Teratosphaeria (Mycosphaerella) nubilosa, the causal agent of Mycosphaerella leaf disease (MLD), recently introduced into Uruguay

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Abstract Mycosphaerella leaf disease on *Eucalyptus* is well known in Uruguay but none of the more serious *Mycosphaerella* spp. and *Teratosphaeria* spp. causing this disease have yet been found. In the autumn of 2007, more severe defoliation than has been known in the past and associated with symptoms resembling *Mycosphaerella* infections was observed on *Eucalyptus globulus*. Isolations and identifications based on morphology and DNA sequence comparisons showed that the causal agent of the defoliation is the well known and serious pathogen *Teratosphaeria nubilosa* (=*Mycosphaerella nubilosa*). This is the first record of the pathogen in South America. Using ten microsatellite loci previously developed for *T. nubilosa*, only one multilocus haplotype was found from 46 *T. nubilosa* collected isolates. Interestingly, this haplotype was the same as one previously found in Portugal and Spain. The results suggest that *T. nubilosa* has recently been introduced into Uruguay and that it most likely originated from the Iberian Peninsula where *E. globulus* is widely planted.

Abbreviations
MLD   Mycosphaerella leaf disease

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Introduction

Commercial forestry in Uruguay using exotic species of *Pinus* and *Eucalyptus* has expanded rapidly during the course of the last two decades. *Eucalyptus* plantations now cover approximately 457,000 ha with >50% of this area being planted to *Eucalyptus globulus* (Petraglia and Dell'Acqua 2006). Other species commonly planted include *E. grandis* and *E. dunnii*. As in other areas of the world, the principal

end-use of these plantations is in the pulp and paper industry, which is growing rapidly in Uruguay.

More than 100 species of *Mycosphaerella* have been identified from *Eucalyptus*, where they primarily cause leaf spots (Burgess et al. 2007; Crous 1998; Crous et al. 2007). Collectively, these leaf diseases are referred to as Mycosphaerella leaf disease (MLD) (Crous 1998). However, Crous et al. (2007) have shown recently that the genus *Mycosphaerella* is polyphyletic and therefore several species occurring on *Eucalyptus* have been transferred to the genus *Teratosphaeria*. Despite the changes in the species names, the common name for the associated disease has not changed. Thus, MLD on *Eucalyptus* is currently caused by both *Mycosphaerella* spp. and *Teratosphaeria* spp. (Crous et al. 2007; Hunter et al. 2009). Mycosphaerella leaf disease reduces the photosynthetic capacity of leaves causing premature defoliation, shoot die-back and decreased growth resulting in significant growth losses (Lundquist and Purnell 1987; Milgate et al. 2005; Mohammed et al. 2003; Park and Keane 1982a).

Despite the fact that many species of *Teratosphaeria* and *Mycosphaerella* infect *Eucalyptus*, few studies have considered the pathogenicity of these fungi. Certain species such as *Teratosphaeria nubilosa* (= *Mycosphaerella nubilosa*) and *Teratosphaeria cryptica* (= *Mycosphaerella cryptica*) act as primary pathogens infecting living leaf tissue and acquiring nutrients from them in a hemibiotrophic fashion (Hunter et al. 2009; Park and Keane 1982b). These two species are considered as the most important causing MLD worldwide (Carnegie and Ades 2002; Hunter et al. 2009; Park and Keane 1982a). In contrast, many *Teratosphaeria* and *Mycosphaerella* species on *Eucalyptus* are apparently mildly pathogenic or secondary colonisers of necrotic tissue, following infection by more pathogenic species, insect damage or from natural leaf senescence (Crous 1998; Crous et al. 2006).

Alfenas et al. (2004) reported four species of *Mycosphaerella* and *Teratosphaeria* in Brazil, namely *M. marksii, M. parkii, T. suberosa* and *T. suttonii*. However, there are no reports of MLD resulting in severe losses either in Brazil (Alfenas et al. 2004) or in the rest of South America. Severe defoliation of *E. globulus* was observed in Chile during 2000 and while *M. grandis, M. lateralis, M. parkii, M. walkeri, T. africana* and *T. molleriana* were associated with lesions, the primary cause of the defoliation was

abiotic stress (Ahumada 2003). In Uruguay, several species of *Mycosphaerella* and *Teratosphaeria* have been reported, including *M. marksii, M. walkeri, M. vespa* and *T. suberosa* (Balmelli et al. 2004), but none are known as aggressive pathogens.

A recent population study of *T. nubilosa* suggests that the natural range of this species is eastern Australia (Hunter et al. 2008; 2009), coinciding with the natural distribution of *E. globulus* and *E. nitens* (Tibbits et al. 1997), two of the most susceptible species to *T. nubilosa* (Carnegie et al. 1998). The introduction of *Eucalyptus* spp. into many Southern Hemisphere countries during the course of the last century has allowed *T. nubilosa* to expand its geographical distribution considerably (Hunter et al. 2009). The first report of *T. nubilosa* (as *Mycosphaerella molleriana*) outside Australia was in 1950 in South Africa, although it is believed that it was present in that country as early as the 1930s (Lundquist and Purnell 1987). By 1982, *T. nubilosa* was known to be broadly distributed in New Zealand (Mohammed et al. 2003). The introduction of *T. nubilosa* into Western Australia most likely occurred between 1994 and 1999 (Maxwell et al. 2001). In 1995, it was reported in Kenya, Tanzania and Zambia (as *Mycosphaerella juvenis*) (Crous 1998) and in 2000 in Ethiopia (Hunter et al. 2008). *Teratosphaeria nubilosa* apparently arrived in Europe around 2001, where it was associated with *E. globulus* in four regions of Spain (Crous et al. 2004) and more recently in Portugal (Hunter et al. 2008). Given its widespread distribution as an alien invasive pathogen, it is interesting that *T. nubilosa* has not previously been reported from any South American country.

During the autumn of 2007, severe defoliation resulting from symptoms resembling those of MLD was observed on young *E. globulus* plantations in Uruguay. The change in the status of MLD in the country and the severity of the defoliation led to the suspicion that a new pathogen had been introduced into Uruguay. The aims of this study are to isolate and identify the causal agent of the disease. Once the pathogen had been identified, an investigation to determine the most probable origin of the pathogen was undertaken using 10 microsatellite loci. In this regard, the three possible areas of origin that were considered included Spain, because this country has acted as a source of seed and clonal material for Uruguayan forestry (Balmelli 2006); South Africa, the country where *E. globulus* was first introduced as a

non-native for forestry in 1853 (Potts et al. 2004) and Australia, which is known to represent the native range of *T. nubilosa* and most *Eucalyptus* grown in Uruguay (Balmelli 2006; Hunter et al. 2008).

Materials and methods

Sample collection and lesion description

Diseased juvenile, intermediate and adult *Eucalyptus* leaves showing symptoms of MLD were collected during December 2007. Juvenile leaves were obtained from young trees, coppice growth and epicormic shoots on older trees. Plantations surveyed included *E. globulus* subsp. *globulus*, *E. globulus* subsp. *maidenii* and *E. dunnii* of different ages. Sampling was conducted in six provinces of Uruguay, namely Colonia, Lavalleja, Maldonado, Paysandú, Río Negro and Rocha. *Eucalyptus* leaves were air-dried and transported to a laboratory for detailed description of the lesions and isolations.

Fungal isolations

Lesions on the affected leaves were typical of those caused by a *Mycosphaerella* sp. or *Teratosphaeria* sp. with pseudothecia abundant on the abaxial surfaces. Fungal strains were isolated using the method described by Crous (1998). Lesions were excised from the leaves, hydrated for 2 h and attached to the undersides of Petri dish lids, with pseudothecia facing downwards over 2% Malt Extract Agar (MEA). After 24 h, germinating ascospores were observed on the surface of the agar, photographed using phase contrast microscopy and classified according to the germination patterns described by Crous (1998). Single germinating ascospores were transferred to fresh 2% MEA plates to obtain pure cultures, initially incubated at 15°C for 2 weeks and then at 20°C for a further 30 days. All cultures obtained in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction, PCR amplification and sequencing

Mycelium was scraped from the surface of actively growing cultures, freeze-dried and ground to a fine powder using sterile metal beads on a Mixer Mill type MM 301 Retsch® tissue lyser (Retsch, Germany) for 3 min at a frequency of 30 cycles s^{-1} DNA was isolated following the protocol described by Cortinas et al. (2006).

The internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene of the rDNA operon were amplified using the primer pairs ITS-1 and ITS-4 (White et al. 1990). A portion of the beta tubulin-2 gene region was amplified using the primers Bt-2a and Bt-2b (Glass and Donaldson 1995). PCR reactions were conducted with the same reaction mixture as those utilised by Cortinas et al. (2006) with an initial denaturation at 95°C for 2 min, followed by 35 cycles of 30s at 94°C, 30 s at 57°C, 1 min at 72°C and a final elongation step of 7 min at 72°C.

For DNA sequencing reactions, PCR products were purified using Sephadex G-50 (Sigma Aldrich) in Centri-Sep spin columns (Princeton Separations) following the manufacturer's instructions. Purified amplicons were sequenced using the ABI Prism Big Dye Terminator Cycle sequencing reaction kit v 3.1 (Applied Biosystems) following the manufacturer's protocols. Both forward and reverse primers were utilised in all cases to sequence both strands.

Phylogenetic analyses

DNA sequences obtained in this study were analysed and edited in MEGA v 3.1 (Kumar et al. 2004). Analysed sequences were compared to representative *Mycosphaerella* and *Teratosphaeria* sequences deposited in GenBank, including *T. nubilosa* sequences reported from different countries (Table 1). All sequences obtained in this study were deposited in GenBank (Table 1).

DNA sequence alignments were conducted in MAFFT v 6. employing the high-accuracy option of local alignment mode L-INS-I (Katoh et al. 2005) and manually aligned by inserting gaps when necessary in MEGA v 3.1 (Kumar et al. 2004). MODELTEST v 3.7 (Posada and Crandall 1998) was utilised to select the best nucleotide substitution model to fit the data. Using the Akaike Information Criterion (AIC), the Transitional Model (TIM+G) with a gamma distribution of the rate variation was then selected for a Bayesian analysis. Gaps were treated as missing data. Four Monte-Carlo Markov chains were run twice in MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003) for

Table 1 *Mycosphaerella* and *Teratosphaeria* isolates used in this study for phylogenetic analyses

Fungal Species	Isolate number[a]	Host	Origin	GenBank number ITS	Beta tubulin-2
Mycosphaerella ambiphylla	CMW 14180; CBS 110499	*Eucalyptus globulus*	Australia	AY725530	
M. vespa	CMW 11558; CBS 117924	*E. globulus*	Australia	DQ267590	
Neofusicoccum parvum	STEU 4438	*Vitis vinifera*	South Africa	AY343467	
Teratosphaeria cryptica	MUCC 561	*Eucalpytus* sp.	Australia	DQ302951	
T. nubilosa (ex-epitype)	CBS 111679	*E. nitens*	Australia	EU301082	
T. nubilosa	CBS 116005	*E. globulus*	Australia	AY725572	DQ992068
T. nubilosa	CMW 9003; CBS 114708	*E. nitens*	South Africa	AF449099	
T. nubilosa	CMW14928	*E. globulus*	New Zealand	DQ923548	
T. nubilosa	CMW 12548	*E globulus*	Spain	DQ923570	DQ992067
T. nubilosa	CMW 18796	*E globulus*	Portugal	DQ923565	
T. nubilosa	CMW 21518	*E nitens*	South Africa	DQ923576	
T. nubilosa	CMW 21603	*E globulus*	Tanzania	DQ923563	
T. nubilosa	CMW 22371	*E globulus*	Australia	DQ923573	
T. nubilosa	CMW 23910	*E. dunnii*	Australia	DQ923558	
T. nubilosa	CMW 23916	*E nitens*	Australia	DQ923561	
T. nubilosa	CMW 11148	*E. globulus*	Ethiopia	AY244409	
T. nubilosa	CMW 28593 CMW 30206 CMW 30207 CMW 30208 CMW 30209 CMW 30210	*E. globulus* subsp. *maidenii*	Paysandú-Uruguay	FJ791788 FJ791789 FJ791790 FJ791791 FJ791792 FJ791793	FJ791808 FJ791809 FJ791810 FJ791811 FJ791812 FJ791813
T. nubilosa	CMW 30211 CMW 30212 CMW 30213 CMW 30214	*E. globulus* subsp. *globulus*	Paysandú-Uruguay	FJ791794 FJ791795 FJ791796 FJ791797	FJ791814 FJ791815 FJ791816 FJ791817
T. nubilosa	CMW 28594 CMW 30215 CMW 30216 CMW 30217 CMW 30218 CMW 30219	*E. globulus* subsp. *globulus*	Lavalleja-Uruguay	FJ791798 FJ791799 FJ791800 FJ791801 FJ791802 FJ791803	FJ791818 FJ791819 FJ791820 FJ791821 FJ791822 FJ791823
T. nubilosa	CMW 28595 CMW 30220	*E. globulus* subsp. *globulus*	Colonia-Uruguay	FJ791804 FJ791805	FJ791824 FJ791825
T. nubilosa	CMW 28596 CMW 30221	*E. globulus* subsp. *globulus*	Rocha-Uruguay	FJ791806 FJ791807	FJ791826 FJ791827
T. molleriana	CMW 4949; CBS 111164	*E. globulus*	Portugal	AF309620	
T. suttonii	CMW 5348	*Eucalyptus* sp.	Indonesia	AF309621	
T. toledana	CMW14457; CBS 113313	*Eucalyptus* sp.	Spain	AY725580	

[a] CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

STEU: Culture collection of Stellenbosch University, South Africa

MUCC: Culture collection of Murdoch University, Australia

two million generations starting from random trees; trees were sampled every 100th generation. The first 3000 trees were discarded (burn-in). The remaining 17,000 trees were utilised to obtain a consensus Bayesian tree (50% majority-rule), rooted using *Neofusicoccum parvum* (= *Botryosphaeria parva*) (AF343467) as the outgroup taxon.

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 and remaining trees were added in single sequence fashion, MaxTrees was set at 100. Parsimony uninformative characters were excluded from the analysis. Finally, 1000 bootstrap replicates were conducted to determine confidence levels of branching points in the phylogenetic tree.

Genescan and population diversity

A total of 46 isolates collected from leaves on different trees from four provinces of Uruguay was selected to consider the diversity of the population (Table 2). Ten microsatellite loci were amplified using the fluorescently-labelled primers developed by Hunter et al. (2006). PCR reactions were conducted as described above, starting with an initial denaturation at 94°C for 2 min, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C and a final elongation step for 45 min at 72°C. An exception was made in those reactions employing the MN-8 primer pair, where the annealing temperature was set at 54°C and not at 60°C. GeneScan-500 LIZ Size Standard (Applied Biosystems) was utilised to determine the allele sizes by electrophoresis on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems).

Genescan and Genemapper software packages (Applied Biosystems) were used for further data analysis.

Results

Sample collection and lesion description

Leaf spot and blotch symptoms were observed in all surveyed areas on *E. globulus* subsp. *globulus* and *E. globulus* subsp. *maidenii*, but they were less evident on *E. dunnii*. Similar leaf spots were observed on both juvenile and intermediate age foliage (Fig. 1). Leaf spots were present on leaves from the previous season's growth as well as on juvenile leaves from the current season. Usually the second or third fully expanded leaf on new branches was already showing symptoms, suggesting the presence of a primary pathogen.

The most common lesions observed were yellow to brown in colour, round to angular in shape, frequently coalescing to form larger blotches across the leaf surface (Fig. 1). As they aged, lesions became darker and pseudothecia became more visible, especially, but not exclusively, on the abaxial leaf surfaces (Fig. 1b, f). Lesion borders were surrounded by raised corky reddish to red-purple margins (Fig. 1f, g). These lesions were typical of MLD.

Evaluation of the severity of the disease was beyond the scope of the current study; however, remarkable differences in the impact of the disease were observed between *Eucalyptus* species. Leaves of both sub-species of *E. globulus* showed several spots per leaf and in severely affected plantations the diseased area covered up to 50% of the leaf lamina (Fig. 1a–d). On *E. dunnii*, however, typically only one to three small lesions were observed per leaf (Fig. 1e). Adult foliage also exhibited symptoms of MLD, but the character-

Table 2 Origin of the Uruguayan *Teratosphaeria nubilosa* isolates used in this study

Host	Province	Area	No. of isolates
Eucalyptus globulus subsp. *maidenii*	Paysandú	Paso de la Cruz	12
E. globulus subsp. *globulus*	Paysandú	Paso de la Cruz	11
E. globulus subsp. *globulus*	Lavalleja	Campanero	14
E. globulus subsp. *globulus*	Colonia	Colonia	2
E. globulus subsp. *globulus*	Rocha	La Pedrera	7
		Total	46

Fig 1 Leaf symptoms, lesions and germinating ascospore of *Teratosphaeria nubilosa* on different hosts. **a** Adaxial surface of juvenile foliage of *E. globulus* subsp. *maidenii* **b** Abaxial surface of the same leaf in **a** **c** Intermediate foliage of *E. globulus* subsp. *maidenii* **d** *E. globulus* subsp. *globulus* (adaxial surface) **e** *E. dunnii* (adaxial surface) **f** Lesion on *E. globulus* subsp. *maidenii* (abaxial surface) **g** Lesion on *E. dunnii* (adaxial surface) **h** Germinating ascospore after 24 h
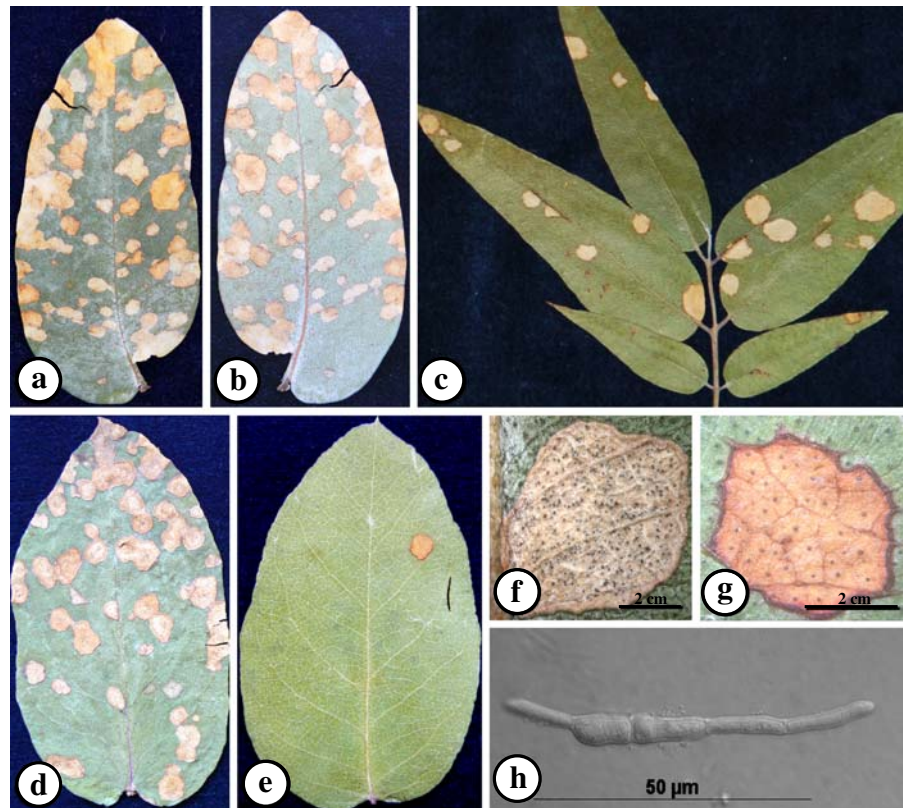
istics of the lesions were different from those found on juvenile and intermediate foliage. Lesions were usually smaller than typical for the pathogen and they did not coalesce to form blotches. These lesions were more frequent on senescing leaves, characteristic of saprophytic or slightly pathogenic *Mycosphaerella* spp. and *Teratosphaeria* spp.

Fungal isolations

Germinating ascospores from leaf spots found on both juvenile and intermediate age foliage showed the type F germination pattern described by Crous (1998). The same germination pattern was observed in ascospores obtained from *E. globulus* subsp. *globulus*, *E. globulus* subsp. *maidenii* and *E. dunnii*. Both cells of the ascospores produced germ tubes that grew parallel to the long axis of the spore. Spores became distorted and slightly constricted at the medium septum and did not darken during germination (Fig. 1h). Conversely, ascospores isolated from adult leaves showed a different germination pattern to those from young and intermediate age foliage.

Phylogenetic analyses

PCR amplifications of the ITS region yielded amplicons of approximately 600 bp for all the isolates. Following alignment, the DNA sequence data contained a total of 580 characters, 397 of which were constant, 119 were variable and parsimony-uninformative and 64 were parsimony-informative. The consensus Bayesian phylogram showed that all isolates from Uruguay grouped together in a well supported clade (Bayesian support=0.99) (Fig. 2). The ex-epitype isolate of *T. nubilosa* was also included in this clade, along with *T. nubilosa* isolates from different countries and hosts. Similarly, parsimony analysis resulted in a phylogenetic tree where all isolates from Uruguay and *T. nubilosa* grouped in a clade showing 100% bootstrap support (data not shown).

PCR amplifications of the beta tubulin-2 locus yielded amplicons of approximately 400 bp for all the isolates. The beta tubulin-2 sequences for all 20 Uruguayan isolates were identical (data not shown but see Table 1 for GenBank accession numbers). In addition, beta tubulin-2 sequences of Uruguayan isolates showed 100% similarity to the sequence for

Fig 2 Bayesian consensus tree obtained from the ITS sequence data. Bayesian support values are shown on the branching points. Uruguayan isolates are shown in bold and the number of isolates per province is provided in brackets. The outgroup branch has been shortened
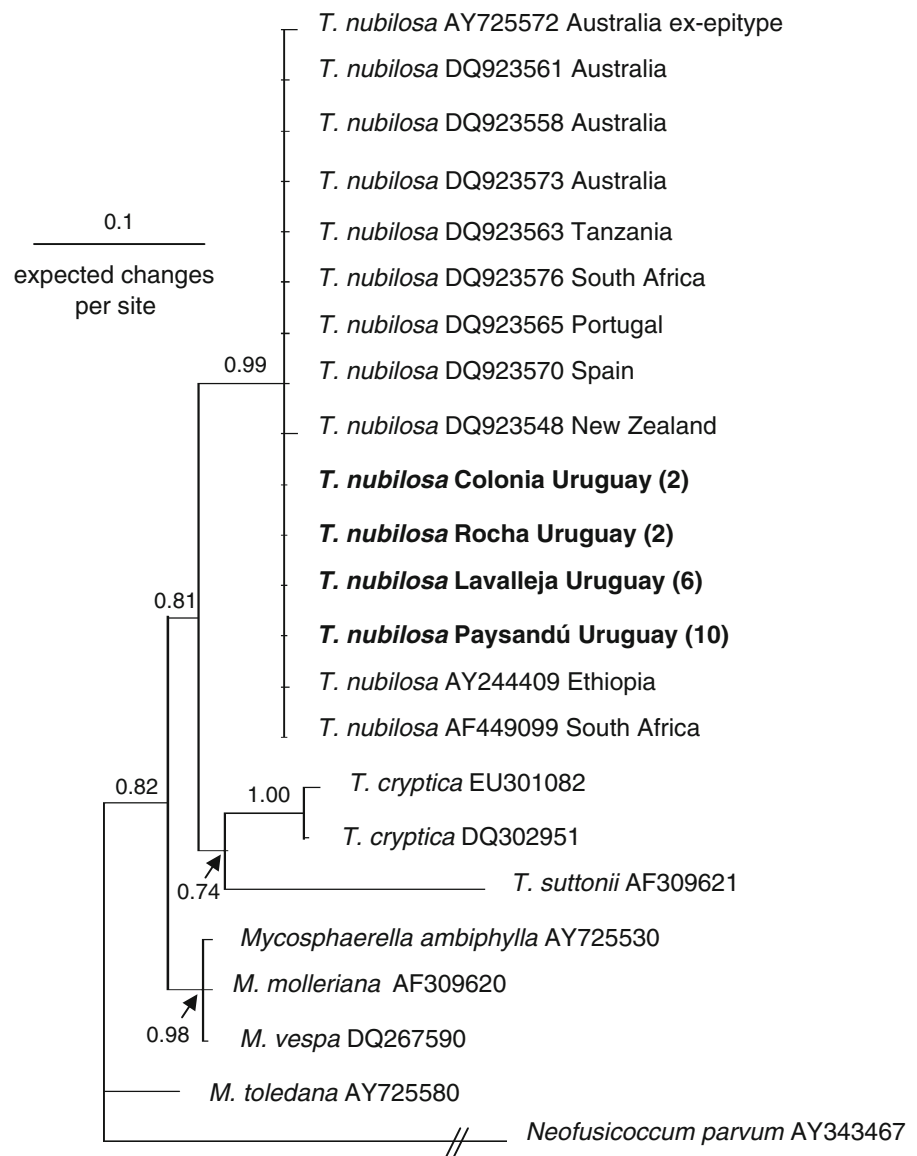
the ex-epitype culture of this species and to the sequence of a previously identified *T. nubilosa* isolate from Spain (data not shown but see Table 1 for GenBank accession numbers).

Genescan and population diversity

All ten microsatellite loci used to describe the genetic diversity of the isolates obtained in this study were monomorphic, showing only one allele per locus. In this way, allele sizes 267, 183, 309, 162, 380, 319, 216, 137, 224 and 100 bp were obtained from the loci MN-1, MN-2, MN-3, MN-4, MN-7, MN-8, MN-9,

MN-10, MN-11 and MN-14 s respectively. This reflects the presence of only one multilocus haplotype in Uruguay. Interestingly, this multilocus haplotype was identical to the unique haplotype found by Hunter et al. (2009) in isolates of *T. nubilosa* from Portugal and Spain and referred to as the Iberian populations. Furthermore, all 10 microsatellite primer pairs developed by Hunter et al. (2006) were species-specific for *T. nubilosa* and amplified the expected DNA fragments in all 46 Uruguayan isolates. These results add substantial confidence to the DNA sequence-based identification of the pathogen in Uruguay as *T. nubilosa*.

Discussion

The nature and severity of the outbreak of MLD, as well as the leaf symptoms observed in the Uruguayan *Eucalyptus* plantations, are consistent with previous descriptions of *T. nubilosa* elsewhere (Crous 1998; Hunter et al. 2009; Park and Keane 1982a). The presence of leaf spots on leaves from the current season's growth is characteristic of *Teratosphaeria* species that are primary pathogens. Moreover, the germination pattern of ascospores from the isolated fungus coincides with the descriptions of the germination pattern of *T. nubilosa* (Crous 1998; Hunter et al. 2009). The Uruguayan isolates were grouped in a clade with 0.99 of Bayesian support and 100% of bootstrap support in the phylogenetic analyses, which included the ex-epitype culture of this species and *T. nubilosa* isolates collected from seven countries on three continents. In addition, the beta tubulin-2 sequences of the Uruguayan isolates showed 100% similarity to the sequence for the ex-epitype culture of this species and to the sequence of a previously identified *T. nubilosa* isolate from Spain (Crous et al. 2004). All 10 microsatellite primer pairs that are species-specific for *T. nubilosa* (Hunter et al. 2006) amplified the expected amplicon successfully and the allele sizes for the Uruguayan and Iberian isolates were identical. These data allowed us to conclusively identify the causal agent of the new and severe outbreak of MLD in Uruguay as *T. nubilosa*.

The discovery of *T. nubilosa* in this study represents the first record of this important pathogen in South America. In a recent population genetic study on *T. nubilosa,* Hunter et al. (2008) suggested that the centre of origin of the pathogen is eastern Australia. They further showed that the pathogen had been introduced into Ethiopia, Kenya, New Zealand, Portugal, South Africa, Spain, Tanzania, Western Australia and Zambia (Hunter et al. 2008; 2009). It is thus intriguing that *T. nubilosa* has taken as long as it has to reach South America.

Teratosphaeria nubilosa was associated with severe leaf disease on *E. globulus*, which is the most highly susceptible species (Carnegie and Ades 2002; Carnegie et al. 1998; Milgate et al. 2005). Leaf disease caused by *T. nubilosa* can be extremely serious as was found in South Africa where commercial forestry with *E. globulus* was abandoned during the 1930s due to the impact of this disease (Hunter et

al. 2009; Lundquist and Purnell 1987). Likewise, two attempts in 1971 and 1997 at afforestation with *E. globulus* have failed due to MLD in north-western Tasmania (Mohammed et al. 2003), and *T. nubilosa* caused almost complete defoliation on *E. globulus* subsp. *maidenii* in northern New South Wales, Australia (Carnegie 2007). *Teratosphaeria nubilosa* has been reported as a primary pathogen of twelve *Eucalyptus* spp. and hybrids, including *E. grandis*, which is widely planted in Uruguay (Crous 1998; Hunter et al. 2009; Park and Keane 1982a). The appearance of the pathogen in Uruguay is, therefore, serious and has the potential to limit forestry in this country.

The appearance of *T. nubilosa* in Uruguay raises substantial concerns regarding further spread of this pathogen to other countries in South America. As in Uruguay, *E. globulus* is extensively planted in several South American countries such as Chile, Colombia, Ecuador and Peru (Potts et al. 2004). The appearance of *T. nubilosa* in Uruguay increases the likelihood that it will now move to neighbouring countries. This could occur by natural spread, given that *T. nubilosa* produces windborne ascospores, a quality that confers high dispersion ability (Hunter et al. 2009; Park and Keane 1982b). Long-distance spread to countries further afield in South America is also likely to be enhanced by the apparent first occurrence of *T. nubilosa* in the region. This would most likely occur through the movement of planting material, which is commonly exchanged by companies owning land in more than one country of the region (Wingfield et al. 2008).

All ten microsatellite loci developed by Hunter et al. (2006) and used to analyse the Uruguayan population of *T. nubilosa* were monomorphic. This is despite the fact that these microsatellite markers have previously been shown as highly variable on a global suite of isolates of the pathogen, with a total of 68 multilocus haplotypes (Hunter et al. 2006; 2008; 2009). There seems little doubt that the pathogen in Uruguay is represented by a single clone. *Teratosphaeria nubilosa* is homothallic, where single individuals can undergo sexual reproduction alone (Park and Keane 1982b). An intriguing result of this study was that the single multilocus haplotype found in Uruguay, is the same haplotype found in Spain and Portugal, where the population of *T. nubilosa* is also represented by a single clone (Hunter et al. 2008;

2009). This result suggests strongly that the pathogen was introduced into Uruguay from one of these countries where its population has a very limited diversity, rather than from Australia, the area of origin of the fungus or an area such as South Africa where *T. nubilosa* is represented by a relatively diverse population (Hunter et al. 2009, 2008). Forestry companies in Uruguay have close associations with those particularly in the Iberian Peninsula and opportunities for an introduction into Uruguay from that area would have been great.

Commercial *Eucalyptus* plantations in Uruguay originated from seed and clonal material imported predominantly from Australia, Chile and Spain (Balmelli 2006). The movement of seed and asymptomatic vegetative material among countries is one of the most important vehicles for plant pathogen movement (Elmer 2001). As far as we know, there is no study showing that *Mycosphaerella* or *Teratosphaeria* species associated with MLD are seedborne; however, the widespread distribution of apparently Australian *Mycosphaerella* spp. on *Eucalyptus* strongly suggests that this is the case (Ciesla et al. 1996; Wingfield et al. 2008). Although there is no evidence to suggest that this has occurred, the introduction of clonal plant material could have also provided a means of introduction of the pathogen, particularly considering that symptoms of *T. nubilosa* are visible only after four weeks of incubation (Park and Keane 1982b).

Annual *Eucalyptus* disease surveys have been conducted in Uruguay during the course of the last ten years (M. J. Wingfield, unpublished data). These surveys have included special efforts to identify *Mycosphaerella* spp. associated with leaf diseases in the country and there has been no evidence to suggest that *T. nubilosa* was present. This suggests strongly that *T. nubilosa* in Uruguay represents a recent introduction of a pathogen that has spread rapidly in the country. The fact that *T. nubilosa* was first reported in the Iberian Peninsula in 2001 (Crous et al. 2004) leads us to believe that this pathogen entered Uruguay during the course of the last seven years.

The only economically viable management strategy for MLD is the development of resistant or tolerant *Eucalyptus* genotypes as has been true for various other diseases of these trees (Tibbits et al. 1997; Wingfield 2003). *Eucalyptus globulus* provenances are known to differ in their susceptibility to diseases,

including MLD in Uruguay (Balmelli et al. 2004). In addition, there are excellent opportunities to plant non-susceptible *Eucalyptus* spp. and hybrids of *E. globulus* with species that have a low level of susceptibility to infection by *T. nubilosa* (Carnegie and Ades 2002; Carnegie et al. 1998; Milgate et al. 2005). Given the serious damage that this pathogen is able to cause, evaluation and development of resistant planting stock in Uruguay should be considered a priority.

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