie *et al.*, 2007; Crous, 1998; Crous *et al.*, 2007). The leaf pathogen *Teratosphaeria nubilosa* is one of the most aggressive species causing MLD (Carnegie, 2007b; Hunter *et al.*, 2009), causing premature leaf abscission and reduction in tree growth in commercial plantations, which can lead to significant economic losses (Carnegie & Ades, 2003; Lundquist & Purnell, 1987; Milgate *et al.*, 2005). *T. 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.*, 2009; Hunter *et al.*, 2008; Pérez *et al.*, 2009a; Pérez *et al.*, 2009b).

It has been suspected that *T. nubilosa* might represent a species complex, since 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 quite common throughout the tree of life (Bickford *et al.*, 2007), and in fungi in particular, since 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). To recognize species within species complexes multiple gene regions are typically sequenced, using an approach also known as Multi

Locus 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 have 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 (Grünig *et al.*, 2007; O'Donnell *et al.*, 2004; 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 (Barber *et al.*, 2008; Carnegie, 2007a; Maxwell *et al.*, 2001; Mohammed *et al.*, 2003). 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 cryptic species genetically isolated in nature. This was achieved 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 (1) determined the most likely center of origin and the human-introduced area of distribution for each population/phylogenetic group, (2) tested whether recombination or hybridization have occurred recently among native and human-introduced pathogen

populations and (3) 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* x *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). *T. 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; Pérez *et al.*, 2009b) (Table 1).

In all cases, sampling was done 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 analyzed, 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 due to repeated genotypes (Grünwald *et al.*, 2003), the matrix of data were clone-corrected using GENCLONE v 2.0 (Arnaud-Haond & Belkhir, 2007), retaining only one representative isolate per genotype for the analyses. A total of 112 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 datasets). An exception was made when comparing genetic diversity among native forests and adjacent commercial plantations because 6 MLHs were shared among those types of forests. Therefore, these 6 MLHs were included in each data subset totaling 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 analyze 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 analyzed in STRUCTURE v 2.2 (Pritchard *et al.*, 2000) to determine the optimal number of populations (K) using a Bayesian Monte Carlo Markov Chain (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 'non-admixture 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 v 2.2 (Pritchard *et al.*, 2000). To further investigate potential population sub-structuring within each *K* group, new sets of MCMC iterations were performed in STRUCTURE v 2.2 (Pritchard *et al.*, 2000). The procedure was essentially the same as described above 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 isolates (or 16 isolates, see below) were chosen to represent groups identified in STRUCTURE and PCA analyses. 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). Phylogenetic reconstructions were based on 29 gene regions. These included the internal transcribed spacers (ITS1 and ITS2) (n=32) (White *et al.*, 1990), portions of the β-tubulin-1 and β-tubulin-2 gene regions (n=32) (Glass, Donaldson, 1995), and 27 polymorphic anonymous loci from the genome of *T. nubilosa* (n=16) (Pérez, 2010). The ITS region was amplified using the primer pair ITS-1 and ITS-4 (White *et al.*, 1990), the β-tubulin-1 and β-tubulin-2 gene regions were amplified using the primers Bt-1a, Bt-1b, Bt-2a and Bt-2b (Glass, Donaldson, 1995) and the 27 anonymous loci using the primer pairs developed recently 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 analyzed, edited and aligned in MEGA v 3.1 (Kumar *et al.*, 2004). All sequences were deposited in GenBank (HQ130795-HQ131328). For parsimony analysis, heuristic searches were conducted in PAUP v 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 was set at 100. Indels were treated as a single evolutionary eventand 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 v 3.1 (Kumar *et al.*, 2004).

Phylogenetic reconstructions were based on 29 gene regions, where each gene region was initially analyzed separately. Additionally, haplotype networks were constructed pooling DNA sequence data from all 29 loci. Haplotype networks were constructed using the software TCS v 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 5^{th} state.

Gene and genotypic diversity

Isolates were assigned to groups using STRUCTURE (Pritchard *et al.*, 2000). In this way, populations were defined according to the STRUCTURE grouping algorithms and the geographic origin of the isolates. The number of Multi Locus Haplotypes (MLHs) per

population was calculated using GENCLONE v 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 v 1.31 for each population (Yeh *et al.*, 1999). In all cases clone-corrected datasets were used. To compare the genetic diversity among geographic 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 v 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 v 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 (Chen *et al.*, 1994; Stoddart, Taylor, 1988). 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).

MLHs are likely to arise in the *T. nubilosa* population through sexual reproduction in [~~both homothallic~~ – BUT homothallic reproduction of a haploid does not

result in any change – I think you need to remove this part? ~~(intra-haploid mating) and~~] heterothallic fashion (outcrossing) (Giraud *et al.*, 2008; Pérez *et al.*, 2010). The contribution of each mechanism to the general genotypic diversity was investigated calculating the P_{sex} index in GENCLONE v 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 sexual events (outcrossing) (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 v 3.11 (Excoffier *et al.*, 2005). An AMOVA test was run for the entire dataset to detremine the hierarchical partitioning of the molecular variation among groups (A, B and C), among geographical populations within groups and within populations. Here groups were defined based on the STRUCTURE grouping algorithms as well as the geographic origin of the isolates. All isolates belonging to the same STRUCTURE group (A, B or C) and collected in the same geographic area (NSW, Tasmania or Victoria) were pooled to perform this AMOVA test regardless of they were collected on different sites on native forests or plantations.

A second AMOVA test aimed to compare *T. nubilosa* populations collected in native forests or commercial plantations. This was possibleonly in Tasmania and Victoria. In this way clone corrected *T. nubilosa* populations collected on native forests (collecting sites 5, 6, 8, 12 and 14, see Table 1) were compared against *T. nubilosa* populations collected on commercial plantations (collecting sites 10, 11, 20 and 22, see

Table 1). Sites with a sample size was lesser than 5 MLHs were excluded from this analysis.

To analyze the genetic differentiation as a function of the geographic distance, Mantel tests were conducted in AIS (Alleles in Space) 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 datasets). 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 (Supplementary Fig. 1a). For the best choice of K, ΔK was calculated and plotted against K, where a clear peak was observed when $K = 3$ (Supplementary Fig. 1b). In addition, every single 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 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 populations.

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 sub-structuring 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 analyses 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 geographic 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 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 analyses (Fig. 3b).

Phylogenetic reconstructions

To interrogate the biological relevance of the three groups recognized by STRUCTURE and PCA analyses, 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). Likewise, DNA sequencing of 27 anonymous loci yielded 4020 characters where 50 of them 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). Isolates from Group A clustered together with those from Group C and isolates in Group B clustered apart (Fig. 4). Identical results were observed on 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) where isolates from Group A clustered together with those from Group C and isolates in Group B clustered separately (TreeBase S12689). To the contrary, in the β-tubulin phylogram isolates from Group A clustered together with those from Group B 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). A cluster accommodated all haplotypes from Groups A and C and another cluster accommodated all haplotypes from Group B (Fig. 5). Haplotypes from Group A grouped together and were separated by seven missing intermediates from those haplotypes of Group C. Haplotypes from Group B grouped apart from those of Groups A and C and were separated by 42 missing intermediates (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, less than one 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, 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 6 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 populations 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, which occurred more than once in the NSW, Tasmanian and Victorian populations (Fig. 6). Alternatively, just 9 (21 %) of 42 MLHs that occurred more than once in Australia showed non-significant 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 dataset 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 populations belonging to each Group (55.5 %) and relatively low variation (15.2 %) was distributed among populations from distinct geographic 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 test, a relatively high percentage of variation (30.7 %) was distributed among Groups B and C (Table 7). The correlation between genetic and geographic distance (Mantel test) was significant ($r=0.559$, $p<0.001$).

Discussion

In this study, three genetically isolated groups were identified amongst 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 either recombination or 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 not gene 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 of isolates treated as the single species, *T. nubilosa*. Very few alleles were shared among groups (on average less than one 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 reflects discrete species (Crous *et al.*, 2006; Goodwin *et al.*, 2001). 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 analyzed and grouped apart in a well supported clade in the haplotype network. Although taxonomic treatment of *T. nubilosa* was not the intention of this study, applying the Genealogical Concordance Phylogenetic Species Concept proposed by Taylor *et al*. (2000), our results shows 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) as is being discovered for many ascomycete fungi via the application of contemporary molecular genetic tools and the GCPSC (O'Donnell *et al.*, 2004; Pavlic *et al.*, 2009; Taylor *et al.*, 2006).

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 analyzed, 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 suggests that Group A is in the process of speciation and has been genetically isolated from Groups B & C since recent times, allowing sufficient time for microsatellite alleles to be fixed in the population but not sufficient time for DNA polymorphisms fixation (Taylor *et al.*, 2000).

Guillermo, I do not like the above paragraph, you are mixing up two things, "one rate of change" and the other "fixation of alleles" – they are not the same thing. I think the paragraph needs to re-written to focus on the one OR the other – or to be clear that these are two different processes

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 due to the sequential founder events, where introduced populations originate from already established populations in non-native areas of distribution (Clegg *et al.*, 2002; Lombaert *et al.*, 2010). While introduced populations can be highly diverse (Estoup & Guillemaud, 2010; Kolbe *et al.*, 2004; Mitchell *et al.*, 2010) due to multiple introductions and subsequent admixture (Dlugosch & Parker, 2007; Dutech *et al.*, 2010; Smart & Fry, 2001), our results suggest that is not the case for *T. nubilosa* were 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 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). Since 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.

Contrasting values of both gene and genotypic diversity were observed among different Australian populations. 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. In this way two clearly introduced populations and consequent biological invasions were identified within Australia.. 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 suggests that *T. nubilosa* was introduced into WA after 1994 (Maxwell *et al.*, 2001) as it was not reported in previous surveys conducted in this area (Carnegie *et al.*, 1997).

The discovery of an introduced population in NSW was surprising. 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. *T. nubilosa* was not commonly observed in early 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; Carnegie, 2007b). It is probable that the introduction of isolates representing Group C in NSW account 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 co-exists with

isolates of native Group A in NSW, even in the same plantation (Tabulam), isolates have neither hybridized nor recombined. 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).

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 due to 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, co-existing 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 align with the native distribution of *E. globulus* in Tasmania and Victoria (Potts *et al.*, 2004).

Genotypic diversity was in general extremely low to moderate. In addition, very few, commonly occurring MLHs were present in all introduced populations from WA, New Zealand, Brazil and Uruguay. Conversely, most MLHs (75 %) occurred once or

twice in the Australian population. The asexual 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). A previous study conducted at micro and macro spatial hierarchical levels in South Africa has shown that while sexual reproduction through selfing (intra-haploid mating sensu Giraud *et al.* (2008)) is the main reproductive strategy of *T. nubilosa*, outcrossing is also common (at low levels) in populations (Pérez *et al.*, 2010). Results presented in this study are fully consistent with those observations. P_{sex} values were significant for all introduced populations ($p << 0.001$) suggesting that sexual reproduction through intra-haploid mating is common in this species as been observed in other plant pathogens (Billiard *et al.*, 2011; Giraud *et al.*, 2008). In addition, those low frequent 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 intra-haploid 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 geographic 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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References

Arnaud-Haond S, Belkhir K (2007) GENCLONE: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Molecular Ecology Notes* **7**, 15-17.

Arnaud-Haond S, Duarte CM, Alberto F, Serrão EA (2007) Standardizing methods to address clonality in population studies. *Molecular Ecology* **16**, 5115-5139.

Barber PA, Carnegie AJ, Burgess TI, Keane PJ (2008) Leaf diseases caused by *Mycosphaerella* species in *Eucalyptus globulus* plantations and nearby native

forest in the Green Triangle Region of southern Australia. *Australasian Plant Pathology* **37**, 372-481.
Bickford D, Lohman DJ, Sodhi NS*, et al.* (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22**, 148-155.
Billiard S, López-Villavicencio M, Devier B*, et al.* (2011) Having sex, yes, but with whom? Inferences from fungi on the evolution of anisogamy and mating types. *Biological Reviews* **86**, 421-442.
Booker MIH, Kleinig DA (1994) Field guide to eucalypts. Inkata, Sydney.
Brasier CM (2008) The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* **57**, 792-808.
Brown JKM, Hovmøller MS (2002) Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. *Science* **297**, 537-541.
Burgess TI, Barber PA, Sufaati S*, et al.* (2007) *Mycosphaerella* spp. on *Eucalyptus* in Asia; new species; new hosts and new records. *Fungal Diversity* **24**, 135-157.
Burgess TI, Sakalidis ML, Hardy GESJ (2006) Gene flow of the canker pathogen *Botryosphaeria australis* between *Eucalyptus globulus* plantations and native eucalypt forests in Western Australia. *Austral Ecology* **31**, 559-566.
Burnett JH (2003) *Fungal populations and species* Oxford University Press, Oxford.
Carnegie AJ (2007a) Forest health condition in New South Wales, Australia, 1996-2005. I. Fungi recorded from eucalypt plantations during forest health surveys. *Australasian Plant Pathology* **36**, 213-224.
Carnegie AJ (2007b) Forest health condition in New South Wales, Australia, 1996-2005. II. Fungal damage recorded in eucalypt plantations during forest health surveys and their management. *Australasian Plant Pathology* **36**, 225-239.
Carnegie AJ, Ades PK (2003) *Mycosphaerella* leaf disease reduces growth of plantation-grown *Eucalyptus globulus*. *Australian Forestry* **66**, 113-119.
Carnegie AJ, Burgess TI, Beilharz V, Wingfield MJ (2007) New species of *Mycosphaerella* from *Myrtaceae* in plantations and native forests in eastern Australia. *Mycologia* **99**, 461-474.
Carnegie AJ, Keane PJ, Podger FD (1997) The impact of three species of *Mycosphaerella* newly recorded on *Eucalyptus* in Western Australia. *Australasian Plant Pathology* **26**, 71-77.
Clegg SM, Degnan SM, Kikkawa J*, et al.* (2002) Genetic consequences of sequential founder events by an island-colonizing bird. *Proceedings of the National Academy of Sciences of the USA* **99**, 8127-8132.
Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657-1659.
Colautti RI, Grigorovich IA, MacIsaac HJ (2006) Propagule pressure: a null model for biological invasions. *Biological Invasions* **8**, 1023-1037.
Crous PW (1998) *Mycosphaerella* spp. and their anamorphs associated with leaf spot diseases of *Eucalyptus*. *Mycologia Memoir* **21**, 1-170.
Crous PW, Braun U, Groenewald JZ (2007) *Mycosphaerella* is polyphyletic. *Studies in Mycology* **58**, 1-32.
Crous PW, Groenewald JZ, Mansilla PJ, Hunter GC, Wingfield MJ (2004) Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. *Studies in Mycology* **50**, 195-214.

Crous PW, Wingfield MJ, Mansilla JP, Alfenas AC, Groenewald JZ (2006) Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. II. *Studies in Mycology* **55**, 99-131.

Chen RS, Boeger JM, McDonald BA (1994) Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology* **3**, 209-218.

Desprez-Loustau M-L, Robin C, Buée M*, et al.* (2007) The fungal dimension of biological invasions. *Trends in Ecology & Evolution* **22**, 472-480.

Dick M (1982) Leaf-inhabiting fungi of eucalypts in New Zealand. *New Zealand Journal of Forestry Science* **12**, 525-537.

Dlugosch KM, Parker IM (2007) Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Molecular Ecology* **17**, 431-449.

Dutech C, Fabreguettes O, Capdevielle X, Robin C (2010) Multiple introductions of divergent genetic lineages in an invasive fungal pathogen, *Cryphonectria parasitica*, in France. *Heredity* **105**, 220-228.

Estoup A, Guillemaud T (2010) Reconstructing routes of invasion using genetic data: why, how and so what? *Molecular Ecology* **19**, 4113-4130.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**, 2611-2620.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47-50.

Ficetola GF, Bonin A, Miaud C (2008) Population genetics reveals origin and number of founders in a biological invasion. *Molecular Ecology* **17**, 773-782.

Fry WE, Goodwin SB, Dyer AT*, et al.* (1993) Historical and recent migrations of *Phytophthora infestans*: Chronology, pathways, and implications. *Plant Disease* **77**, 653-661.

Gilbert GS (2002) Evolutionary ecology of plant diseases in natural ecosystems. *Annual Review of Phytopathology* **40**, 13-43.

Giraud T, Roxana Y, López-Villavicencio M, Refrégier G, Hood ME (2008) Mating system of the anther smut fungus *Microbotryum violaceum*: selfing under heterothallism. *Eukaryotic Cell* **7**, 765-775.

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**, 1323 -1330.

Goodwin SB, Dunkle LD, Zismann VL (2001) Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the Internal Transcribed Spacer region of ribosomal DNA. *Phytopathology* **91**, 648-658.

Grünig CR, Brunner PC, Duò A, Sieber TN (2007) Suitability of methods for species recognition in the *Phialocephala fortinii-Acephala applanata* species complex using DNA analysis. *Fungal Genetics and Biology* **44**, 773-788.

Grünwald NJ, Goodwin SB, Milgroom MG, Fry WE (2003) Analysis of genotypic diversity data for populations of microorganisms. *Phytopathology* **93**, 738-746.

Hayden HL, Carlier J, Aitken EAB (2003) Genetic structure of *Mycosphaerella fijiensis* populations from Australia, Papua New Guinea and the Pacific Islands. *Plant Pathology* **52**, 703-712.

Hunter GC, Cortinas MN, Wingfield BD, Crous PW, Wingfield MJ (2006) Development of polymorphic microsatellite markers for the *Eucalyptus* leaf pathogen *Mycosphaerella nubilosa*. *Molecular Ecology Notes* **6**, 900-903.

Hunter GC, Crous PW, Carnegie AJ, Wingfield MJ (2009) *Teratosphaeria nubilosa*, a serious leaf disease pathogen of *Eucalyptus* spp. in native and introduced areas. *Molecular Plant Pathology* **10**, 1-14.

Hunter GC, van der Merwe NA, Burgess TI*, et al.* (2008) Global movement and population biology of *Mycosphaerella nubilosa* infecting leaves of cold-tolerant *Eucalyptus globulus* and *E. nitens*. *Plant Pathology* **57**, 235-242.

Jackson SL, Maxwell A, Burgess TI, Hardy GES, Dell B (2008) Incidence and new records of *Mycosphaerella* species within a *Eucalyptus globulus* plantation in Western Australia. *Forest Ecology and Management* **255**, 3931-3937.

Kolar CS, Lodge DM (2001) Progress in invasion biology: predicting invaders. *Trends in Ecology & Evolution* **16**, 199-204.

Kolbe JJ, Glor RE, Rodriguez-Schettino L*, et al.* (2004) Genetic variation increases during biological invasion by a Cuban lizard. *Nature* **4**, 177-181.

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* **5**, 150-163.

Lockwood JL, Cassey P, Blackburn TM (2005) The role of propagule pressure in explaining species invasions. *Trends in Ecology & Evolution* **20**, 223-228.

Lombaert E, Guillemaud T, Cornuet J-M*, et al.* (2010) Bridgehead effect in the worldwide invasion of the biocontrol harlequin ladybird. *PLoS ONE* **5**, e9743.

Lundquist JE, Purnell RC (1987) Effects of Mycosphaerella leaf spot on growth of *Eucalyptus nitens*. *Plant Disease* **71**, 1025-1029.

Maxwell A, Hardy GESJ, Dell B (2001) First record of *Mycosphaerella nubilosa* in Western Australia. *Australasian Plant Pathology* **30**, 65.

McDonald BA, Linde C (2002) Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology* **40**, 349-379.

Milgate AW, Vaillancourt RE, Mohammed C, Powell M, Potts BM (2005) Genetic structure of a *Mycosphaerella cryptica* population. *Australasian Plant Pathology* **34**, 345-354.

Milgroom MG (1996) Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology* **34**, 457-477.

Miller MP (2005) Alleles In Space: Computer software for the joint analysis of interindividual spatial and genetic information. *Journal of Heredity* **96**, 722-724.

Mitchell CE, Blumenthal D, Jarosik V, Puckett EE, Pysek P (2010) Controls on pathogen species richness in plants' introduced and native ranges: roles of residence time, range size and host traits. *Ecology Letters* **13**, 1525-1535.

Mohammed CL, Wardlaw T, Smith A*, et al.* (2003) Mycosphaerella leaf diseases of temperate eucalypts around the Southern Pacific Rim. *New Zealand Journal of Forestry Science* **33**, 362-372.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the USA* **70**, 3321-3323.

Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution* **29**, 1-10.

Nichols JD, Smith RG, Grant J, Glencross K (2010) Subtropical eucalypt plantations in eastern Australia. *Australian Forestry* **73**, 53-62.

O'Donnell K, Ward TJ, Geiser DM, Kistler HC, Aoki T (2004) Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology* **41**, 600-623.

Pavlic D, Slippers B, Coutinho TA, Wingfield MJ (2009) Multiple gene genealogies and phenotypic data reveal cryptic species of the Botryosphaeriaceae: A case study on the *Neofusicoccum parvum/N. ribis* complex. *Molecular Phylogenetics and Evolution* **51**, 259-268.

Pérez G (2010) Global genetic diversity of the *Eucalyptus* leaf pathogen *Teratosphaeria nubilosa* species complex in native forests and commercial plantations. *University of Pretoria, Pretoria, South Africa*, p260.

Pérez G, Hunter GC, Slippers B*, et al.* (2009a) *Teratosphaeria* (*Mycosphaerella*) *nubilosa*, the causal agent of Mycosphaerella Leaf Disease (MLD), recently introduced into Uruguay. *European Journal of Plant Pathology* **125**, 109-118.

Pérez G, Slippers B, Wingfield BD, Finkenauer E, Wingfield MJ (2009b) Mycosphaerella leaf disease (MLD) outbreak on *Eucalyptus globulus* in Brazil caused by *Teratosphaeria (Mycosphaerella) nubilosa*. *Phytopathologia Mediterranea* **48**, 302-306.

Pérez G, Slippers B, Wingfield BD, Hunter GC, Wingfield MJ (2010) Micro- and macro spatial scale analyses illustrates mixed mating strategies and extensive geneflow in populations of an invasive haploid pathogen. *Molecular Ecology* **19**, 1801-1813.

Philibert A, Desprez-Loustau M-L, Fabre B*, et al.* (2011) Predicting invasion success of forest pathogenic fungi from species traits. *Journal of Applied Ecology* **48**, 1381-1390.

Potts BM, Pederick LA (2000) Morphology, phylogeny, origin, distribution and genetic diversity of eucalypts. In: *Diseases and pathogens of eucalypts* (eds. Keane PJ, Kile GA, Podger FD, Brown BN), pp. 11-32. CSIRO Publishing, Australia.

Potts BM, Vaillancourt RE, Jordan G*, et al.* (2004) Exploration of the *Eucalyptus globulus* gene pool. *Proceedings IUFRO Conference*, 46-61.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.

Smart CD, Fry WE (2001) Invasions by the late blight pathogen: renewed sex and enhanced fitness. *Biological Invasions* **3**, 235-243.

Stoddart JA, Taylor JF (1988) Genotype diversity: estimation and prediction in samples. *Genetics* **118**, 705-711.

Stukenbrock EH, Banke S, Javan-Nikkhah M, McDonald BA (2007) Origin and domestication of the fungal wheat pathogen *Mycosphaerella graminicola* via sympatric speciation. *Molecular Biology and Evolution* **24**, 398-411.

Stukenbrock EH, McDonald BA (2008) The origins of plant pathogens in agro-ecosystems. *Annual Review of Phytopathology* **46**, 75-100.

Suarez AV, Tsutsui ND (2008) The evolutionary consequences of biological invasions. *Molecular Ecology* **17**, 351-360.

Swofford DL (2002) PAUP*: phylogenetic analysis using parsimony (*and other methods). *Version 4.0b10. Sinauer Associates, Sunderland, MA*.

Taylor JW, Fisher MC (2003) Fungal multilocus sequence typing - it's not just for bacteria. *Current Opinion in Microbiology* **6**, 351-356.

Taylor JW, Jacobson DJ, Fisher MC (1999) The evolution of asexual fungi: Reproduction, speciation and classification. *Annual Review of Phytopathology* **37**, 197-246.

Taylor JW, Jacobson DJ, Kroken S*, et al.* (2000) Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**, 21-32.

Taylor JW, Turner E, Townsend JP, Dettman JR, Jacobson D (2006) Eukaryotic microbes, species recognition and the geographic limits of species: examples from the kingdom Fungi. *Philosophical Transactions of the Royal Society Biology* **361**, 1947-1963.

Tibbits WN, Boomsma DB, Jarvis S (1997) Distribution, biology, genetics and improvement programs for *Eucalyptus globulus* and *E. nitens* around the world, 81-95.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (eds. Innis MA, Gelfand DH, Snindky JJ, White TJ), pp. 315-322. Academic Press, San Diego.

Wingfield MJ (2003) Increasing threat of diseases to exotic plantation forests in the Southern Hemisphere: Lessons from Cryphonectria canker. *Australasian Plant Pathology* **32**, 133-139.

Wingfield MJ, Slippers B, Hurley BP*, et al.* (2008) Eucalypt pests and diseases: Growing threats to plantation productivity. *Southern Forests* **70**, 139-144.

Yeh FC, Yang RC, Boyle T (1999) POPGENE. Version 1·31. Microsoft Windows Based Freeware for Population Genetic Analysis.

Zhan J, Pettway RE, McDonald BA (2003) The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. *Fungal Genetics and Biology* **38**, 286-297.

Zhou X, Burgess TI, De Beer W*, et al.* (2007) High intercontinental migration rates and population admixture in the sapstain fungus *Ophiostoma ips*. *Molecular Ecology* **16**, 89-99.

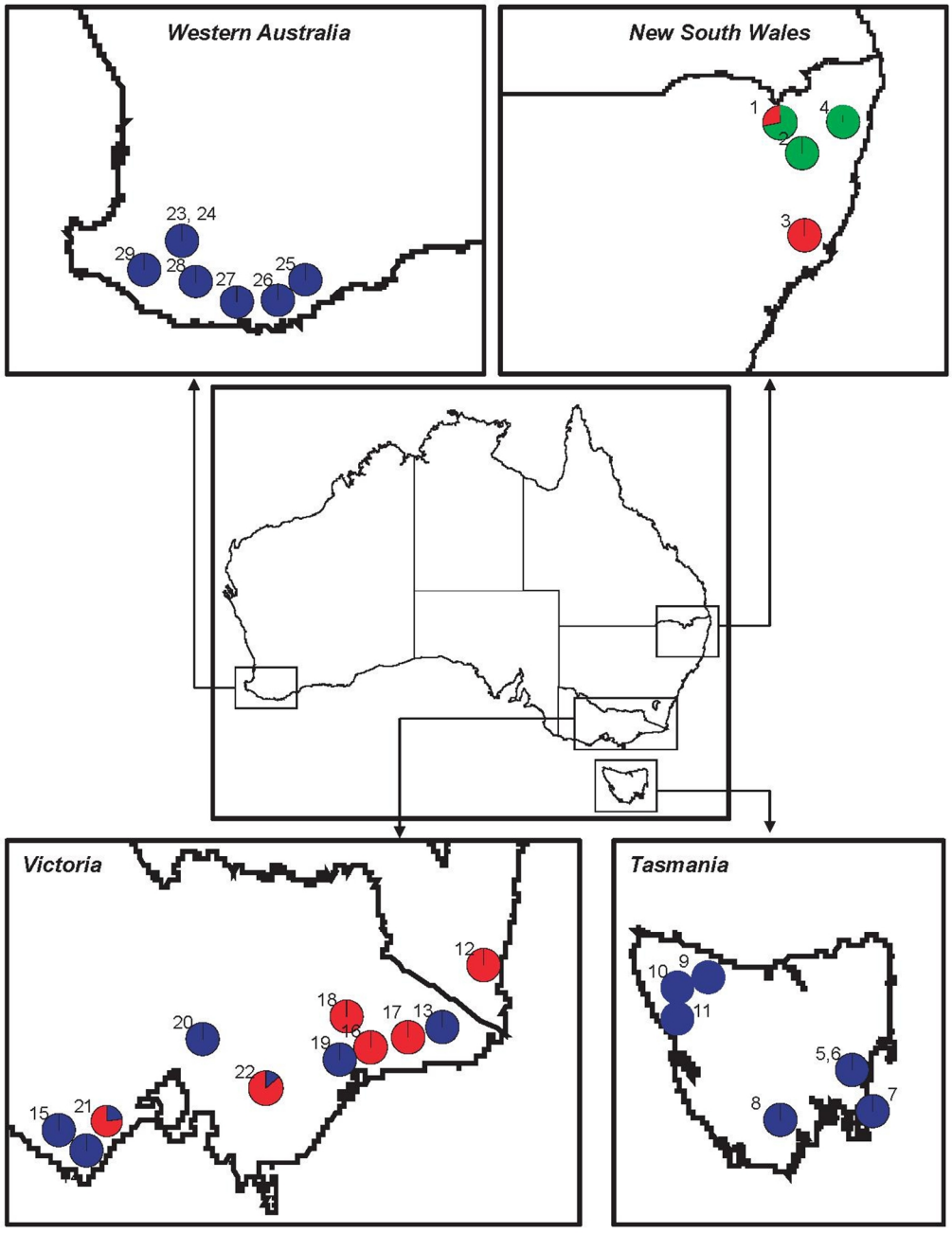

Figure 1. *Teratosphaeria nubilosa* populations collected from native forests and *Eucalyptus* plantations in 29 locations in Australia. Pie charts show the relative proportion of isolates belonging to Group A (green), Group B (blue) and Group C (red) in each location. See Table 1 for reference numbers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

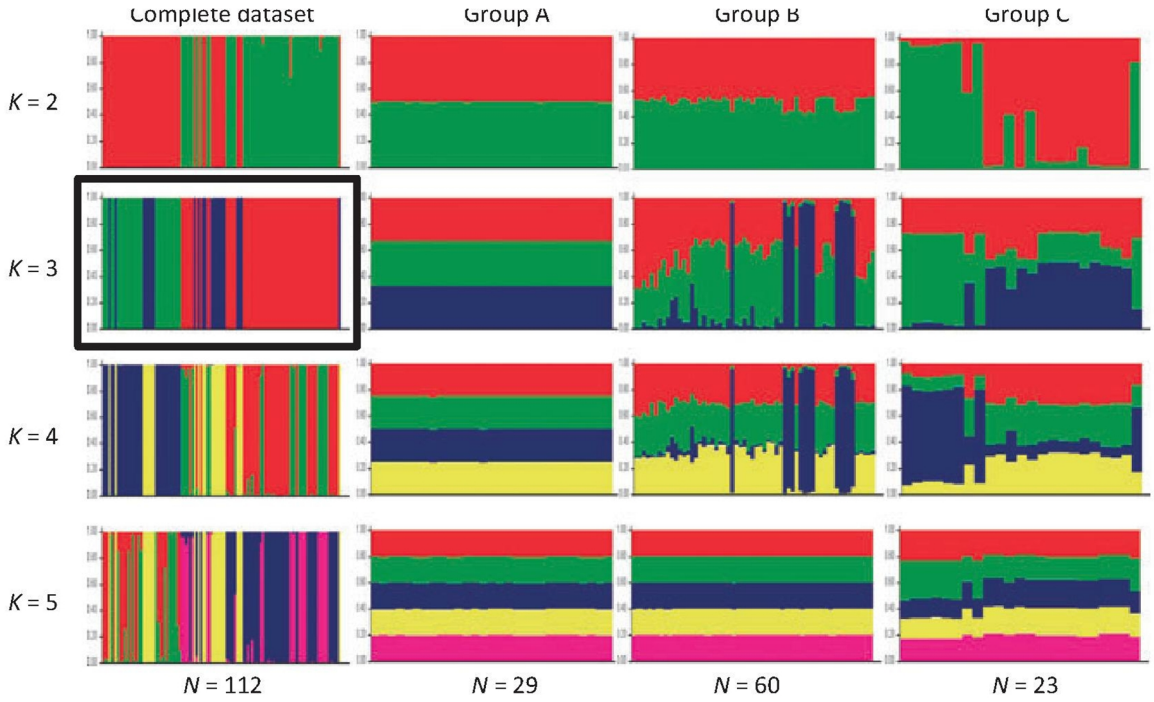

Figure 2. Assignment of *T. nubilosa* MLHs into growing *K* populations using STRUCTURE for the complete dataset and for each group separately. The best estimate for the real number of distinct populations *K=3* is boxed.

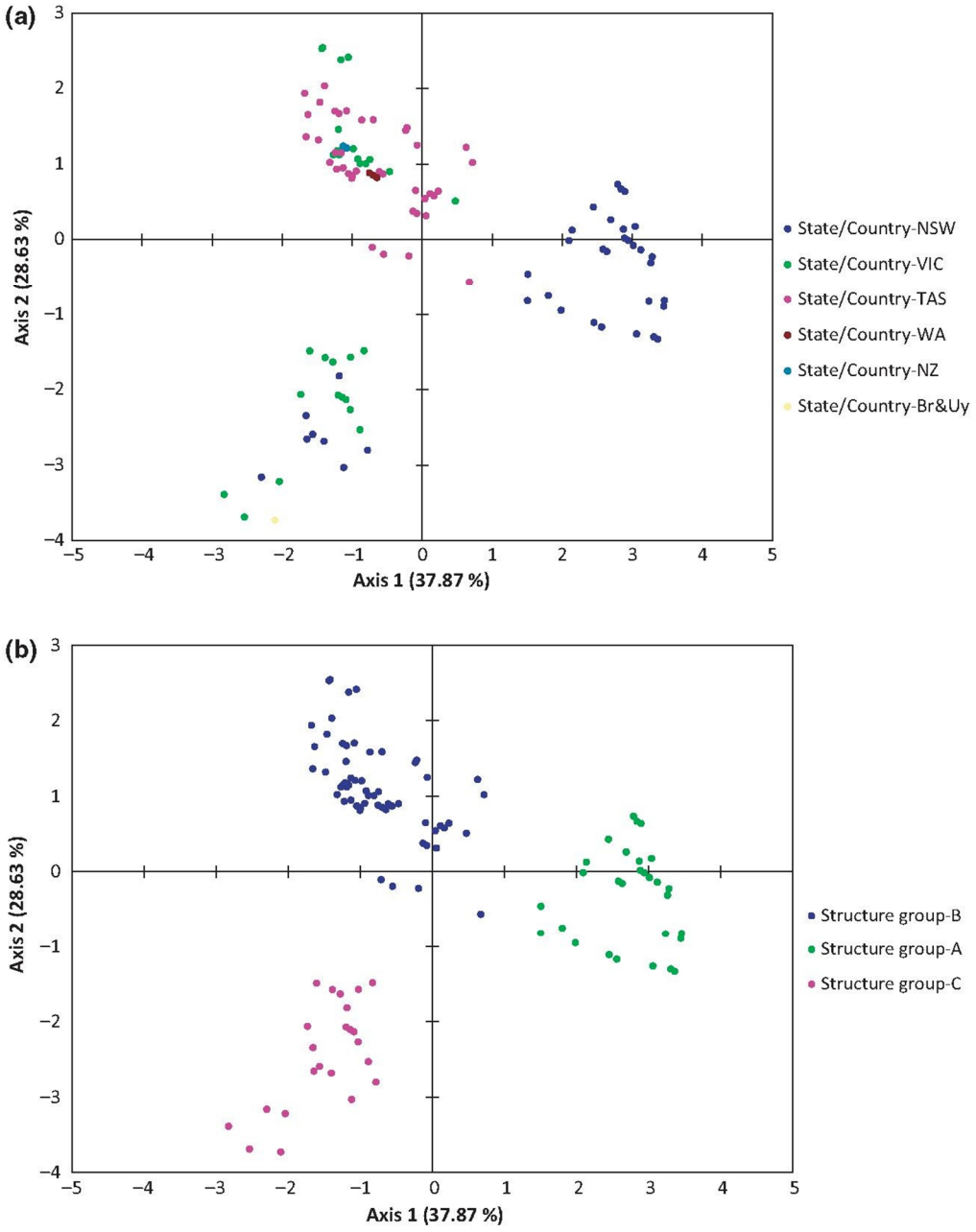

Figure 3. Principal Component Analysis (PCA) performed on the 112 MLHs of *T. nubilosa* based on eight microsatellite markers. Each point represents a MLH. **a.** Point colors representing the geographical origin of isolates. **b.** Same PCA plot where point colors represent the STRUCTURE grouping of isolates.

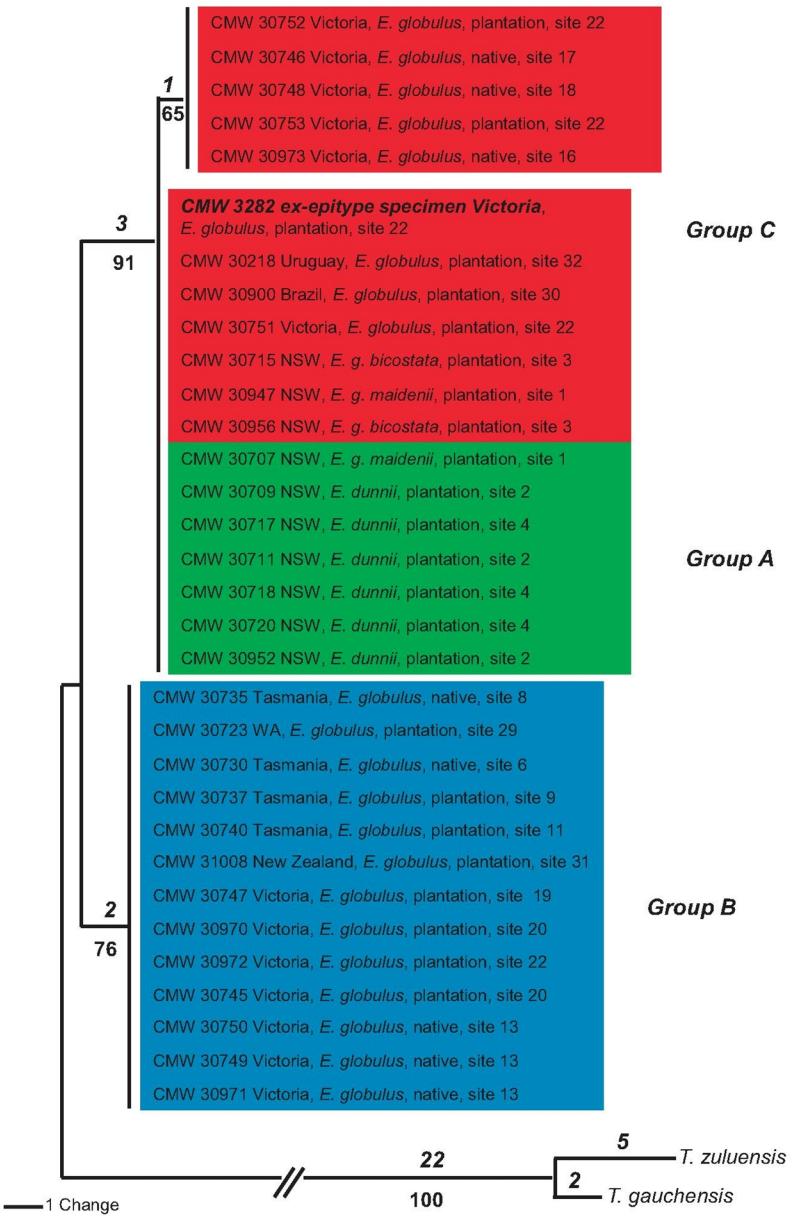

Figure 4. Consensus ITS phylogram obtained using parsimony and heuristic analysis. Branch length and bootstrap support values after 1000 randomizations are shown above and below the branching points, respectively. The geographic origin, type of forest (native or commercial plantation), collecting site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

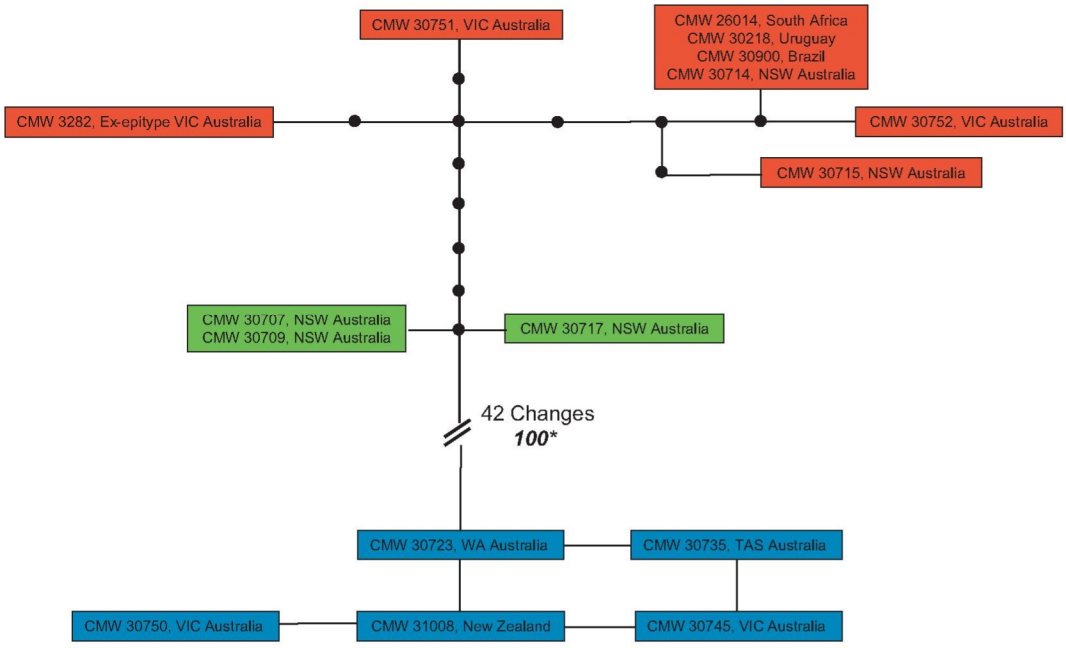

Figure 5. Parsimony haplotype network for the 29 loci analyzed. A total of 5348 characters were analyzed, 62 characters were variable, 55 of which were parsimony informative. Indels were treated as the 5th state. Dots indicate hypothetical missing intermediate haplotypes. (*) Bootstrap support value after 1000 randomizations. The geographic origin and STRUCTURE grouping of isolates are also provided; Group A (green), Group B (blue) and Group C (red).

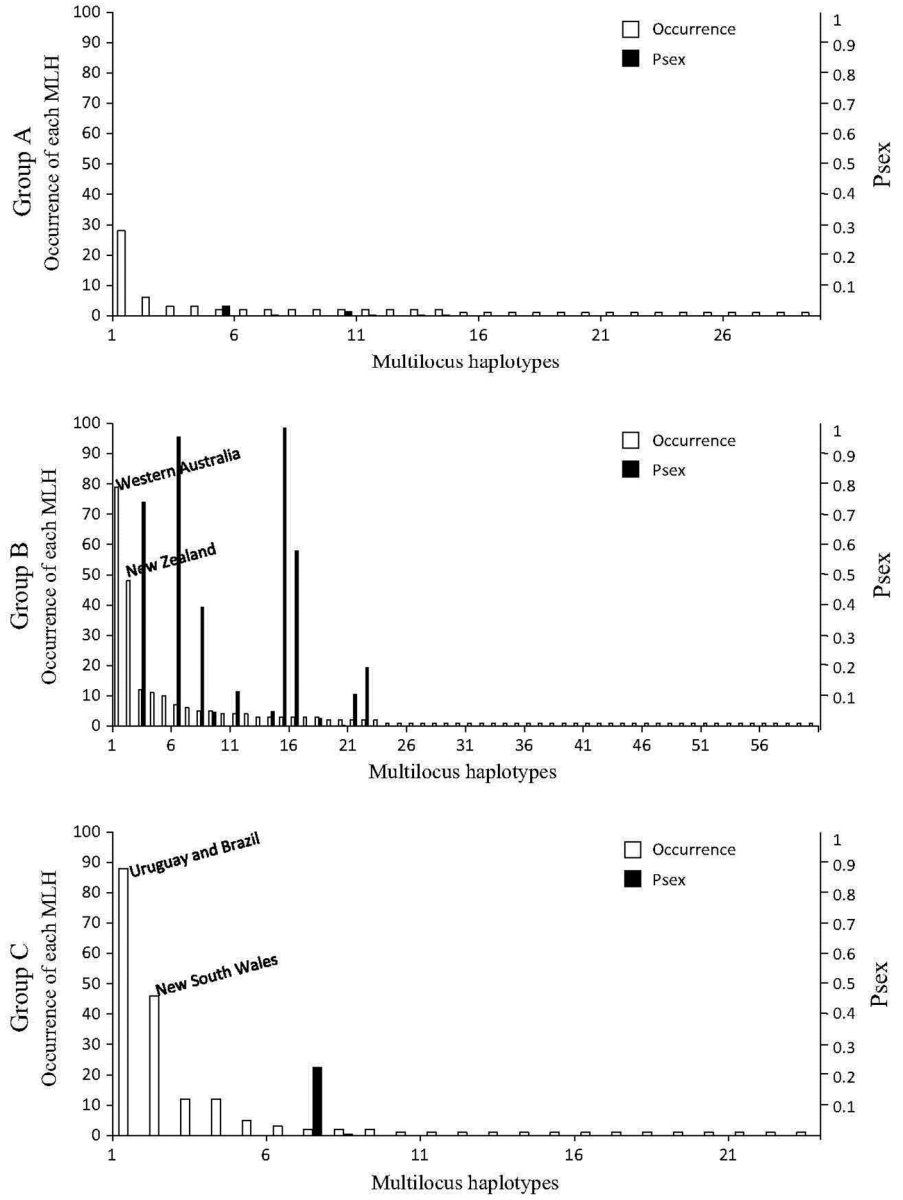

Figure 6. Occurrence of the 112 *T. nubilosa* MLHs (white) assigned to Group A (n=29) Group B (n=60) and Group C (n=23) and P_{sex} probabilities associated to each MLH (black).

Table 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.

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.

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	6 105	T6 125
C	Victoria	CMW 3282[t]	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.

[t]*Teratosphaeria nubilosa* ex-epitype culture.

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.

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.

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.

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.