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Species of Mycosphaerellaceae and Teratosphaeriaceae on native Myrtaceae in Uruguay: evidence of fungal host jumps

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ABSTRACT

Mycosphaerella species are well-known causal agents of leaf diseases on many economically and ecologically important plant species. In Uruguay, a relatively large number of Mycosphaerellaceae and Teratosphaeriaceae are found on *Eucalyptus*, but nothing is known of these fungi on native Myrtaceae. The aim of this study was to identify Mycosphaerellaceae and Teratosphaeriaceae species associated with leaf diseases on native Myrtaceae in Uruguay and to consider whether host jumps by the pathogen from introduced *Eucalyptus* to native Myrtaceae have occurred. Several native forests throughout the country were surveyed with special attention given to those located close to *Eucalyptus* plantations. Five species belonging to the Mycosphaerellaceae and Teratosphaeriaceae clades were found on native Myrtaceous trees and three of these had previously been reported on *Eucalyptus* in Uruguay. Those occurring both on *Eucalyptus* and native Myrtaceae included *Pallidocercospora heimii*, *Pseudocercospora norchiensis*, and *Teratosphaeria aurantia*. In addition, *Mycosphaerella yunnanensis*, a species known to occur on *Eucalyptus* but not previously recorded in Uruguay, was found on leaves of two native Myrtaceous hosts. Because most of these species occur on *Eucalyptus* in countries other than Uruguay, it appears that they were introduced in this country and have adapted to be able to infect native Myrtaceae. These apparent host jumps have the potential to result in serious disease problems and they should be carefully monitored.

Introduction

A diverse group of Mycosphaerellaceae and Teratosphaeriaceae has been associated with *Mycosphaerella* leaf disease (MLD), which are considered particularly important in *Eucalyptus* plantations worldwide (Park *et al.* 2000; Maxwell *et al.* 2003; Crous *et al.* 2004; Crous *et al.* 2006; Cortinas *et al.* 2006; Hunter *et al.* 2006; Summerell *et al.* 2006; Crous *et al.* 2009; Perez *et al.* 2009a; Hunter *et al.* 2011). These fungi cause leaf spots, leaf blotches, or petiole and stem cankers that often result in stressed and stunted trees, adversely affecting commercial forestry operations (Lundquist & Purnell 1987; Carnegie *et al.* 1994; Carnegie *et al.* 1998; Park *et al.* 2000; Sánchez Márquez *et al.* 2011).

Although most studies on MLD have focused primarily on *Eucalyptus*, species of Mycosphaerellaceae and Teratosphaeriaceae

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have also been found infecting species of the Myrtaceae other than those on *Eucalyptus* genus. At least 23 species have been found on non-*Eucalyptus* species in the Myrtaceae worldwide (Crous 1999; Sivanesan & Shivas 2002; Carnegie *et al.* 2007). Increased sampling over the last decade has shown that certain species included in these taxa can be found on a wide range of different hosts including different plant orders such as *Myrtales*, *Proteales*, *Fabaes* and *Apiales* (Hunter *et al.* 2011). The apparent ease of movement in these fungi between hosts is thus of considerable concern.

Most *Eucalyptus* species are native to Australia and have been moved extensively around the world. Where *Eucalyptus* spp. are grown as non-natives, they have largely been separated from their natural enemies (Burgess & Wingfield 2002; Wingfield 2003). This is a situation that is gradually changing with pathogens and pests being brought back into contact with their hosts due to accidental introductions resulting in serious disease problems (Wingfield *et al.* 2008).

Eucalypts are threatened not only by pathogens that are known to attack them in their native environment but there is also growing evidence of pathogens from native Myrtaceae undergoing host shifts to infect them (Slippers *et al.* 2005). The best-known example of such a host shift linked to *Eucalyptus* is that of the *Eucalyptus* rust pathogen *Puccinia psidii*. This rust disease is native on Myrtaceae in South and Central America and has adapted to infect *Eucalyptus* in that region (Coutinho *et al.* 1998; Glen *et al.* 2007). In addition, there are many recent examples of members of the Cryphonectriaceae, that are native on members of the Myrtales, adapted to infect *Eucalyptus* in Africa (Heath *et al.* 2006) as well as South and Central America and Asia (Hodges *et al.* 1986; Myburg *et al.* 2003; Rodas *et al.* 2005; Gryzenhout *et al.* 2006).

Where pathogens have been introduced into new areas, they also have the potential to cause serious diseases on related native plants. It is for this reason that the severe impact of the recent introduction of *P. psidii* to Australia has been of such great concern (Grgurinovic *et al.* 2006; Glen *et al.* 2007; Carnegie *et al.* 2010).

Eucalyptus is widely planted in Uruguay and these trees have already been seriously affected by many diseases thought to have been introduced from other areas. Yet almost nothing is known regarding the pathogens of native Myrtaceae in Uruguay, whether these trees might be threatened by *Eucalyptus* pathogens or possibly the pathogens on the Myrtaceae causing new disease problems on *Eucalyptus*. Uruguay has a large resource of native Myrtaceae (Brussa & Grela 2007) and the aim of this study was to identify Mycosphaerellaceae and Teratosphaeriaceae species associated with MLD on native Myrtaceae species. Furthermore, we considered their relationships with those species currently affecting *Eucalyptus* plantations in Uruguay.

Materials and methods

Samples and isolations

Between 2005 and 2008, trees belonging to the Myrtaceae were surveyed in native forests throughout Uruguay and special attention was placed on those located close to *Eucalyptus* plantations. Leaves showing MLD symptoms were recorded photographically, collected and taken to the laboratory for further study. Samples were collected from a total of 199 trees belonging to 20 native species residing in the Myrtaceae (Table 1). Sampled trees were distributed over the main areas where *Eucalyptus* is planted (Fig 1).

Lesions on leaves bearing pseudothecia were processed for isolation following the procedure described by Crous (1998). Parts of lesions with mature pseudothecia were soaked in sterile water for 2 h. The leaf pieces were then dried on sterilized paper and adhered 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). Petri dishes were incubated at 17–18 °C in the dark for 24–48 h. Ascospores that had been ejected onto the media and had germinated were observed under a microscope to record the germination patterns as described by Crous (1998). Individual germinating ascospores were lifted from the medium and transferred to new plates to generate monosporic cultures.

Where pseudothecia were not observed, pieces of leaf from the edges of the lesion were cut, surface-disinfested in 70 % ethyl alcohol for 30 s, and 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 to minimize bacterial contamination. Plates were then incubated at room temperature and emerging colonies were sub-cultured on fresh 2 % MEA plates. Only those cultures with colony morphologies resembling those of species of Mycosphaerellaceae/Teratosphaeraceae were included in further studies.

Table 1 – List of Myrtaceae species native to Uruguay sampled in this study. Tree species in bold indicate those where Mycosphaerellaceae or Teratosphaeriaceae species were found associated with MLD symptoms.

Tree Species	Fungal species	Province
Acca sellowiana*	*Pseudocercospora norchiensis*	Rivera
	Passalora loranthi	Rivera
Agariota eucalyptides		
Blepharocalyx salicifolius*	*Mycosphaerella yunnanensis*	Rivera
	Pseudocercospora norchiensis	Rivera
	Teratosphaeria aurantia	Rivera
Calyptranthes concinna		
Eugenia involucrata		
E. mansoni		
E. repanda		
E. uniflora		
E. uruguayensis		
Gomidesia palustris		
Hexachlamis edulis		
Myrceugenia euosma		
Myrce. glaucescens*	*Pallidocercospora heimii*	Río Negro
Myrcianthes cisplatensis		
Myrci. pungens		
Myrciaria tenella		
Myrrhinium atropurpureum var. octandrum*	*Mycosphaerella yunnanensis*	Rivera
Psidium luridum		
P. incanum		
P. pubifolium		

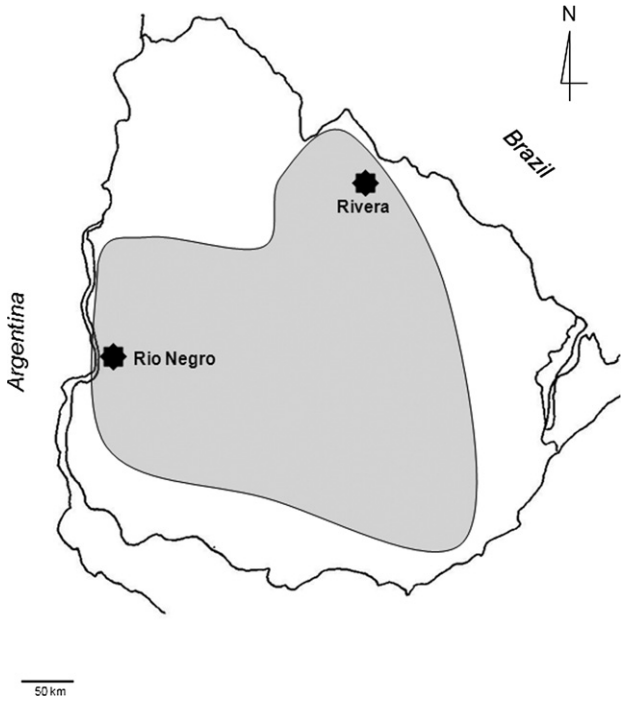

Fig 1 – Map of Uruguay. The shaded area indicates the geographic distribution of *Eucalyptus* plantations in the country. Stars indicate those locations where Mycosphaerellaceae and Teratosphaeriaceae species were found occurring on native trees.

For these isolates, pure cultures were made by transferring hyphal tips to clean culture media and thus ensuring that isolates represented a single genotype. Cultures were grouped based on ascospore germination pattern, conidial and ascospore morphology, and colony morphology. These morphological characteristics were then used to confirm grouping that emerged from the phylogenetic analyses.

DNA extraction, polymerase chain reaction (PCR), sequencing, and phylogenetic analysis

DNA was extracted from isolates representing each morphological group. Mycelium was scrapped directly from the surface of colonies grown on 2 % MEA plates at room temperature for 30 d and transferred to Eppendorf tubes (1.5 ml) with 3-mm glass beads and extraction buffer (Qiagen Inc., Valencia, CA, USA). These were shaken vigorously using a vortex mixer and placed in a water bath at 60 °C for 1 h. DNA extraction was performed using the Qiagen Plant DNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's instructions.

The entire ribosomal DNA internal transcribed spacer (ITS) regions (ITS1 and ITS2) including the 5.8S gene of the rDNA were amplified using the primers ITS1 and ITS4 (White *et al.* 1990). PCR amplifications were performed in an MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV, USA) with the following conditions: initial denaturation for 5 min at 94 °C, then 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C, followed by a final elongation step of 5 min at 72 °C, and hold at 10 °C. The PCRs 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 products were stained with SYBR Green nucleic acid dye (MBL International, Woburn, MA, USA) and visualized on 1.5 % agarose gels under UV light. Amplicons were prepared for sequencing using ExoSAP-IT PCR clean-up kit (USB Corp., Cleveland, OH, USA) following the manufacturer's instructions. For sequencing reactions, the same primer pairs were used 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. Forward and reverse sequences were assembled using ChromasPro software version 1.33 (Technelysium Pty. Ltd., Eden Prairie, MN, USA).

Sequences were subjected to BLAST searches in NCBI Genbank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, verified 26 Jun. 2012), and those of the most closely matching species were downloaded from GenBank. Where available, sequences that represented the ex-type cultures of the closely matching species were used and all species of Mycosphaerellaceae and Teratosphaeriaceae previously reported from *Eucalyptus* were also included. Following a first preliminary phylogenetic analysis, the alignment was trimmed, discarding those species only distantly related to the sequences under investigation and populating the remainder of the data set with at least two sequences per taxon when possible (Table 2). In addition, sequences of species residing in the Mycosphaerellaceae and Teratosphaeriaceae obtained from *Eucalyptus* spp. in Uruguay (Perez *et al.* 2009b) were included in the alignment for comparison. 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/, verified 4 May 2012) (Katoh *et al.* 2005).

Neighbour-joining (NJ) and Maximum parsimony (MP) analyses were performed using PAUP v. 4.0b10 (Swofford 2002). The best substitution model for NJ analysis was determined using Modeltest v. 3.7 (Posada & Crandall 1998) 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 from the Akaike information criterion (AIC; proportion of invariable sites (I) = 0.3375; gamma distribution shape parameter (G) = 0.8871; base frequencies: π_A = 0.2015, π_C = 0.2991, π_G = 0.2800, π_T = 0.2194). Gaps were treated as missing data and all characters were treated as unordered and of equal weight. MP analysis was performed using the heuristic search option with simple addition of taxa and tree bisection and reconnection (TBR) as the branch-swapping algorithm. Support for the nodes of the shortest trees was determined by analyses of 1000 bootstrap replicates (Hillis & Bull 1993) and tree length (TL), consistency index (CI), retention index (RI), and homoplasy index (HI) were calculated.

Part of the translation elongation factor 1-α (EF1-α) region and the Actin (ACT) gene was also amplified for selected isolates to conduct a multiple gene genealogy analysis and thus

Table 2 – List of sequences used in the phylogenetic analysis including those obtained in this study and reference sequences obtained from Genbank. Cultures from Uruguay are indicated with the prefix 'UY'. Cultures from native Myrtaceae sequenced in this study are in bold.

Culture ID	Species	Host	GenBank accession no.		
			ITS	EF-1α	ACT
UY322	*Pallidocercospora heimii*	*Myrceugenia glaucescens*	EU853466	JX444995	JX445002
UY372	*Teratosphaeria aurantia*	*Eucalyptus grandis*	EU851913		
UY423	*Pal. heimii*	*Euc. dunnii*	EU851921	JX444996	JX445003
UY1382	*T. aurantia*	*B. salicifolius*	EU853472	JX444997	JX445004
UY1436	*Pseudocercospora norchiensis*	*Acca sellowiana*	EU853474	JX444998	JX445005
UY1462	*Mycosphaerella yunnanensis*	*Myrrhinium atropurpureum* var. *octandrum*	EU853475		
UY1483	*M. yunnanensis*	*B. salicifolius*	EU853477		
UY1484	*Ps. norchiensis*	*B. salicifolius*	EU853478	JX444999	JX445006
UY1506	*Passalora loranthi*	*Acca sellowiana*	EU853479		
UY1528	*Ps. norchiensis*	*Euc. dunnii*	EU851936	JX445000	JX445007
UY1561	*Ps. norchiensis*	*Euc. grandis*	EU851938	JX445001	JX445008
CBS120735 T	*M. elongata*	*E. camaldulensis* × *E. urophylla*	EF394833		
CBS111519 T	*M. endophytica*	*Eucalyptus* sp.	DQ267579		
CBS111001 T	*M. keniensis*	*Euc. grandis*	AF309601		
CBS326.52	*'M'. laricina*	*Larix decidua*	AY152590		
Lari01.03	*'M'. laricina*	*n/a*	DQ019342		
CBS118493 T	*M. scytalidii*	*Eucalyptus* sp.	DQ303016		
STE-U2769	*M. walkeri*	*Eucalyptus* sp.	AF309616		
CBS119975 T	*M. yunnanensis*	*Eucalyptus* sp.	DQ632686		
CBS119976	*M. yunnanensis*	*Eucalyptus* sp.	DQ632687		
CMW23445	*M. yunnanensis*	*Eucalyptus* sp.	DQ632688		
CPC3837 T	*Pal. acaciigena*	*Acacia mangium*	AY752143		
CBS681.95 T	*Pal. crystallina*	*Euc. bicostata*	AY490757		
CBS110682 T	*Pal. heimii*	*Eucalyptus* sp.	DQ239992	DQ211667	DQ147638
CBS120743	*Pal. heimii*	*Euc. urophylla*	EF394838		
CMW5719	*Pal. heimii*	*Eucalyptus* sp.	AF452516		
CPC13371	*Pal. heimii*	*Euc. urophylla*	EF394840		
CBS111190 T	*Pal. heimioides*	*Eucalyptus* sp.	AF309609		
CBS111364	*Pal. heimioides*	*Eucalyptus* sp.	DQ267586		
CBS114774 T	*Pal. irregulariramosa*	*Euc. saligna*	AF309607		
STE-U2123	*Pal. konae*	*Leucadendron* sp.	AY260086		
STE-U2125	*Pal. konae*	*Leucadendron* sp.	AY260085		
n/a	*Pas. loranthi*	*n/a*	AY348311		
CPC11258	*Pas. sequoiae*	*Juniperus virginiana*	GU214667		
CMW5148 T	*Ps. basiramifera*	*Euc. pellita*	AF309595		
CBS111280	*Ps. basitruncata*	*Euc. grandis*	DQ267601	DQ211676	DQ147621
CBS114664	*Ps. basitruncata*	*Euc. grandis*	DQ267600		
CBS110969 T	*'Ps.' colombiensis*	*Euc. urophylla*	AY752149		
CBS682.95 T	*'Ps.' epispemogonia*	*Euc. grandis*	DQ267587		
CBS110777 T	*Ps. eucalyptorum*	*Eucalyptus* sp.	AF309598		
CMW13586 T	*Ps. flavomarginata*	*Euc. camaldulensis*	DQ155657		
STE-U2556	*Ps. luzardii*	*Hancornia speciosa*	AF362057		
CBS111069 T	*Ps. natalensis*	*Eucalyptus* sp.	DQ303077		
CBS120738 T	*Ps. norchiensis*	*Eucalyptus* sp.	EF394859		
STE-U1458	*Ps. paraguayensis*	*Eucalyptus* sp.	AF309596		
CBS120029 T	*Ps. schizolobii*	*Schizolobium parahybum*	DQ885903		
CPC10547 T	*'Ps.' thailandica*	*Acacia mangium*	AY752156	AY840478	AY752219
CBS680.95 T	*Teratosphaeria africana*	*Euc. viminalis*	AF309602	DQ235099	DQ147609
CMW3025	*T. africana*	*Euc. viminalis*	AF283690		
CBS110500 T	*T. aurantia*	*Euc. globulus*	AY725531	DQ235097	DQ147610
MURU151	*T. aurantia*	*Euc. globulus*	AY150331		
MURU152	*T. aurantia*	*Euc. globulus*	AY509742		
MURU222	*T. aurantia*	*Euc. globulus*	AY509744		
CBS120146 T	*T. molleriana*	*Eucalyptus* sp.	EF394844		
CBS116005 T	*T. nubilosa*	*Euc. globulus*	AF309618		
CBS110949 T	*T. ohnowa*	*Euc. grandis*	AY725575		
CBS118508 T	*T. pluritubularis*	*Euc. globulus*	DQ303007		
CBS120303 T	*T. gauchensis*	*Euc. grandis*	EU019290		
CBS114238 T	*Uwebraunia commune*	*Euc. globulus*	AY725541		
CBS110748 T	*U. dekkeri*	*Eucalyptus* sp.	AF309624		

(*continued on next page*)

Table 2 – (continued)

Culture ID	Species	Host	GenBank accession no.		
			ITS	EF-1α	ACT
STE-U348	*Zasmidium marasasii*	*Syzygium* sp.	AF309591		
CMW3358 T	*Zasmidium parkii*	*Eucalyptus* sp.	AF309590		
CMW7773	*Neofusiccocum ribis*	*Ribes* sp.	AY236936		

T: ex-type cultures.

to confirm the identity of those species found on both native Myrtaceae and *Eucalyptus*. EF-1α was amplified using the primers EF1-728F (5′-CAT CGA GAA GTT CGA GAA GG-3′) and EF1-986R (5′-TAC TTG AAG GAA CCC TTA CC-3′) (Carbone & Kohn 1999). Reaction conditions were: an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s and primer extension at 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 7 min. For the ACT gene, the primers ACT-512F (5′-ATG TGC AAG GCC GGT TTC GC-3′) and ACT-783R (5′-TAC GAG TCC TTC TGG CCC AT-3′) were used (Carbone & Kohn 1999). PCR reaction conditions were: an initial denaturation step at 96 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s and extension at 72 °C for 45 s. This was followed by 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C and elongation at 72 °C for 45 s with an increase of 5 s per cycle. The reaction was completed with a final elongation step at 72 °C for 7 min.

ITS, EF1-α, and ACT sequence datasets were examined for congruence using the Partition Homogeneity Test in PAUP (Farris *et al.* 1995; Huelsenbeck *et al.* 1996). Selected isolates of *Pallidocercospora heimii*, *Pseudocercospora norchiensis* and *Teratosphaeria aurantia*, obtained from *Eucalyptus* (Perez *et al.* 2009b), were included as references to confirm genetic similarity. A combined data set for the ITS, EF1-α, and ACT sequence was analyzed using NJ and MP. Phylogenetic analyses were performed using PAUP Version 4.0b10. Best models for NJ analysis was determined from the AIC Modeltest version 3.7 (Posada & Crandall 1998) as GTR + G for the combined dataset (proportion of invariable sites (I) = 0; gamma distribution shape parameter (G) = 0.2668; base frequencies: $\pi_A = 0.2136$, $\pi_C = 0.2966$, $\pi_G = 0.2583$, $\pi_T = 0.2315$). Gaps generated in the alignment process during the comparison were treated as missing data and all characters were treated as unordered and of equal weight. Ties were broken randomly when found.

All the sequences obtained in this study were deposited in GenBank (Table 1). Sequence alignments and trees of the ITS alone and combined analysis have been deposited in TreeBASE (accession number: 13107).

Results

Samples and isolations

Twenty species of Myrtaceae from a wide range of different collection sites in Uruguay were evaluated and sampled during this study. Symptoms resembling MLD were observed on four species namely *Acca sellowiana*, *Blepharocalyx salicifolius*, *Myrceugenia glaucescens* and *Myrrhinium atropurpureum* var. *octandrum* (Table 1). A total of 45 isolates were obtained from lesions on leaves of these trees. Isolates were grouped by culture and conidial morphology, ascospore germination pattern and host species. One isolate was then selected from each of the resulting seven groups for further investigation using DNA sequence comparisons.

DNA comparison and phylogenetic analyzes

ITS analysis

ITS sequences were generated in both directions and DNA amplicons of ~550 nucleotides were obtained after assemblage. Sequences were deposited in GenBank and accession numbers are shown in Table 2. Following BLAST searches, the 11 sequences obtained in this study were aligned with closest taxa available in GenBank or other species of Mycosphaerellaceae and Teratosphaeriaceae that have been previously reported on *Eucalyptus*. The alignment consisted of 63 ingroup sequences and *Neofusicoccum ribis* as the outgroup taxon.

Sequence alignment resulted in a total of 708 characters of which 233 were constant, 111 variable characters were parsimony-uninformative and 364 were parsimony informative. NJ and MP resulted in trees of identical topology. The heuristic search analysis of the data resulted in four most parsimonious trees (TL = 1129 steps; CI = 0.648; RI = 0.841; HI = 0.352). The genetic distance consensus tree obtained with NJ analysis based on the ITS region of the rDNA operon analysis is shown (Fig 2) with the bootstrap values of 1000 replicates of NJ and MP analyses displayed on the branches.

Combined data set analysis (ITS, EF-1α, ACT)

The five sequences of those species previously found on *Eucalyptus* in Uruguay were subject to a combined analysis to fully verify their identity. The sequence alignment consisted of 12 ingroup sequences and *Teratosphaeria nubilosa* as the outgroup. A combined dataset of a total of 984 characters was analyzed (474, 227, 283 characters for ITS, EF-1α, and ACT, respectively), of which 532 were constant, 147 were parsimony-uninformative and 305 were parsimony informative. Both NJ (NJ) and MP analyses resulted in trees of identical topology. Heuristic search analysis of the data resulted in one most parsimonious tree (TL = 811 steps; CI = 0.874; RI = 0.905; HI = 0.126). The distance tree obtained from NJ analysis of the combined ITS, EF1-α, and ACT with the bootstrap values of 1000 replicates from NJ and MP analyses displayed on the branches is shown (Fig 3).

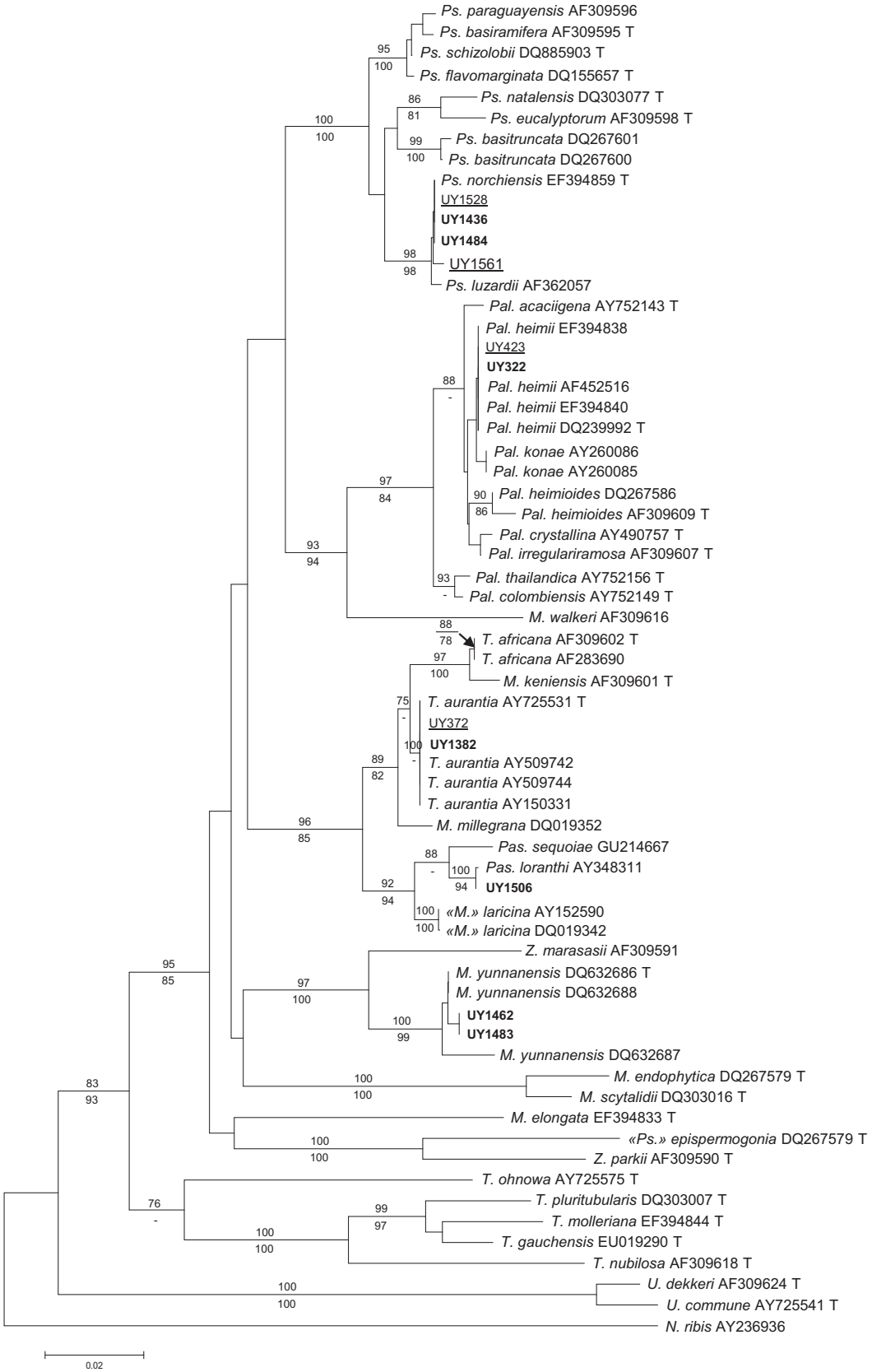

Fig 2 – Genetic distance consensus tree obtained with NJ analysis based on the ITS region of the rDNA operon. Species name and GenBank accession number are shown for each sequence. Sequences labelled with a 'T' at the end correspond to the ex-type culture. Bootstrap values of 1000 replicates of NJ and MP analyses are shown above and below branches, respectively. Only bootstrap values higher than 75 % are shown. *Neofusicoccum ribis* was used as outgroup taxon. Uruguayan isolates are indicated with the prefix 'UY' and sequences corresponding to isolates obtained from native Myrtaceous trees are in bold. Underlined sequences correspond to isolates obtained from *Eucalyptus* spp. in Uruguay, and included for reference.

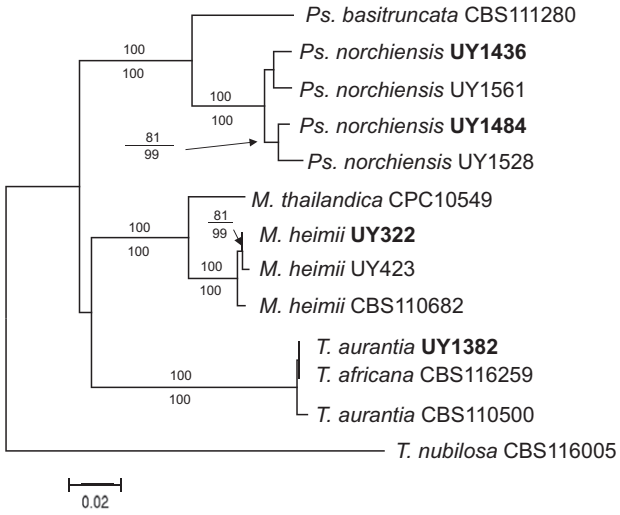

Fig 3 – NJ tree from the combined analysis based on partial ITS, Actin, and EF1-α sequence data. Bootstrap values of 1000 replicates of NJ and MP analyses are shown above and below branches, respectively. Only bootstrap values higher than 75 % are shown. Sequences corresponding to isolates obtained from native Myrtaceous trees are in bold.

Species identified

A diverse group of Mycosphaerellaceae and Teratosphaeriaceae were found to occur on diseased leaves of native Myrtaceae in this study. Phylogenetic analyses revealed a total of five distinct species residing in the Mycosphaerellaceae and Teratosphaeriaceae. These included *Mycosphaerella yunnanensis*, *Pallidocercospora heimii*, *Passalora loranthi*, *Pseudocercospora norchiensis*, and *Teratosphaeria aurantia*.

Three of these species were previously found on *Eucalyptus* by Perez *et al.* (2009b). *Teratosphaeria aurantia* was found associated with leaf spots on *Blepharocalyx salicifolius*, in the northern region (i.e. province of Rivera). The sequence of this isolate was identical to that of UY372 obtained from *Eucalyptus grandis* by Perez *et al.* (2009a,b,c) and was confirmed in the multigene analysis.

Pallidocercospora heimii was associated with leaf spots only on *Myrceugenia glaucescens* in Rio Negro, and the sequence of this isolate was identical to that for the isolate obtained from *Eucalyptus dunnii* by Perez *et al.* (2009b), whereas *Ps. norchiensis* was found on leaves of two native tree species, *Acca sellowiana* and *B. salicifolius*, also in the province of Rivera, and obtained sequences grouped with sequences UY1528 and UY1561 obtained from *E. dunni* and *E. grandis* also in Rivera by Perez *et al.* (2009b). *Passalora loranthi* was found on leaf spots on *A. sellowiana* in the province of Rivera. Surprisingly, *M. yunnanensis*, not previously reported in Uruguay, was found on leaves of *B. salicifolius* and *Myrrhinium atropurpureum* var. *octandrum* in Rivera. Phylogenetic grouping was strongly supported by both NJ and MP analyses and sequences differed only at one base from the sequence for the ex-type culture of this fungus.

Discussion

Results of this study clearly showed that there is a relatively diverse group of species belonging to the Mycosphaerellaceae and Teratosphaeriaceae associated with leaf spots on native Myrtaceae in Uruguay. Three species, *Pallidocercospora heimii*, *Pseudocercospora norchiensis* and *Teratosphaeria aurantia*, are well-known *Eucalyptus* leaf spot associated fungi, previously reported to infect *Eucalyptus* in Uruguay (Balmelli *et al.* 2004; Crous *et al.* 2006; Perez *et al.* 2009b). An important and intriguing aspect of this study was the clear evidence of fungi previously thought to be specific to *Eucalyptus*, occurring on the leaves of native trees in Uruguay. These fungi are all known on *Eucalyptus* leaves in countries other than Uruguay and it seems most likely that they were introduced into Uruguay on *Eucalyptus* and have subsequently undergone a host shift to native tree species. Such host shifts have recently been shown in Uruguay for *Quambalaria* leaf disease caused by *Quambalaria eucalypti* (Perez *et al.* 2008) and *Neofusicoccum eucalyptorum* (Perez *et al.* 2009c). This is, however, the first evidence of species associated with MLD of *Eucalyptus* undergoing such host shifts.

Teratosphaeria aurantia (syn. *Mycosphaerella aurantia*) was found associated with leaf spots on *Blepharocalyx salicifolius*. Although there has been some confusion regarding the identification of this species, with Hunter *et al.* (2006) suggesting that it is likely the same as *Teratosphaeria africana*, isolate UY1382 obtained from *B. salicifolius* grouped strongly with other *T. aurantia* isolates, including that of the ex-type (Maxwell *et al.* 2003). Morphological characteristics of ascospores observed in this study also showed they did not have constrictions at the median septum, as described for *T. aurantia* (Maxwell *et al.* 2003). To date, this species has only been known to occur in Australia and Uruguay (Andjic *et al.* 2010; Hunter *et al.* 2011; Perez *et al.* 2009b) and this study represents the first report on a host other than *Eucalyptus*.

Pallidocercospora heimii was found associated with leaf lesions on *Myrceugenia glaucescens*. *Pallidocercospora* is a novel genus to accommodate former '*Mycosphaerella*' species with pale brown Cercospora-like conidia, and includes *Pal. heimii* (syn. *Mycosphaerella heimii*) (Crous *et al.* 2013). Although Hunter *et al.* (2006) considered *Pal. heimii* to represent a member of a species complex due to the difficulty differentiating this species from *Pallidocercospora heiminoides*, *Pallidocercospora crystallina* and *Pallidocercospora irregulariramosa*, we found that isolate UY322 consistently grouped with *Pal. heimii* sequences and it was clearly separate from other related species of this complex. The ITS sequence for this isolate was also identical to isolate UY423 obtained from *Eucalyptus dunnii* in Uruguay by Perez *et al.* (2009b).

Pallidocercospora heimii is known from Australia, Brazil, Madagascar, Portugal, Uruguay, and Venezuela (Hunter *et al.* 2004; Crous *et al.* 2006; Crous *et al.* 2007; Perez *et al.* 2009b) where it has been found only on *Eucalyptus*. However, it was also found on *Acacia auriculiformis* and *Acacia* sp. in Thailand (Crous & Groenewald 2005). Our results support this data and demonstrate that it is able to cross hosts and all indications are that in Uruguay, it has moved from *Eucalyptus* onto native Myrtaceae. *Pseudocercospora norchiensis*,

found on *Acca sellowiana* and *B. salicifolius* in this study, was very recently described by Crous *et al.* (2007) on leaves of *Eucalyptus* collected in Italy. Very little is known regarding this fungus but Perez *et al.* (2009b) found it on *E. dunnii*, *Eucalyptus globulus* and *Eucalyptus grandis* in the northern region of Uruguay. Although *Pseudocercospora luzardii* grouped closely with *Ps. norchiensis*, the similarity in morphological features of isolates UY1436 and UY1484 and those described for *Ps. norchiensis* as well as the DNA sequence data (100 % of similarity with the ex-type sequence of *Ps. norchiensis*) supports the identification of these isolates as *Ps. norchiensis*. In addition, the reference sequence of *Ps. luzardii* (AF362057) in GenBank differed from *Ps. norchiensis* (EF394859) at three nucleotides in the ITS2 region. The former species has been reported only on *Hancornia speciosa* (Apocynaceae) in Brazil (Furnaletto & Dianese 1999) and it probably represents a distinct species.

Mycosphaerella yunnanensis found on native Myrtaceae in this study has been described by Burgess *et al.* (2007) from *Eucalyptus urophylla* in China. We found this species associated with leaf spots on the native *B. salicifolius* and *Myrrhinium atropurpureum* var. *octandrum* in Uruguay. To the best of our knowledge, this is the first report of *M. yunnanensis* outside China. Although it has not been found on *Eucalyptus* in Uruguay, it seems likely that its origin is on that host.

Passalora loranthi appears to be a species in the Mycosphaerellaceae with a wide host range. The fungus has been previously recorded in two unrelated hosts, namely *Citrus* sp. and *Musa* (Arzanlou *et al.* 2008). We found *Pa. loranthi* associated with leaf disease on *A. sellowiana*. This finding adds a member of the Myrtaceae to the list of hosts that can be infected by this fungus.

This study is the first to broadly consider the MLD on native Myrtaceae growing in association with non-native *Eucalyptus* plantations. Four species previously only known from *Eucalyptus* were found on native Myrtaceae. This suggests strongly that these fungi are moving from non-native *Eucalyptus* to native trees. Our findings support the views of Crous & Groenewald (2005) and Hunter *et al.* (2011) that some leaf-infecting fungi previously thought to be specific to *Eucalyptus*, have wider host ranges than was thought in the past. Almost nothing is known regarding the etiology and impact of these species, other than the fact that they are associated with leaf spot diseases on native trees. There is currently no evidence to suggest that they are causing serious disease problems on the native trees on which they were found, but their potential to result in serious disease situations such as those observed on *Eucalyptus* in Uruguay and around the world must be considered. Continued monitoring of these disease situations is essential.

While there are growing numbers of examples of pathogens of native Myrtaceae moving to *Eucalyptus* where these trees are grown as exotics (Rodas *et al.* 2005; Glen *et al.* 2007; Coutinho *et al.* 2011), there are far fewer examples of movement of apparently introduced *Eucalyptus* pathogens to native plants (Crous & Groenewald 2005). Results of this study provide important new information that this movement is far more common than has been previously thought. Although the consequences have yet to be realized, the results illustrate the danger of moving crop plants between countries together with pathogenic fungi that are poorly understood.

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