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Mycosphaerellaceae and Teratosphaeriaceae associated with *Eucalyptus* leaf diseases and stem cankers in Uruguay

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Summary

Mycosphaerella leaf diseases represent one of the most important impediments to *Eucalyptus* plantation forestry. Yet they have been afforded little attention in Uruguay where these trees are an important resource for a growing pulp industry. The objective of this study was to identify species of Mycosphaerellaceae and Teratosphaeriaceae resulting from surveys in all major *Eucalyptus* growing areas of the country. Species identification was based on morphological characteristics and DNA sequence comparisons for the Internal Transcribed Spacer (ITS) region of the rDNA operon. A total of ten Mycosphaerellaceae and Teratosphaeriaceae were found associated with leaf spots and stem cankers on *Eucalyptus*. Of these, *Mycosphaerella aurantia*, *M. heimii*, *M. lateralis*, *M. scytalidii*, *Pseudocercospora norchiensis*, *Teratosphaeria ohnowa* and *T. pluritubularis* are newly recorded in Uruguay. This is also the first report of *M. aurantia* occurring outside of Australia, and the first record of *P. norchiensis* and *T. pluritubularis* in South America. New hosts were identified for *Kirramyces gauchensis*, *M. aurantia*, *M. marksii*, *M. lateralis*, *M. scytalidii*, *P. norchiensis*, *T. molleriana*, *T. ohnowa* and *T. pluritubularis*. Interestingly *K. gauchensis,* which has been known only as a stem pathogen, was isolated from leaf spots on *E. maidenii* and *E. tereticornis*. The large number of Mycosphaerellaceae and Teratosphaeriaceae occurring in Uruguay is disturbing and raises concerns regarding the introduction of new pathogens that could threaten not only *Eucalyptus* plantations but also native forests.

1 Introduction

Eucalyptus is one of the most important hardwood crops in the world, planted primarily for pulp and timber production (Turnbull 2000). The success of *Eucalyptus* plantations in areas outside Australia, where most species are native, has been attributed to many factors including the absence of pests and pathogens that affect these trees in their areas of origin (Burgess and Wingfield 2002; Wingfield et al. 2001).

A diverse group of fungi threatens *Eucalyptus* production worldwide and amongst these, *Mycosphaerella* leaf diseases (MLD) are considered particularly important (Park et al. 2000; Summerell et al. 2006). To date, more than 90 species of Mycosphaerellaceae and Teratosphaeriaceae residing in *Mycosphaerella*, *Teratosphaeria*, and several anamorph genera where the teleomorph is unknown (Crous et al. 2007a) have been recorded on *Eucalyptus* (Burgess et al. 2007; Cortinas et al. 2006b; Crous et al. 2004a, 2006; Hunter et al. 2006). This group of fungi may cause leaf spots, leaf blotch, and stem cankers and various species have the capacity to reduce tree growth (Park et al. 2000). Carnegie et al. (1994) found a negative correlation between tree height and diameter and severity of MLD in *Eucalyptus globulus* plantations and Carnegie et al. (1998) reported that even a 10% infection resulted in a 17% reduction in height of *E. globulus* in plantations. Other

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investigations found a significant reduction in growth rate when >25% of the juvenile foliage of *E. nitens* was lost due to MLD (Lundquist and Purnell 1987).

In Uruguay, the area planted to *Eucalyptus* has tripled in the last 10 years, increasing from 175 000 ha in 1995 to ca. 500 000 ha in 2005 (MGAP 2005). This explosive increase in the planted area has also been associated with an increase in disease problems. Despite this, very little research has been done on *Eucalyptus* pathogens and almost nothing is known regarding the identity of the fungi associated with MLD. Prior to the present study, seven Mycosphaerellaceae and Teratosphaeriaceae species had been reported on *Eucalyptus* in Uruguay. These include *Kirramyces gauchensis*, *K. epicoccoides*, *Mycosphaerella marksii*, *M. walkeri*, *Teratosphaeria molleriana*, *T. pseudosuberosa*, and *T. suberosa* (Balmelli et al. 2004; Crous et al. 2006; Cortinas et al. 2006b). However, symptoms suggested that other species were present and the objective of this study was to gain a comprehensive view of the Mycosphaerellaceae and Teratosphaeriaceae species occurring on *Eucalyptus* plantations in the country.

2 Materials and methods

2.1 Collection of specimens and isolation

Several surveys were conducted throughout Uruguay and these were arranged to cover all major *Eucalyptus* growing areas and the widest possible number of species including *E. camaldulensis*, *E. cinerea*, *E. dunnii*, *E. ficifolia*, *E. globulus*, *E. grandis*, *E. maidenii*, *E. robusta*, *E. tereticornis* and *E. viminalis*. Diseased leaves were collected and taken to the laboratory for examination. Symptoms were described and photographed for future reference.

Isolations from lesions with pseudothecia were conducted following the procedure described by Crous (1998). Briefly, leaf pieces cut from the lesions bearing pseudothecia were soaked in sterile water for 2 h. Leaf pieces were then dried on sterilized paper and attached with adhesive tape to the undersides of Petri dish lids with the pseudothecia facing the surface of 2% malt extract agar (MEA) (2% malt extract, 1.5% agar; Oxoid, Basingstoke, England, UK). Petri dishes were incubated in the dark at 17–18°C. After 24–48 h, ascospores that had been ejected onto the surface of the medium and had germinated were observed under a dissecting microscope. Germinated ascospores were then lifted from the medium, mounted on microscope slides and observed under a light microscope for germination patterns and recorded as described by Crous (1998). Individual germinating ascospores were also transferred to fresh plates of 2% MEA medium to generate monosporic cultures.

In cases where pseudothecia were not observed on the lesions, pieces of leaf tissue were cut from the edges of the lesions, surface-disinfected in 70% ethyl alcohol for 30 s, rinsed twice in sterile distilled water, blotted dry on sterile filter paper, and plated on 2% MEA amended with 0.01 g of streptomycin per litre. Plates were then incubated at room temperature for 2–3 days and emerging colonies were sub-cultured onto fresh 2% MEA plates. Only those cultures with colony morphologies resembling those of Mycoshaerellaceae species were retained for further study. Selected colonies were purified by making single hyphal tip transfers to fresh media.

Isolation from twig cankers was done following the methods described by Cortinas et al. (2006b). Single-conidial cultures were obtained from mature pycnidia taken from twig lesions. Two pycnidia from each lesion were suspended in 100 μl of sterile distilled water to allow conidial release. After 30 mins, the conidial suspension was spread onto the surface of 2% MEA. After 24–36 h, germinating conidia were transferred to new MEA plates. Cultures were grouped based on host species, ascospore germination patterns as described by Crous (1998), conidial and ascospore morphology and/or colony morphology.

Table 1. **List of cultures isolated in this study and used in the phylogenetic analysis.**

Culture ID	Teleomorph	Anamorph	Host	Location of collection	Genbank accession no.
UY23	Unknown	*Kirramyces gauchensis*	*Eucalyptus grandis*	Tacuarembo	EU851910
UY186	Unknown	*Pseudocercospora norchiensis*	*E. globulus*	Paysandú	EU851911
UY214	Unknown	*K. gauchensis*	*E. globulus*	Paysandú	EU851912
UY372	*Mycosphaerella aurantia*	Unknown	*E. grandis*	Río Negro	EU851913
UY379	*M. scytalidii*	Unknown	*E. grandis*	Río Negro	EU851914
UY386	*M. aurantia*	Unknown	*E. grandis*	Río Negro	EU851915
UY387	*M. scytalidii*	Unknown	*E. grandis*	Río Negro	EU851916
UY400	*M. scytalidii*	Unknown	*E. dunnii*	Río Negro	EU851917
UY414	*M. marksii*	Unknown	*E. dunnii*	Río Negro	EU851918
UY418	*M. lateralis*	*Dissoconium dekkeri*	*E. dunnii*	Río Negro	EU851919
UY422	Unknown	*Ps. norchiensis*	*E. dunnii*	Río Negro	EU851920
UY423	*M. heimii*	*Ps. heimii*	*E. dunnii*	Río Negro	EU851921
UY440	*M. marksii*	Unknown	*E. grandis*	Río Negro	EU851922
UY604	Unknown	*K. gauchensis*	*E. tereticornis*	Paysandú	EU851923
UY1122	*M. marksii*	Unknown	*E. globulus*	Durazno	EU851924
UY1126	*Teratosphaeria pluritubularis*	Unknown	*E. globulus*	Durazno	EU851925
UY1155	*M. marksii*	Unknown	*E. dunnii*	Durazno	EU851926
UY1156	*M. scytalidii*	Unknown	*E. globulus*	Durazno	EU851927
UY1158	*T. molleriana*	*Colletogloeopsis molleriana*	*E. globulus*	Durazno	EU851928
UY1163	*M. marksii*	Unknown	*E. dunnii*	Durazno	EU851929
UY1192	*M. marksii*	Unknown	*E. globulus*	Florida	EU851930
UY1196	*M. marksii*	Unknown	*E. maidenii*	Florida	EU851931
UY1197	*T. molleriana*	*C. molleriana*	*E. maidenii*	Florida	EU851932
UY1199	Unknown	*K. gauchensis*	*E. maidenii*	Florida	EU851933
UY1240	*T. ohnowa*	Unknown	*E. viminalis*	Lavalleja	EU851934
UY1522	Unknown	*K. gauchensis*	*E. tereticornis*	Rivera	EU851935
UY1528	Unknown	*Ps. norchiensis*	*E. dunnii*	Rivera	EU851936
UY1530	Unknown	*K. gauchensis*	*E. tereticornis*	Rivera	EU851937
UY1561	Unknown	*Ps. norchiensis*	*E. grandis*	Rivera	EU851938

2.2 DNA extraction, PCR, sequencing and phylogenetic analysis

Genomic DNA was extracted from 29 isolates representing the different morphological forms emerging from the survey (Table 1). Cultures were grown on 2% MEA at 25°C for 30 days. Mycelium scrapped directly from the colonies was transferred to microfuge tubes (1.5 ml) with 3-mm glass beads and extraction buffer of the Qiagen Plant DNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA), vigorously shaken using a vortex mixer and placed in a water bath at 60°C for 1 h. DNA was extracted from the mycelial slurry using the Qiagen Plant DNeasy Mini Kit following the manufacturer's instructions.

The primers ITS1 and ITS4 (White et al. 1990) were used to amplify the entire internal transcribed spacer region 1 and 2 (ITS1 and ITS2) plus the 5.8S gene of the ribosomal DNA operon. The polymerase chain reactions (PCR) had a total volume of 25-μl containing 1X of Amplitaq Gold PCR Master-Mix (Applied Biosystems, Foster City, CA, USA), 0.2 μM of each primer and approx. 10 ng μl^{-1} of DNA template. Deionized-distilled water was added to a final volume of 25 μl. PCR amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV, USA) using the

following PCR cycling conditions: initial denaturation for 5 mins at 94°C; 1 min at 94°C; 1 min at 50°C; 1 min at 72°C; repeated 35 times; followed by a final elongation step of 5 mins at 72°C.

The PCR products stained with SYBR Green nucleic acid dye (MBL International, Woburn, MA, USA) were visualized on 1.5% agarose gels under UV light. ExoSAP-IT PCR clean-up kit (USB Corp., Cleveland, OH, USA) was used to purify PCR amplicons for sequencing following manufacturer's instructions. Sequencing reactions were performed using the same primers as those for the PCR with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 377 automated DNA sequencer. Sequences were obtained in both directions and assembled using ChromasPro software version 1.33 (Technelysium Pty. Ltd., Eden Prairie, MN, USA).

The BLAST searches in NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), were conducted with the sequences obtained in this study (Table 1). Sequences for the ex-type cultures of the closest matching species were downloaded from Genbank where available, along with other representative sequences of Mycosphaerellaceae species reported on *Eucalyptus* from studies of Burgess et al. (2007), Cortinas et al. (2006a,b), Crous et al. (2001, 2004a,b, 2006, 2007b), Hunter et al. (2006, 2007), Maxwell et al. (2003, 2005), Slippers et al. (2004), Summerell et al. (2006), Taylor et al. (2003), Verkley et al. (2004) and Wingfield et al. (2006). Multiple sequence alignments were made online using the E-INS-i strategy in MAFFT version 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh et al. 2005).

Phylogenetic analyses were performed using PAUP Version 4.0b10 (Swofford 2002) for neighbour-joining (NJ) and maximum parsimony (MP) analyses, and Mr Bayes v3.1.2 (Ronquist and Huelsenbeck 2003) for Bayesian analysis. The best model for neighbour-joining and Bayesian analyses was determined in Modeltest version 3.7 (Posada and Crandall 1998) and MrModeltest v2.2 (Nylander 2004), respectively, from which a general time reversible substitution model including a proportion of invariant sites and gamma-distributed substitution rates of the remaining sites (GTR + I + G) was selected for both analyses using Akaike information criterion [AIC; proportion of invariable sites (I) = 0.3039; gamma distribution shape parameter (G) = 0.8768; base frequencies: π_A = 0.1895, π_C = 0.3346, π_G = 0.2805, π_T = 0.1954)]. Gaps generated in the alignment process were treated as missing data and all characters were treated as unordered and of equal weight. Ties were broken randomly when found. Maximum parsimony analysis was performed using the heuristic search option with simple addition of taxa and tree bisection and reconnection as the branch-swapping algorithm. The confidence levels of the tree branch nodes were determined by analysis of 1000 bootstrap replicates (Hillis and Bull 1993).

For the Bayesian analysis, four Markov Chain Monte Carlo (MCMC) chains starting from random tree topology were run over 10 million generations. Trees were sampled every 100th generation and the burn-in value was set at 200 since the likelihood values were stationary after 20 000 generations. To obtain the estimates for the posterior probabilities, a 50% majority rule consensus of the remaining 99 711 trees was computed from a total of 199 512 sampled trees.

3 Results

3.1 Isolates

A total of 68 isolates were obtained from six different *Eucalyptus* species collected throughout Uruguay. Isolates grouped by host species, ascospore germination pattern, and spore and colony morphology resulted in 29 isolates representing each group. These were further identified using DNA sequence comparisons (Table 1).

3.2 DNA comparisons and phylogenetic inference

The PCR amplicons of approximately 600–650 bp were produced with the selected primers for all 29 isolates. Sequences were deposited in Genbank and accession numbers are shown in Table 1. The ITS sequence alignment consisted of 104 ingroup sequences and *Neofusicoccum ribis* as the outgroup taxon. Aligned DNA sequences of 724 total characters included the complete ITS region (ITS1, 5.8 and ITS2), of which 212 were constant, 79 variable characters were parsimony-uninformative and 433 were parsimony informative. The heuristic search analysis of the data resulted in a single most parsimonious tree (TL = 1425 steps; CI = 0.614; RI = 0.927; HI = 0.386). Neighbour-joining, MP and Bayesian analyses resulted in trees of similar topology. The NJ tree is shown in Fig. 1 with bootstrap values of the NJ and MP analyses and the posteriori probabilities obtained in the Bayesian analysis shown at branch nodes. The aligned sequence data was deposited in TreeBASE (SN3972).

Ten distinct species residing in the Mycosphaerellaceae and Teratosphaeriaceae emerged from these analyses (Fig. 1) and identity was confirmed by microscopic observations of morphological characteristics. All of these species were found associated with MLD symptoms, including *K. gauchensis,* which was also found on stem and twig cankers on several different *Eucalyptus* species. The species identified from isolates emerging from the survey included *K. gauchensis*, *M. aurantia*, *M. heimii*, *M. lateralis*, *M. marksii*, *M. scytalidii*, *Pseudocercospora norchienses*, *Teratosphaeria molleriana, T. ohnowa* and *T. pluritubularis* (Fig. 1).

Mycosphaerellaceae and Teratosphaeriaceae were found to occur in most of the major areas where *Eucalyptus* is grown (Table 2, Fig. 2). Five Mycosphaerellaceae species were associated with a single *Eucalyptus* species and these were *M. aurantia*, *M. heimii*, *M. lateralis*, *T. ohnowa* and *T. pluritubularis*. *M. aurantia* was isolated only from diseased leaves of *E. grandis* plantations in Río Negro whereas *M. heimii* and *M. lateralis* were found only on *E. dunnii* in plantations also located in Río Negro. *Teratosphaeria ohnowa* was associated with symptoms on *E. viminalis* in Lavalleja, whereas *T. pluritubularis* was found only on *E. globulus* planted in Durazno.

Some Mycosphaerellaceae species were found occurring on several *Eucalyptus* species. For example, *M. marksii* was found associated with lesions on *E. dunnii, E. globulus, E. grandis* and *E. maidenii* in Durazno, Florida, Lavalleja and Río Negro. *T. molleriana* was isolated from diseased *E. globulus* and *E. maidenii* leaves in Durazno and Florida. *Kirramyces gauchensis* was isolated from stem cankers on *E. globulus*, *E. grandis*, *E. maidenii,* and *E. tereticornis*, and also from specks on leaves of *E. maidenii* and *E. tereticornis*. This pathogen was found on *Eucalyptus* in plantations in four different provinces including Florida, Paysandú, Rivera and Tacuarembó. *Mycosphaerella scytalidii* and *P. norchiensis* were found on lesions on *E. dunnii*, *E. globulus* and *E. grandis* in Río Negro and Durazno, and Río Negro, Paysandú and Rivera, respectively.

4 Discussion

Results of this study revealed ten species of Mycosphaerellaceae and Teratosphaeriaceae infecting *Eucalyptus* in Uruguay. Of these, *M. aurantia*, *M. heimii*, *M. lateralis*, *M. scytalidii*, *P. norchiensis*, *T. ohnowa* and *T. pluritubularis* are recorded in Uruguay for the first time. This is also the first report of *M. aurantia* occurring outside of Australia and the first record of *P. norchiensis* and *T. pluritubularis* in South America. New hosts and an expanded geographical distribution for several species associated with MLD in *Eucalyptus* plantations are further provided.

The Mycosphaerellaceae and Teratosphaeriaceae represent a taxonomically complex group and many species have yet to be identified (Crous et al. 2006, 2007b). The

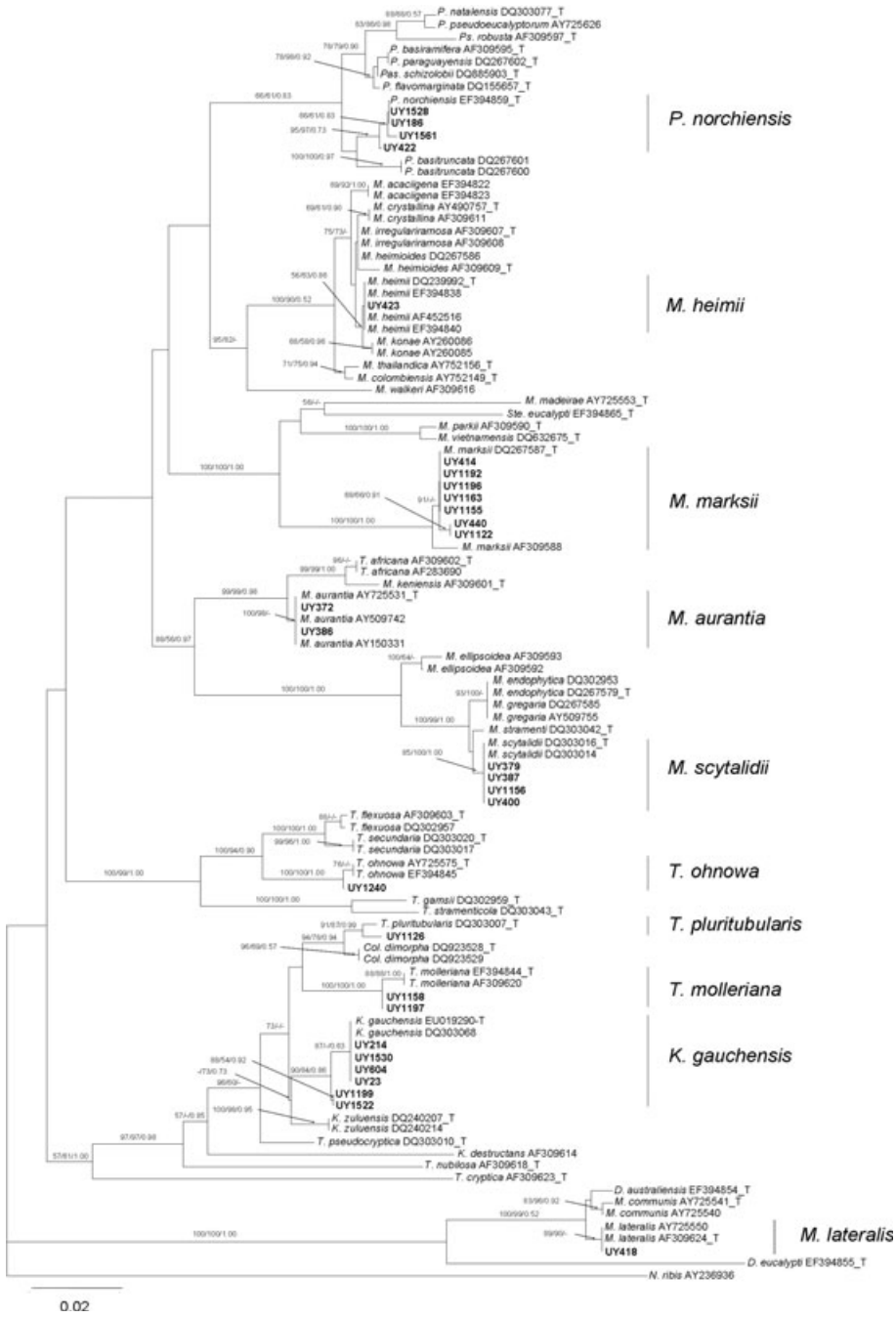
P. natalensis DQ303077_T
P. pseudoeucalyptorum AY725626
Ps. robusta AF309597_T
P. basiramifera AF309595_T
P. paraguayensis DQ267602_T
Pas. schizolobii DQ855903_T
P. flavomarginata DQ155657_T
P. norchiensis EF394859_T
UY1528
UY186
UY1561
UY422
P. basitruncata DQ267601
P. basitruncata DQ267600
P. norchiensis
M. acaciigena EF394822
M. acaciigena EF394823
M. crystallina AY490757_T
M. crystallina AF309611
M. irregulariramosa AF309607_T
M. irregulariramosa AF309608
M. heimioides DQ267586
M. heimioides AF309609_T
M. heimii DQ239992_T
M. heimii EF394838
UY423
M. heimii AF452516
M. heimii EF394840
M. konae AY260086
M. konae AY260085
M. thailandica AY752156_T
M. colombiensis AY752149_T
M. wakeri AF309616
M. heimii
M. madeirae AY725553_T
Ste. eucalypti EF394865_T
M. parkii AF309590_T
M. vietnamensis DQ832675_T
M. marksii DQ267587_T
UY414
UY1192
UY1196
UY1163
UY1155
UY440
UY1122
M. marksii AF309588
M. marksii
T. africana AF309602_T
T. africana AF283690
M. keniensis AF309601_T
M. aurantia AY725531_T
UY372
M. aurantia AY509742
UY386
M. aurantia AY150331
M. aurantia
M. ellipsoidea AF309593
M. ellipsoidea AF309592
M. endophytica DQ302953
M. endophytica DQ267579_T
M. gregaria DQ267585
M. gregaria AY509755
M. stramenti DQ303042_T
M. scytalidii DQ303016_T
M. scytalidii DQ303014
UY379
UY387
UY1156
UY400
M. scytalidii
T. flexuosa AF309603_T
T. flexuosa DQ302957
T. secundaria DQ303020_T
T. secundaria DQ303017
T. ohnowa AY725575_T
T. ohnowa EF394845
UY1240
T. ohnowa
T. gamsii DQ302959_T
T. stramenticola DQ303043_T
T. pluritubularis DQ303007_T
UY1126
Col. dimorpha DQ923528_T
Col. dimorpha DQ923529
T. pluritubularis
T. molleriana EF394844_T
T. molleriana AF309620
UY1158
UY1197
T. molleriana
K. gauchensis EU019290-T
K. gauchensis DQ303068
UY214
UY1530
UY804
UY23
UY1199
UY1522
K. gauchensis
K. zuluensis DQ240207_T
K. zuluensis DQ240214
T. pseudocryptica DQ303010_T
K. destructans AF309614
T. nubilosa AF309618_T
T. cryptica AF309623_T
D. australiensis EF394854_T
M. communis AY725541_T
M. communis AY725540
M. lateralis AY725550
M. lateralis AF309624_T
UY418
M. lateralis
D. eucalypti EF394855_T
N. ribis AY236936
0.02

Table 2. **Location of each Mycosphaerellaceae and Teratosphaeriaceae species and host association found in Uruguay.**

	Eucalyptus dunnii	*E. globulus*	*E. grandis*	*E. maidenii*	*E. tereticornis*	*E. viminalis*
Kirromyces gauchensis	–	Paysandú	Tacuarembó	Florida	Paysandú, Rivera	–
Mycosphaerella aurantia	–	–	Río Negro	–	–	–
M. heimii	Río Negro	–	–	–	–	–
M. lateralis	Río Negro	–	–	–	–	–
M. marksii	Durazno, Río Negro	Durazno, Florida	Río Negro	Florida	–	–
M. scytalidii	Río Negro	Durazno	Río Negro	–	–	–
Pseudocercospora. norchiensis	Río Negro, Rivera	Paysandú	Río Negro	–	–	–
Teratosphaeria molleriana	–	Durazno	–	–	–	–
T. ohnowa	–	–	–	–	–	Lavalleja
T. pluritubularis	–	Durazno	–	–	–	–

availability of DNA sequence comparisons has led to the identification of many cryptic species or morphologically similar species. As cultures become available, more are likely to appear. While many new names are appearing in this group, it is widely accepted that very little is known about geographic distribution and host range of most Mycosphaerellaceae and Teratosphaeriaceae species. This study contributes to a better and more comprehensive understanding of the distribution and host range of this important group of fungi on *Eucalyptus*. The known geographic distribution of several species is expanded with this study and, except for *M. heimii*, an expanded host range for every species found is also reported.

Pathogenicity is an issue that has not been well resolved for most species. That is the case for *M. scytalidii*, *P. norchiensis*, *T. ohnowa* and *T. pluritubularis,* which have been very recently described and nothing is known regarding their etiology. *Mycosphaerella scytalidii* has been only reported in Colombia causing leaf disease on *E. urophylla* and in Brazil infecting *E. globulus* (Crous et al. 2006). *Pseudocercospora norchiensis* has been very recently described in Italy causing leaf spots on *Eucalyptus* (Crous et al. 2007b). In addition, *T. ohnowa* has been reported in South Africa infecting *E. grandis* and *E. smithii* (Crous et al. 2004a) and was recently found in Australia on *E. dunnii* (Crous et al. 2007b) whereas *T. pluritubularis* is known to occur only in Spain causing leaf spots on *E. globulus* (Crous et al. 2006). Further investigation to determine the economic importance of these fungi, along with their geographical distribution in the country is warranted.

Hunter et al. (2006) concluded that *M. aurantia* and *T. africana* represent a single phylogenetic species supported by multi-gene phylogeny analyses, and that they may be distinguished from each other based on morphology. However, a clear distinction in

Fig. 1. Neighbour-joining tree from the ITS sequence data. Species name and Genbank accession number is shown for each sequence. Sequences labelled with a 'T' at the end correspond to the ex-type culture. Bootstrap values of 1000 replicates of neighbour-joining and maximum parsimony analyses and posteriori probabilities of the Bayesian analysis are shown at the branches, respectively. Only bootstrap values higher than 50% are shown. *Neofusicoccum ribis* was used as outgroup taxon. Those sequences obtained in this study are shown in bold and the species where they grouped are indicated with a right brace. Branch lengths are scaled and scale bar is 0.02 nucleotide substitutions per site.

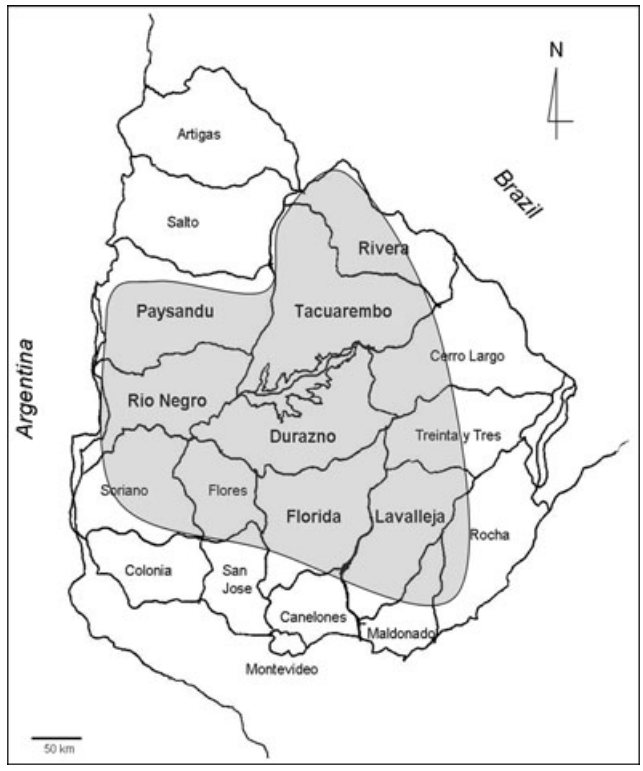

Fig. 2. Map of Uruguay showing in bold those provinces where Mycosphaerellaceae species were found infecting *Eucalyptus*. Shaded area indicates major *Eucalyptus* growing areas.

morphology was not evident in our study. Ascospores size and morphology observed in isolates UY372 and UY386 were similar to those described for both *M. aurantia* and *T. africana* according to Maxwell et al. (2003) and Crous (1998), respectively. Ascospore germination was from both ends of the spore growing in parallel to its long axis. The only distinctive characteristic was that spores of Uruguayan isolates remained hyaline as described for *M. aurantia* by Maxwell et al. (2003), whereas spores of *T. africana* tend to become medium brown after germination according to Crous (1998). In the three phylogenetic analyses reported here the two isolates (UY372 and UY386) were strongly grouped with *M. aurantia* and separated from *T. africana* and *M. keniensis* (Fig. 1). *Mycosphaerella aurantia* along with *T. molleriana* are considered primary pathogens in Australia (Maxwell et al. 2003) and their importance in Uruguay as pathogens must be determined because they were found infecting *E. globulus* and *E. grandis.* These *Eucalyptus* species make up 90% of the *Eucalyptus* area planted. *T. molleriana* has been found infecting adult and juvenile foliage of *E. globulus* plantations in Australia, Portugal, Tasmania, United States and Uruguay (Balmelli et al. 2004; Carnegie and Keane 1998; Hunter et al. 2006; Maxwell et al. 2003) and less commonly infecting *E. viminalis* in Australia (Carnegie and Keane 1998). In contrast, *M. aurantia* was first described in Australia infecting juvenile leaves of *E. globulus* and it is known only from that country (Maxwell et al. 2003). Finding outside Australia raises concern about its current geographic distribution and potential threat to *Eucalyptus* in regions other than Australia.

Mycosphaerella heimii is known to be widely distributed in Australia, Brazil, Madagascar, Indonesia, Portugal, Thailand and Venezuela (Crous et al. 2006, 2007b; Hunter et al. 2006; Whyte et al. 2005) and it is also considered a primary pathogen that can affect as much as 70% of the foliage of susceptible trees (Whyte et al. 2005). Also *M. lateralis* was confirmed as a primary pathogen able to infect *E. globulus* leaves via stomata (Jackson et al. 2005). *Mycosphaerella lateralis* was first described in South Africa (Crous and Wingfield 1996) and later in Australia and Zambia (Crous 1998; Maxwell et al. 2000). This species is particularly interesting because it was recently reported as causing leaf disease on a *Musa* cultivar (Arzanlou et al. 2008). Previously this species was known only from *Eucalyptus* and it is possible that it may also undergo a host shift in Uruguay to infect other agronomic crops as seen by Arzanlou et al. (2008) in Mauritius.

Mycosphaerella marksii has been considered a minor pathogen (Park et al. 2000), however, it seems to be prevalent in *Eucalyptus* plantations in Uruguay and there is some evidence that it contributes to disease. It was first reported in Australia infecting adult and juvenile leaves of several *Eucalyptus* spp. (Carnegie and Keane 1994) and later in Portugal (Crous 1998), China and Indonesia (Burgess et al. 2007; Crous 1998), South Africa (Crous and Wingfield 1996), Ethiopia (Gezahgne et al. 2006), and Uruguay causing leaf disease on *E. globulus* (Balmelli et al. 2004; Crous et al. 2006). We found this fungus associated with leaf blotches on many *Eucalyptus* species but always on adult leaves located in the lower section of the canopy. In addition, it was found associated with leaf blotches on *E. dunnii* on the same leaves where *M. heimii* and *M. lateralis* were isolated. The occurrence of more than one species in the same leaf and even in the same lesion has been previously reported (Crous 1998; Glen et al. 2007) and recognition of this fact is particularly important when undertaking surveys.

Kirramyces gauchensis together with *M. marksii* were the most widely distributed species, occurring on diverse *Eucalyptus* spp. over a broad geographical distribution in Uruguay. *K. gauchensis* is an important stem canker pathogen that was first described from Uruguay and Argentina on *E. grandis* (Cortinas et al. 2006b) and in the current study this species was found associated with stem cankers on *E. globulus*, *E. grandis*, *E. maidenii* and *E. tereticornis*. Interestingly the pathogen was associated with leaf specks whereas it has previously been known only from stem cankers. *Kirramyces gauchensis* is a well-known pathogen of *E. grandis* in Argentina, Hawaii, Uganda and Uruguay, and it has been found on *E. camaldulensis* in Ethiopia (Cortinas et al. 2006b). Our results add *E. globulus*, *E. maidenii* and *E. tereticornis* to the host range of *K. gauchensis* and they provide the first evidence that it can occur on *Eucalyptus* leaves. Its wide distribution in *Eucalyptus* plantations in Uruguay along with its apparent ability to cause diseases on several species of *Eucalyptus* suggests that *K. gauchensis* requires further study. Although, the economic impact of this fungus has not been determined, it is likely to cause damage similar to that attributed to its sibling species *K. zuluensis* (Wingfield et al. 1997). *Kirramyces zuluensis* has been associated with a serious canker disease that hinders bark removal, reduces log value at pulp mills and it may kill trees.

It was surprising that despite intensive surveys, *T. pseudosuberosa*, *T. suberosa* and *M. walkeri* were not found in this study. These fungi are known to occur in the area (Balmelli et al. 2004; Crous et al. 2006) and their absence could indicate a very low prevalence in the main areas planted to *Eucalyptus* in Uruguay. Another common species of Mycosphaerellaceae, *Kirramyces epicoccoides* previously reported by Balmelli et al. (2004), was commonly observed on adult leaves of different species of *Eucalyptus* but isolates were not made and the pathogen was thus not included in the phylogenetic analyses.

The large number of Mycosphaerellaceae species on *Eucalyptus* spp. found in Uruguay during our investigations is disturbing. A similar scenario has been previously observed in *Eucalyptus* plantations in other regions such as Western Australia, China, Indonesia, South Africa and Vietnam (Burgess et al. 2007; Hunter et al. 2004; Jackson et al. 2008). All the

species currently found in Uruguay have been previously reported in other countries and it is likely that most species have been introduced into Uruguay. This likely occurred with the importation of *Eucalyptus* seeds and it raises concerns about the effectiveness of current quarantine procedures. These new introductions and the potential of other devastating pathogens entering Uruguay not only threaten *Eucalyptus* plantations but also may have negative impact on native forest trees that can serve as hosts (Pérez et al. 2008). Continued monitoring is needed to provide an ongoing view of which Mycosphaerellaceae species are infecting *Eucalyptus* in Uruguay plantations.

This research has provided the first comprehensive information regarding the Mycosphaerellaceae associated with MLD in Uruguay and provides a foundation for further work. The impact of these Mycosphaerellaceae species in Uruguay is unknown and many of the species are most likely only weak pathogens. Epidemiology and pathogenicity studies have been conducted on only a few species of the Mycosphaerellaceae (Park 1988a,b; Park and Keane 1982; Milgate et al. 2001; Jackson et al. 2005) and additional investigations should concentrate on understanding the pathology and ecology of the species collected in this study.

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