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Research Papers

Mycosphaerella and *Teratosphaeria* species associated with leaf diseases on *Eucalyptus globulus* in southern Brazil

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Summary. Leaf blight and defoliation caused by *Teratosphaeria* species is one of the most important leaf diseases of *Eucalyptus globulus*. Due to the importance of this tree species for the production of pulp and paper, and recent reports of severe leaf disease symptoms in Brazil, the present study was conducted to identify the pathogen(s) involved. Symptomatic leaves were collected in the Brazilian states of Paraná and Rio Grande do Sul, single ascospore cultures established, and isolates were investigated using DNA-based molecular tools. A species-specific PCR and sequence data from the nuclear ribosomal DNA operon were used for species identification. The following seven species were identified: *Mycosphaerella scytalidii*, *Dissoconium dekkeri* (=*M. lateralis)*, *Teratosphaeria ohnowa*, *T. perpendicularis*, *T. pseudafricana*, *T. flexuosa* and *T.nubilosa.* Of the recorded species, *T. nubilosa* is regarded as the most serious threat to the cultivation of *E. globulus* in the states surveyed.

Key words: forest pathology, ITS, LSU, *Teratosphaeria nubilosa*.

Introduction

Eucalyptus globulus Labill is one of the main tree species planted for pulp and paper production, especially in temperate-climates (Eldridge *et al*., 1993). The wood of *E. globulus* has a high cellulose content, low lignin content, high density and, consequently, requires a low concentration of chlorine in the pulp bleaching process (Kibblewhite *et al*., 2000; Fonseca *et al*., 2010; Cardoso *et al*., 2011). Due to these characteristics, *E. globulus* has been considered a strategic species for the pulp producing industry in Brazil, mainly in hybridization with the species adapted to Brazilian conditions, such as *E. grandis* Hill ex Maiden and *E. urophylla* S.T. Blake (Bison *et al*., 2007). However, the high susceptibility of *E. globulus* to some diseases (Alfenas *et al*., 2009) poses a serious limiting factor to the successful expansion of this species in Brazil.

Of the diseases that affect *E. globulus*, leaf diseases caused by species of *Mycosphaerella* (Mycosphaerella Leaf Disease; MLD) and *Teratosphaeria* (Teratosphaeria Leaf Disease; TLD) are considered to be among the most important (Crous 2009; Hunter *et al*., 2009; 2011). The intensity of the disease varies with the phenological stage of *E. globulus* leaves, affecting juvenile leaves more intensely than older leaves (Márquez *et al*., 2011). TLD was first reported in Australia, from where it probably dispersed to *Eucalyptus*-growing countries, such as South Africa, Spain (Crous *et al*., 2004), Portugal (Hunter *et al*., 2008) and Uruguay (Pérez *et al*., 2009a). Recently, the disease was reported on *E. globulus* in the State of Rio Grande do Sul, southern Brazil, and was attributed to *T. nubilosa* (Cooke) Crous & Braun (Pérez *et al*., 2009b).

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Teratosphaeria nubilosa (= *Mycosphaerella nubilosa*) and *T. cryptica* (Cooke) Crous & Braun (= *M. cryptica*) are the most important pathogenic species in the *Teratosphaeria* disease complex on *Eucalyptus*; however, *T. molleriana* (Thüm.) Crous & Braun, *M. marksii* Carnegie & Keane, *M. fori* Hunter, Crous & M.J. Wingf. (anamorph: *Pseudocercospora fori*) and several other species have also been associated with leaf blight of *E. globulus* (Park and Keane, 1982; Crous and Wingfield, 1997; Hunter *et al.*, 2004, 2009). Furthermore, more than one species may be found on a single leaf and even in the same lesion (Crous and Wingfield, 1996; Crous and Groenewald, 2005; Crous *et al.*, 2009). Therefore, multiple infections of different species may occur, which increases the impact of the disease, and complicates the identification of the primary causal agent and application of potential disease control strategies (Gezahgne *et al.*, 2006). In the present study we report on species of *Mycosphaerella* and *Teratosphaeria*, in addition to *T. nubilosa*, which we have found to be associated with leaf disease of *E. globulus* in southern Brazil.

Material and methods

Sample collection and isolation procedures

Samples of infected *E. globulus* leaves were collected from Paraná and Rio Grande do Sul (Brazil) between January and December 2009, and used to isolate the causal agents of disease as previously described (Crous *et al.*, 1991; Crous, 1998). Excised lesions were soaked in water for 2 h and attached to the undersides of Petri dish lids (6 cm), with pseudothecia facing downwards over Malt Extract Agar (MEA; 1.8 gL^{-1} malt extract, 1.8 gL^{-1} agar). Single germinating ascospores were transferred to fresh MEA plates to obtain pure cultures that were initially incubated at 15°C with a photoperiod of 12 h for 15 days, and then at 20°C with a photoperiod of 12 h for a further 30 days.

DNA isolation

Genomic DNA was isolated from fungal mycelium grown on MEA plates for 2 mo (20°C, photoperiod of 12 h). Mycelium used for DNA extraction was scraped directly from the surfaces of cultures on MEA plates and crushed under liquid nitrogen until the formation of fine powder, following the protocol of Murray and Thompson (1980), or an UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Solana Beach, CA, USA) according to the manufacturer's protocols.

PCR-identification of *Teratosphaeria nubilosa*

A PCR based on the ITS domain of the nrDNA operon was used for identification and detection of *T. nubilosa*. The species-specific primers MNF (5′-cgtcggagtaatacaacc-3′), MNR (5′-aggctggagtggtgaaatg-3′) (Geetha *et al.*, 2004), MN1F (5′- gcgccagcccgacctcc-3′) and MN1R (5′-ggtccccgtcagcgaaacagt-3′) (Maxwell *et al.*, 2005) were used. PCR reactions were conducted with the same reaction mixture as those utilized by Geetha *et al.* (2004) and Maxwell *et al.* (2005), respectively. PCR products were visualized on 2% agarose gels (w/v) stained with ethidium bromide and viewed under UV light. Sizes of PCR products were determined against a 1 Kb molecular weight marker (Invitrogen®).

DNA amplification and sequencing

The primers ITS1 (5′-tccgtaggtgaacctgcgg-3′) and ITS4 (5′-tcctccgcttattgatatgc-3′) were used to amplify the internal transcribed spacer 1 (ITS1), the 5.8S nrRNA gene and the internal transcribed spacer 2 (ITS2) (White *et al.*, 1990). The primers LR0R (5′-accgctgaacttaagc-3′) (Rehner and Samuels, 1994) and LR5 (5′-tcctgagggaaacttcg-3′) (Vilgalys and Hester, 1990) were used to amplify the first 900 bp of the 5′ end of the 28S nrRNA gene (LSU). Each reaction (25 μL) contained 2 μL template DNA (10–20 ng μL^{-1}), 1 × reaction buffer (Promega Inc.), 2.0 mM $MgCl_2$, 200 μM of each dNTP (dATP, dTTP, dCTP and dGTP), 0.2 μM of each primer and 1.0 U GoTaq® DNA polymerase (Promega Inc.). PCR amplifications were performed using a 96-well thermal cycler (PTC-100; MJ Research Inc.). Cycling conditions consisted of an initial denaturing step of 96°C for 5 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 90 s, and a final elongation step at 72°C for 4 min for primers ITS1 and ITS4. For primers LR0R and LR5, the protocol was 4 min at 95°C, with an additional 35 cycles of 94°C for 60 s, 53°C for 90 s and 72°C for 2 min, with a final extension at 72°C for 4 min. PCR products were visualized on 2% agarose gels (w/v) stained with ethidium bromide and viewed under UV light. Sizes of PCR products were determined against a 1 Kb molecular weight marker (Invitro-

gen®). PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics®) following the manufacturer's instructions.

Purified PCR products were used as template DNA for sequencing reactions using the DYEnamicTM ET Terminator Cycle Sequencing Kit (GE Healthcare, Freiburg, Germany) and separation of the sequenced products was by capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer (Applied Bio Systems Inc., Foster City, CA, USA). Sequencing reactions were conducted with the same primers used for the PCR amplification (ITS1-ITS4 and LR0R-LR5). Additionally, two internal primers LR3R (5′-gtcttgaaacacggacc-3′) (www.biology.duke.edu/fungi/mycolab/primers.htm) and LR16 (5′-ttccacccaaacactcg-3′) (Moncalvo *et al*., 1993) were used to completely sequence both DNA strands of the LSU region. Sequences were analyzed using Sequence Navigator v. 1.0.1 (Applied Bio Systems Inc., Foster City, CA).

Phylogenetic analyses

Separate alignments were made for the ITS and LSU regions using MAFFT v. 6 (http://mafft.cbrc.jp/alignment/server/index.html). The phylogenetic analyses included sequences of 19 isolates from this study, as well as 28 and 35 ITS sequences respectively for the *Mycosphaerella* and *Teratosphaeria* alignments retrieved from Gen Bank (Figures 1 and 2) and 43 for LSU (Figure 3). The taxa used for outgroups of the LSU alignment were *Dothidea insculpta* Wallr. and *D. sambuci* (Pers.) Fr., and *Cladosporium bruhnei* Linder was used as the outgroup for the ITS analyses. DNA sequences were assembled, added to the outgroups with additional GenBank sequences, and aligned using Sequence Alignment Editor v. 2.0a11 (Rambaut, 2002), and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were conducted in PAUP v. 4.0b10 (Swofford, 2003). For the parsimony analysis, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed using the heuristic search option with 100 random taxon additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replicates (Hillis and Bull, 1993). Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated and the resulting trees were printed with TreeView v. 1.6.6 (Page, 1996) and edited for layout in Adobe Illustrator CS 5.1. All novel sequences generated in this study were lodged with NCBI's Gen Bank nucleotide database (Table 1), and the alignments deposited in Tree BASE.

Results

Molecular identification

Nineteen single-ascospore isolates were obtained, 11 from Paraná state and eight from Rio Grande do Sul (Table 1). Specific primers for *T. nubilosa* amplified fragments of approximately 200 bp (MNF-MNR) and 400 bp (MN1F-MN1R) for the isolates PM08, PM09, PM10, PM11, PM12, PM13, PM14, PM18 and PM19 (Table 1).

Approximately 1700 bases, spanning the ITS and LSU regions, were obtained from the sequenced cultures. The ITS region was used to determine species-level relationships (Figures 1 and 2) and LSU for the generic placement of the novel cultures (Figure 3). Due to problematic alignment positions in the ITS regions across all included genera, the alignment was split according to the broader fungal genera, namely *Mycosphaerella*/*Dissoconium* and *Teratosphaeria*. The manually adjusted ITS alignment of the *Mycosphaerella*/*Dissoconium* dataset contained 31 taxa (including the outgroup sequence). In total, 548 characters were used in the phylogenetic analysis, of which 231 were parsimony-informative, 65 were variable and parsimony-uninformative and 252 were constant. Three equally most parsimonious trees were retained from the heuristic search, and the first of these is shown in Figure 1 (TL = 624, CI = 0.766, RI = 0.914, RC = 0.700). The manually adjusted ITS alignment of the *Teratosphaeria* dataset contained 53 taxa (including the outgroup sequence). In total, 494 characters were used in the phylogenetic analysis, of which 175 were parsimony-informative, 86 were variable and parsimony-uninformative and 233 were constant. Fifteen equally most parsimonious trees were retained from the heuristic search, and the first of these is shown in Figure 2 (TL = 694, CI = 0.618, RI = 0.880, RC = 0.544).

The manually adjusted LSU alignment contained 53 taxa (including the two outgroup sequences) and, of the 754 characters used in the phylogenetic analy-

Table 1. Species of *Mycosphaerella* and *Teratosphaeria*, associated with *Eucalyptus globulus* leaf disease in Brazil.

Isolate[a]	PCR MN[b]	PCR MN1[c]	Identification[d]	GenBank ITS/LSU	Clone	State[e]
PM01 = CPC 18605	-	-	*M. scytalidii*	JN232408/JN232427	Anselmo Rama	PR
PM02 = CPC 18606	-	-	*T. ohnowa*	JN232409/JN232428	Anselmo Rama	PR
PM03 = CPC 18607	-	-	*T. pseudafricana*	JN232410/JN232429	Anselmo Rama	PR
PM04	-	-	*T. ohnowa*	JN232411/JN232430	Batman	PR
PM05	-	-	*D. dekkeri*	JN232412/JN232431	Batman	PR
PM06 = CPC 18610	-	-	*T. flexuosa*	JN232413/JN232432	Batman	PR
PM07 = CPC 18611	-	-	*T. ohnowa*	JN232414/JN232433	Batman	PR
PM08 = CPC 18612	+	+	*T. nubilosa*	JN232415/JN232434	Batman	PR
PM09 = CPC 18613	+	+	*T. nubilosa*	JN232416/JN232435	Seed	RS
PM10 = CPC 18614	+	+	*T. nubilosa*	JN232417/JN232436	Seed	RS
PM11 = CPC 18615	+	+	*T. nubilosa*	JN232418/JN232437	Seed	RS
PM12 = CPC 18616	+	+	*T. nubilosa*	JN232419/JN232438	Seed	RS
PM13	+	+	*T. nubilosa*	JN232420/JN232439	Seed	RS
PM14 = CPC 18618	+	+	*T. nubilosa*	JN232421/JN232440	Seed	RS
PM15	-	-	*T. ohnowa*	JN232422/JN232441	Rivera	PR
PM16 = CPC 18620	-	-	*T. pseudafricana*	JN232423/JN232442	Anselmo Rama	PR
PM17 = CPC 18621	-	-	*T. perpendicularis*	JN232424/JN232443	Rivera	PR
PM18 = CPC 18622	+	+	*T. nubilosa*	JN232425/JN232444	Seed	RS
PM19 = CPC 18623	+	+	*T. nubilosa*	JN232426/JN232445	Seed	RS

[a] PM, cultures maintained at the Department of Plant Pathology, University of Viçosa, Brazil; CPC, working collection of P.W. Crous maintained at CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands.
[b] Species-specific PCR for *T. nubilosa*, using NMF and NMR primers (Geetha *et al.*, 2004)
[c] Species-specific PCR for *T. nubilosa*, using NM1F and NM1R primers (Maxwell *et al.*, 2005)
[d] Based on the phylogenetic analysis of the ITS1-5.8S nrRNA-ITS2 gene region
[e] PR, Paraná State and RS, Rio Grande do Sul State (Brazil)

sis, 170 were parsimony-informative, 45 were variable and parsimony-uninformative and 539 were constant. Only 16 equally most parsimonious trees were retained from the heuristic search, the first of these is shown in Figure 3 (TL = 520, CI = 0.575, RI = 0.851, RC = 0.490).

Discussion

To date only *M. marksii*, *M. parkii* Crous, M.J. Wingf., F.A. Ferreira & Alfenas, *M. scytalidii*, *T. nubilosa*, *T. suberosa* (Crous, F.A. Ferreira, Alfenas & M.J. Wingf.) Crous & U. Braun and *T. suttonii* (Crous & M.J. Wingf.) Crous & U. Braun have been reported to be associated with MLD and TLD of eucalypts in Brazil (Crous *et al.*, 2006, Alfenas *et al.*, 2009, Pérez *et al.*, 2009c). In the present study we used DNA sequence data from the ITS and LSU gene regions for phylogenetic comparisons and identification of isolates collected in southern Brazil. *Dissoconium dekkeri* de Hoog & Hijwegen (= *M. lateralis* Crous & M.J. Wing.), *M. scytalidii* and five species of *Teratosphaeria* were identified. New reports of fungi for Brazil include *D. dekkeri*, *T. ohnowa*, *T. perpendicularis*, *T. pseudafricana* and *T. flexuosa*.

Presently there are three reports of *M. scytalidii* associated with leaf disease of *Eucalyptus*, namely on *E. globulus* in Colombia and Brazil (Crous *et al.*, 2006),

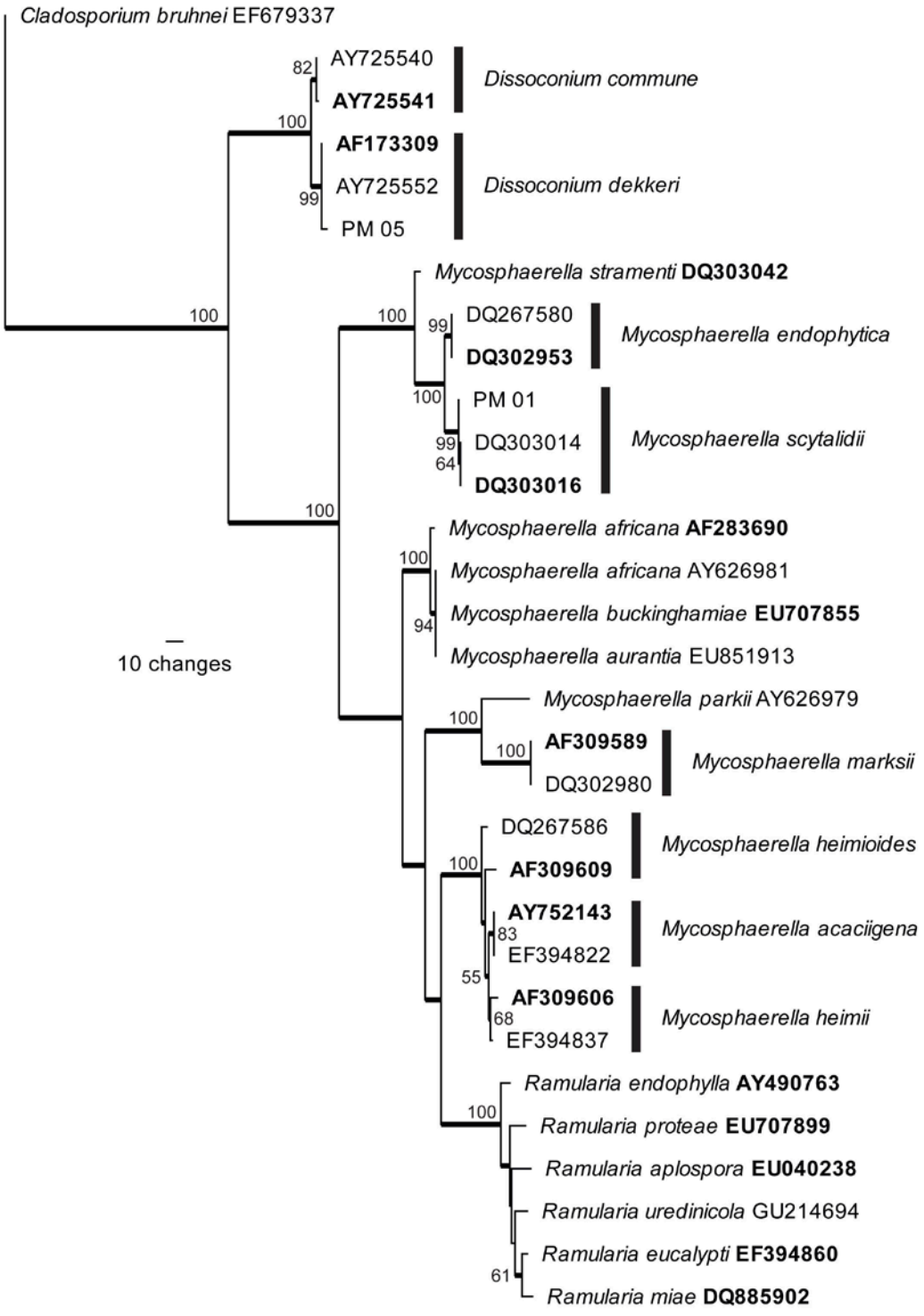

Figure 1. The first of three equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the *Mycosphaerella*/*Dissoconium* ITS sequence alignment. The scale bar shows ten changes, and bootstrap support values from 1000 replicates are shown at the nodes. The sequences from ex-type strains are shown in bold face and branches present in the strict consensus tree are thickened. The tree was rooted to *Cladosporium bruhnei* (GenBank EF679337).

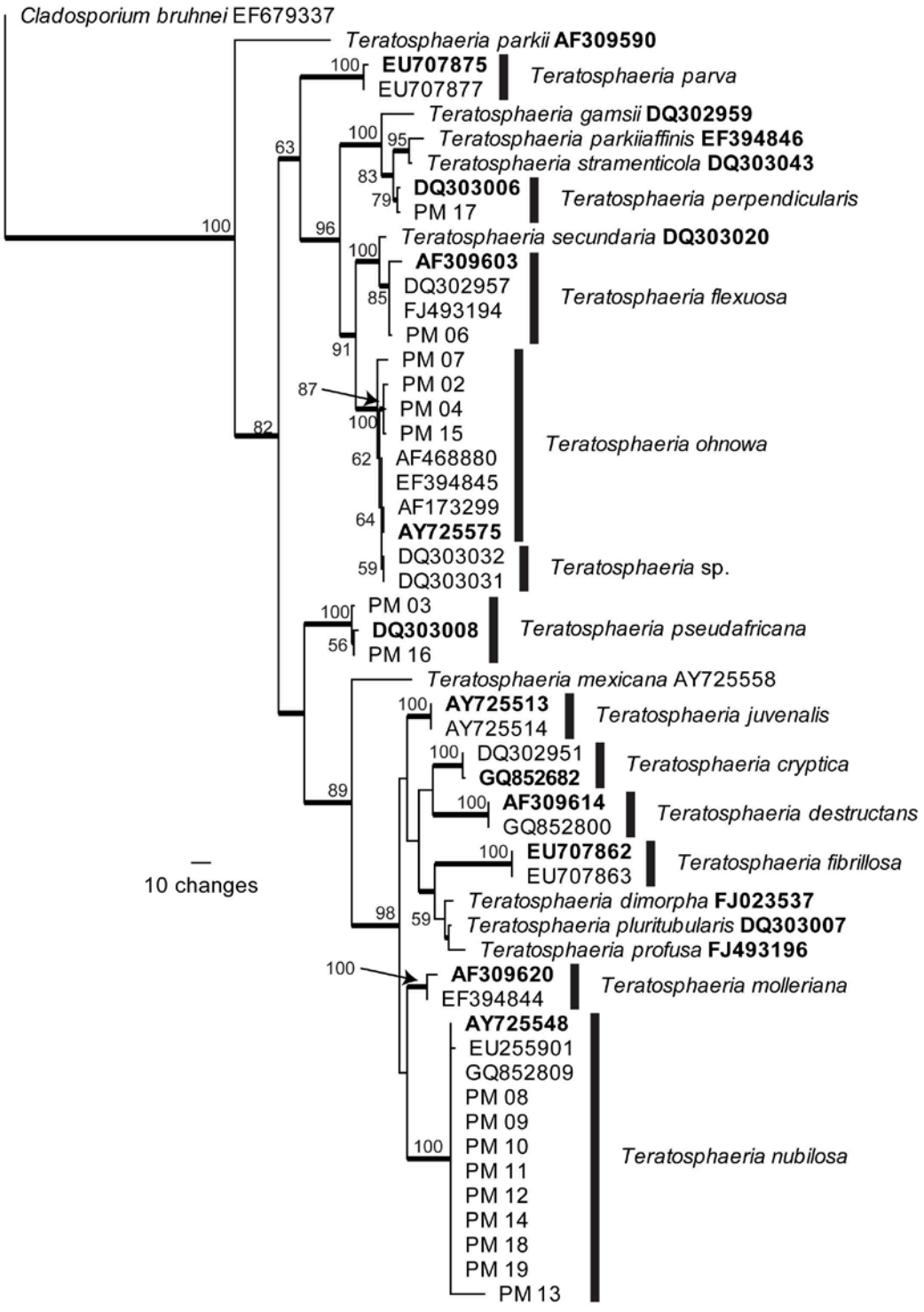

Figure 2. The first of 15 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the *Teratosphaeria* ITS sequence alignment. The scale bar shows ten changes, and bootstrap support values from 1000 replicates are shown at the nodes. The sequences from ex-type strains are shown in bold face and branches present in the strict consensus tree are thickened. The tree was rooted to *Cladosporium bruhnei* (GenBank EF679337).

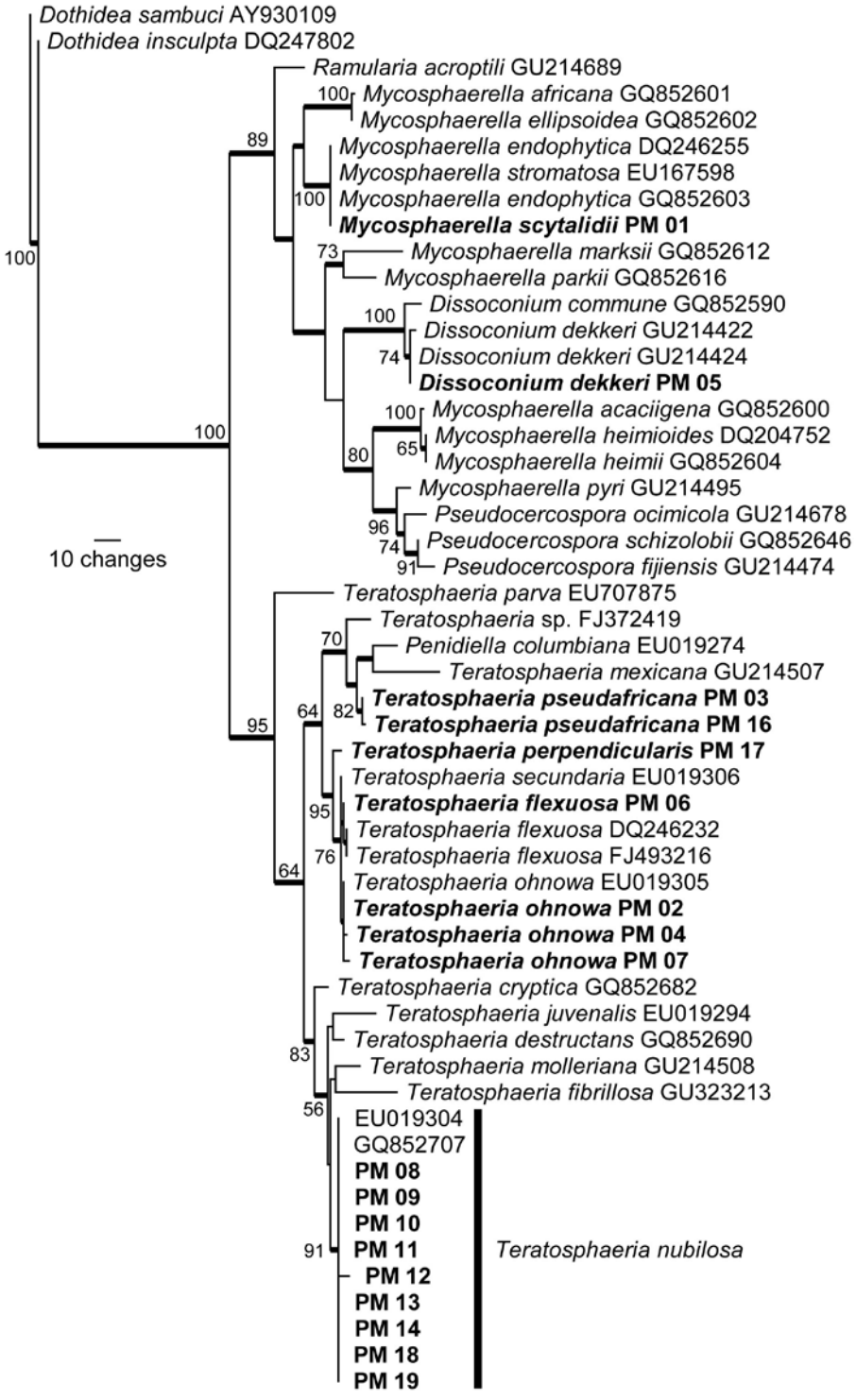

Figure 3. The first of 16 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the LSU sequence alignment. The scale bar shows ten changes, and bootstrap support values from 1000 replicates are shown at the nodes. The novel sequences generated for this study are shown in bold face and branches present in the strict consensus tree are thickened. The tree was rooted to sequences of two *Dothidea* species.

and on *E. grandis, E. dunnii* Maiden, and *E. globulus* in Uruguay (Pérez *et al*., 2009a). *Dissoconium dekkeri* was originally described from the Netherlands on *Juniperus chinensis* (de Hoog *et al*., 1991), and has since been reported in Germany, New Zealand and Malaysia as a hyperparasite on Erysiphaceae on hosts such as *Abies*, *Picea*, *Tusga* (Pinaceae), *Berberis* (Berberidaceae), *Buxus* (Buxaceae), *Juniperus* (Cupressaceae) and others (Crous *et al.*, 1999). It was also reported as *M. lateralis* in Australia, South Africa, Zambia, Bolivia, Spain, Portugal, and Uruguay on *E. grandis*, *E. globulus*, *E. saligna* Smith, *E. nitens* (Deane & Maiden) Maiden, *E. maidenii* F. Muell and on hybrids of *E. grandis* × *E. saligna* (Crous, 1998; Maxwell *et al*., 2000; Crous *et al*., 2006; Pérez *et al*., 2009a). Although *D. dekkeri* is frequently isolated in association with *T. nubilosa* and *T. cryptica,* it was also proven to be a pathogen in its own right (Jackson *et al.*, 2005).

Of the five *Teratosphaeria* species identified, only *T. nubilosa* has recently been reported from Brazil (Pérez *et al*., 2009c). *Teratosphaeria ohnowa* was reported in South Africa and Australia on *E. grandis* and in Uruguay on *E. viminalis* Labill (Crous *et al*., 2004; Crous *et al*., 2007; Pérez *et al*., 2009a). *Teratosphaeria perpendicularis* was reported in Colombia on hybrids of *E. urophylla*×*E. grandis* (Crous *et al*., 2006) and *T. pseudafricana* was reported in Zambia and *T. flexuosa* in Colombia on *E. globulus* (Crous, 1998; Crous *et al*., 2006). However, the pathogenicity of *T. pseudafricana* and *T. flexuosa* has not been proven to date.

Among the species identified in the present study, *T. nubilosa* was the most frequently isolated, and it is considered to be the most important species causing intense defoliation of *E. globulus* in several countries where this host is commercially propagated (Hunter *et al*., 2009; 2011). Recently this fungus was reported in plantations of *E. globulus* and *E. dunnii* in Uruguay and on *E. globulus* in Brazil (Pérez *et al*., 2009b; c). In the present study, *T. nubilosa* was found in the States of Rio Grande do Sul and Paraná. The two species-specific primer pairs for *T. nubilosa* used in the present study proved to be extremely useful tools for identification of the pathogen.

In a population genetic study in Uruguay, Pérez *et al.* (2009b), using 10 microsatellite loci, found a single haplotype of *T. nubilosa* identical to the haplotype found in Spain and Portugal, where the population of this pathogen is also clonal. This suggests recent introduction of the fungus into those countries, probably on imported plant material. It is possible that this pathogen has been introduced to Brazil from Uruguay, due to the close proximity of planting areas and the exchange of plant genotypes between the two countries. Further research would be required, however, to determine if populations in Brazil represent the same haplotype as that found in Uruguay and Europe.

Planting resistant host genotypes is the only effective long-term control strategy for MLD and TLD (Alfenas *et al*., 2009). Recent studies have shown the existence of genetic variability for resistance to leaf blight and defoliation of *E. globulus* caused by *Mycosphaerella* and *Teratosphaeria* spp., making genetic resistance a possible method for controlling the disease (Milgate *et al*., 2005; Freeman *et al*., 2008). Additionally, in Brazil, *E. globulus* has been introduced mainly for hybridization with species adapted to the Brazilian climatic conditions (Fonseca *et al*., 2010). It is expected that the inter-specific hybrids obtained will be more resistant to TLD than the *E. globulus* parents. Thus, interbreeding of *E. globulus* with other species resistant to TLD, adapted to the Brazilian climatic conditions, should be carried out to begin to implement this disease management strategy.

For the selection of resistant genotypes by inoculation under controlled conditions, it is essential to develop pure culture methods for mass spore production of the pathogen, as well as to establish protocols for inoculation and quantification of the disease it causes. Species of *Mycosphaerella* and *Teratosphaeria* are generally slow-growing and typically do not sporulate on culture media (Crous, 1998), which could pose considerable difficulty for such genetic selection research.

The diversity of *Mycosphaerella* and *Teratosphaeria* species found in the present study, on a relatively small number of *E. globulus* leaves sampled, suggests that there may be many other species associated with *Eucalyptus* in Brazil. However, it is now necessary to demonstrate the pathogenicity of these species isolated from *Eucalyptus*, as most research to date has chiefly focused on their identification, host range and distribution.

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