Relationship between pathogens of *Eucalyptus* and native Myrtaceae in Uruguay

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ABSTRACT

Eucalyptus (Myrtaceae) is one of the most important hardwood crops in the world, planted primarily for pulp and timber production. In Uruguay, the area planted to *Eucalyptus* has tripled in the last 10 years. The explosive increase in the area planted has been associated with increases in disease problems. Despite this, very few investigations have been carried out to study *Eucalyptus* pathogens and knowledge regarding the etiology, biology and epidemiology of these diseases is limited. Eucalypts are exotics species in Uruguay and pathogens affecting their production could be exotics too. However, it has been demonstrated that different species of native trees could be host to some pathogens affecting eucalypts. Many species belonging to the Myrtaceae have been reported as potential hosts of *Eucalyptus* pathogens. Since Myrtaceae are dominant species in Uruguayan natural forests, the aim of this study was to determine the relationship between pathogens occurring on *Eucalyptus* and those occurring on native Myrtaceae. Between 2005 and 2008 several surveys were made to examine fungal infections on both *Eucalyptus* and native forest trees located geographically close to *Eucalyptus* plantations. Fungal identification was based on morphological characteristics and confirmed with DNA sequence comparisons. *Puccinia psidii*, *Quambalaria eucalypti*, and several species residing in the Botryosphaeriaceae and Mycosphaerellaceae were found occurring in both hosts countrywide. Interestingly, results suggest that most likely host jumps are occurring from native trees to *Eucalyptus* plantations (eg. *P. psidii*) and vice versa (eg. *Q. eucalypti* and *N. eucalyptorum*). These results raise concern about the host speciation of these pathogens and illustrate the danger of moving crop plants between countries, together with fungi that are poorly understood. The negative impact of host jumping events in plant pathology has been well documented and many examples have been cited in the literature. Biotic exchanges are expected to increase as the planted area and age of plantation increase. This study provides a better understanding of the biology and ecology of these pathogens in Uruguay and will assist breeding programs in attempts to obtain disease resistant *Eucalyptus* plantations. The results also establish new concerns for the threat of these pathogens to native trees.

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THESIS STRUCTURE

This thesis was written in manuscript format where each chapter is a separate work that contributes to the aim of this study, which was to determine the relationship between pathogens occurring on *Eucalyptus* and those occurring on native Myrtaceae.

Between 2005 and 2008 several surveys were conducted throughout Uruguay in *Eucalyptus* plantations and native forests located geographically close to plantations. Symptomatic and asymptomatic material was collected and analyzed in the laboratory, from which several endophytes and lesion-associated fungi were isolated and studied resulting in the following chapters of this thesis.

Chapter 1 consists of information about *Puccinia psidii*, the causal agent of *Eucalyptus* rust, which is native to South America and represents a quarantine threat to Australia and South Africa. It was found on both native and introduced Myrtaceous hosts. Interestingly it was found occurring on *Myrrhinium atropurpureum* var. *octandrum*, being the first record of *P. psidii* occurring on that species. Cross pathogenicity tests indicate that isolates from native trees are able to infect and sporulate on *E. grandis* and *E. globulus*.

Quambalaria eucalypti is considered a serious *Eucalyptus* pathogen in Brazil and South Africa, and was reported in Uruguay in 1999, although no epidemic due to this pathogen has been observed. This fungus is confined to the eucalypts. However, in one of the surveys symptoms resembling *Quambalaria* leaf spots occurring on *Myrcianthes pungens* were observed. Samples were collected and examined in the laboratory, where the identity was confirmed. This study is presented in **Chapter 2** and represents the first record of *Q. eucalypti* occurring in a non-*Eucalyptus* host, and provides evidence of a host-jump event.

To date, more than 90 species of Mycosphaerellaceae residing in *Mycosphaerella*, *Teratosphaeria*, and several anamorph genera where the teleomorph is unknown have been recorded on *Eucalyptus.* Prior to the present study, seven Mycosphaerellaceae species had been reported on *Eucalyptus* in Uruguay associated with leaf and stem canker diseases. However, the varied symptoms observed suggested that other species were also present. In **Chapter 3** a diverse group of Mycosphaerellaceae species occurring in *Eucalyptus* plantations is reported from Uruguay including seven species that are new records for the country along with expanded host ranges and geographic distribution of certain species.

After the studies of the Mycosphaerellaceae diversity occurring in *Eucalyptus* plantations its occurrence on native Myrtaceae trees was examined. **Chapter 4** is the first report to broadly consider the *Mycosphaerella* spp. on native Myrtaceae growing in association with non-native *Eucalyptus* plantations. Various *Mycosphaerella* species, previously only known from *Eucalyptus* were encountered on native Myrtaceae. 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 disease situations that are more serious than those observed on *Eucalyptus* is of concern.

Similarly to *Q. eucalypti*, *Neofusicoccum eucalyptorum* is known to occur only on *Eucalyptus*. This fungus has been associated with stem canker in stressed trees. Interestingly, this fungus was found as an endophyte and associated with stem cankers in three different species of Myrtaceae native to Uruguay. This is the first record of *N. eucalyptorum* occurring in hosts other than *Eucalyptus*. This study along with a preliminary assessment of the genetic and phenotypic variability of the collected isolates is presented in **Chapter 5.**

Neofusicoccum eucalyptorum along with other Botryosphaeriaceae have been reported to cause serious diseases on *Eucalyptus* worldwide. In Uruguay three species of Botryosphaeriaceae have been previously found on *Eucalyptus*, and very little is known about their occurrence on native trees. In **Chapter 6**, both native and introduced species of Myrtaceae were investigated for Botryosphaeriaceae infections. Three species were found occurring in both groups of hosts. In addition, *Lasiodiplodia pseudotheobromae,* which has not been found on *Eucalyptus* in the country, was found associated with a stem canker in a native Myrtaceae. The results emphasize the importance of considering native hosts for early detection of potential threats and when assessing the population structure of known *Eucalyptus* pathogens and potentially new pathogens that could affect *Eucalyptus* plantations.

In summary, a strong relationship among pathogens of *Eucalyptus* and native species of Myrtaceae was found. This study raises additional concerns regarding these pathogens not only in Uruguay but in other regions where *Eucalyptus* has been introduced. The negative impact of host-jump events in plant pathology has been well documented and many examples have been repeatedly cited in the literature. In Uruguay, host-jumps appear to have occurred in both directions, from native hosts to introduced plant species and vice versa, and biotic exchanges between both hosts are expected to increase as the planted area and the age of plantations increase. This thesis provides needed information to better understand the economical and ecological impact of these events on both native and introduced Myrtaceae.

Chapter 1: *Puccinia psidii* on Exotic *Eucalyptus* and Native Myrtaceae in Uruguay

ABSTRACT

Eucalyptus rust caused by *Puccinia psidii,* a species native to South and Central America, is a serious disease of *Eucalyptus* spp. and other Myrtaceae. The pathogen was first discovered in 1884 on *Psidium pomiferum* in Brazil. In Uruguay, it was found on *Psidium brasiliensis* in 1981 and later on *Eucalyptus globulus.* Almost nothing is known regarding the importance or occurrence of *P. psidii* on other *Eucalyptus* species or native Myrtaceae in Uruguay. In this study, the presence of *P. psidii* was recorded on *Eucalyptus* species and native Myrtaceae trees in Uruguay and the pathogenicity of specimens from native Myrtaceous hosts was evaluated on *E. globulus* and *E. grandis.* Phylogenetic analyses based on the internal transcribed spacer (ITS) region of the ribosomal DNA operon were used to confirm pathogen identity and to obtain more information on diversity of *P. psidii* isolates in Uruguay. Comparisons of ITS sequences confirmed the identity of *P. psidii* on *Eucalyptus globulus*, *E. grandis*, *Myrcianthes pungens*, and *Myrrhinium atropurpureum* var. *octandrum.* This is the first report of *P. psidii* on *M. atropurpureum var. octandrum*. Pathogenicity tests showed that isolates from native Myrtaceae could infect both *Eucalyptus* species tested.

INTRODUCTION

Eucalyptus rust caused by *Puccinia psidii* Winter*,* a species native to South and Central America, is a serious disease of *Eucalyptus* spp. and other Myrtaceae. It was first found in 1884 on *Psidium pomiferum* L. (syn. *Psidium guajava* L.) in Brazil (Winter, 1984) and was first found on eucalypts [(*Corymbia citriodora* (Hook) Hill & Johnson syn: *Eucalyptus citriodora* Hook)], in the same country in 1944 (Joffily, 1944). This was the first record of the rust having undergone a host jump from a native to a non-native tree (De Castro *et al*., 1983). Subsequent to its first discovery, *P. psidii* has been recorded on many species in the Myrtaceae from the Americas and Hawaii (Acuña and Garran, 2004; Dianese *et al*., 1984; Ferreira, 1981; Ferreira, 1983; MacLachlan, 1938; Marlatt and Kimbrough, 1979; Rayachhetry *et al*., 2001; Uchida *et al.,* 2006; Walker, 1983). Artificial inoculation tests have also shown that *Heteropyxis natalensis* Harv., a species of native Myrtaceae in South Africa is highly susceptible to *P. psidii* (Alfenas *et al.,* 2005; Wilson *et al*., 2005).

Puccinia psidii is considered a devastating pathogen of *Eucalyptus* in Brazil, causing severe damage on *Eucalyptus* trees younger than two-years-old (Alfenas *et al*., 2004; Coutinho *et al*., 1998). This rust is unique because of its exceedingly wide host range, for which Simpson *et al*. (2006) cite 71 host species. Its wide host range and aggressiveness on certain hosts make this rust a major threat to *Eucalyptus* and other Myrtaceae species throughout the world (Coutinho *et al*., 1998, Glen *et al.,* 2007; Grgurinovic *et al*., 2006; Langrell *et al.,* 2008). In Uruguay, *P. psidii* was first found on *Psidium brasiliensis* L. (Koch de Brotos *et al*., 1981). The rust was more recently reported on plantation-grown *Eucalyptus globulus* Labill. subsp. *globulus* (hereafter *E. globulus*) when it caused severe damage to 1- year-old trees (Telechea *et al.,* 2003). This was the first record of *P. psidii* on *E. globulus* and it raised concern that the rust could threaten the growing and important *Eucalyptus* forestry industry in Uruguay.

Since little is known regarding the occurrence or importance of *P. psidii* on *Eucalyptus* spp. or native Myrtaceae trees in Uruguay, information on host range on *Eucalyptus* spp. and other Myrtaceae in the country is fundamental to develop an effective disease management program. The aim of this study was, therefore, to determine the host range of *P. psidii* on *Eucalyptus* species and native Myrtaceae trees in Uruguay and evaluate pathogenicity of isolates obtained from native Myrtaceous hosts on the two most important *Eucalyptus* spp. (*E. globulus* and *E. grandis* (Hill) Maiden) grown in commercial plantations in the country.

MATERIALS AND METHODS

Rust collections

During 2005 to 2007, *Eucalyptus* plantations and natural forest growing in close proximity (<1 km) to *Eucalyptus* were scouted throughout Uruguay for rust infections. Surveys included the provinces of Cerro Largo, Durazno, Florida, Lavalleja, Maldonado, Paysandu, Rio Negro, Rivera, Tacuarembo, Treinta y Tres and Rocha. A total of 21 Myrtaceae species native to Uruguay and 10 species of *Eucalyptus* were exhaustively examined (Table 1.1). Rust pustules were observed only on four tree species, *Eucalyptus globulus*, *Eucalyptus grandis*, *Myrrhinium atropurpureum* Schott var. *octandrum* Benth and *Myrcianthes pungens* (Berg) Legrand. The rust on native trees was very rare and after examining several trees of native Myrtaceae species during two years of surveys, this rust was found only on two native species. All *Eucalyptus* trees were one-year-old, whereas the native trees were adult specimens of unknown age.

Samples of infected leaves were collected in plastic bags, and transported in a cooler at 8ºC to the laboratory. Each rust sample was divided in the laboratory, where a small amount of leaf tissue bearing pustules was dried in small paper envelopes for later analysis. Urediniospores were collected from fresh pustules and stored at -80ºC in glass capsules until they could be used in pathogenicity tests.

Rust morphology

Teliospores and urediniospores were compared using standard light microscope techniques. Teliospores were germinated on a slide with free water for 180 min and observed under the microscope to examine promycelia and cell number. In addition, urediniospore morphology was observed using a Hitachi S-3500N Variable Pressure Scanning Electron Microscope (SEM) at the Imaging Center, College of Biological Science, University of Minnesota. For each sample, spores were attached to stubs with a thin layer of adhesive. Stubs were coated with gold and placed in the low-vacuum, variable pressure Environmental SEM and photographed with a digital camera at approximately 2,000X magnification.

DNA extraction, PCR, sequencing and phylogenetic analysis

DNA was extracted from dried infected host leaf tissue (~20 mg) containing uredinial pustules. Dried host tissue with spores was pulverized by shaking samples in tubes with sterile 1-mm glass beads (Lysing matrix C; Bio 101, Carlsbad, CA) and 25 mg of sterile diatomaceous earth (Sigma-Aldrich, St. Louis) in a Savant FastPrep shaker (FP120, Holbrook, NY) for 20 s at a speed setting of 5 (Zambino, 2002). DNA extraction from the pulverized samples was performed using OmniPrep™ DNA Extraction Kit (Biosciences, Saint Louis, MO) following manufacturer's instructions.

The internal transcribed spacer region of the ribosomal DNA (ITS) was amplified using the primers ITS-1F (5' CTT GGT CAT TTA GAG GAA GTA A 3') and ITS-RUST1 (5' GCT TAC TGC CTT CCT CAA TC 3') (Kroop *et al.*, 1995). Polymerase Chain Reaction (PCR) was performed in a 50-µl reaction mixture of 5.0 µl of 0.05% Casein, 5.0 µl of 10X PCR Buffer, 1.5 µl of 50 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs, 1.0 µl of 20 mM ITS-1F, 1.0 µl of 20 mM ITS-RUST1, 0.2 µl of Platinum® Taq DNA Polymerase (Applied Biosystems, Foster City, CA), 30.3 µl of ddH_2O, 5.0 µl of DNA template. PCR amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) with the following parameters: 2 min at 94ºC; 30 sec at 94ºC; 30 sec at 44ºC; 2 min at 72ºC; cycle to step 2, 30 times; 10 min at 72ºC; hold at 4ºC.

PCR products were visualized on an agarose gel, purified and prepared for sequencing using EXO-SAP-IT PCR clean-up kit (USB Inc., Cleveland, OH) following the manufacturer's instructions. Sequencing reactions were performed using the same primers as for the PCR and the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on an ABI Prism 377 automated DNA sequencer. Sequences were obtained in both directions and assembled using ChromasPro software (Technelysium Pty. Ltd., Eden Prairie, MN). Sequences were deposited in GenBank and accession numbers are shown in Table 1.2. Assembled sequences were subject to BLAST search in NCBI GenBank. Phylogenetic analysis was performed to confirm species identification. Thus, the six *Puccinia psidii* sequences available in GenBank (30 June 2008) and sequences of the rust species that showed the closest match with *P. psidii* were downloaded and aligned along with sequences of representative species residing in the Uredinineae and Melampsorineae according to Aime (2006) (Table 1.2). *Helicobasidium purpureum* was chosen as the outgroup taxon. Multiple sequence alignments were made online using MAFFT version 6 (Katoh *et al.,* 2005). Sequence alignment was deposited in TreeBASE (accession SN3784).

Phylogenetic analysis was performed using PAUP Version 4.0b10 (Swofford, 2002) for neighbor-joining and maximum parsimony analysis, and Mr. Bayes v3.1.2 (Ronquist and Huelsenbeck, 2003) for Bayesian analysis. Best model for Neighbor-joining analysis was determined in Modeltest version 3.7 (Posada and Crandall, 1998) from which the TVM+G model from the Akaike information criterion was selected. 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. Maximum parsimony analysis was performed using the heuristic search option with simple taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. Support for the nodes of the shortest trees was determined by analysis of 1,000 bootstrap replicas (Hillis and Bull, 1993). Tree length (TL), consistency index (CI), retention index (RI), and homoplasy index (HI) were calculated.

The best nucleotide substitution model for the Bayesian analysis was selected using MrModeltest v2.2 (Nylander, 2004) from which a general time reversible substitution model with gamma parameter (GTR+G) was selected using Akaike information criterion. Four MCMC chains starting from random tree topology were run over 10 million generations. Trees were sampled every 100th generation and burn-in was set at 5,000 generations after which the likelihood values were stationary. To obtain the estimates for the posterior probabilities, a 50% majority rule consensus of the remaining 2,663 trees was computed.

Pathogenicity tests

To assess pathogenicity of the rust samples collected on native Myrtaceae trees to *Eucalyptus, E. grandis* and *E. globulus* seedlings were inoculated with a suspension of urediniospores with each of the two rust collections (UY 220 and UY221) under controlled conditions. Three clones of *E. globulus* (”A”, “B” and “C”) and three clones of *E. grandis* (”D”, “E” and “F”) were inoculated with each rust sample, using the urediniospores that were collected from fresh pustules and that had been stored at -80ºC. In addition to *Eucalyptus*, *Syzygium jambos* (L) Alston plants were inoculated, since this host species has been shown to be highly susceptible to *P. psidii* and it is frequently used for inoculum preservation and multiplication (Junghans *et al*., 2003).

One 4-month-old seedling of each host was inoculated with each rust sample. Inoculation was conducted using a pipette to apply five drops of suspension per leaf on five leaves per plant. Each drop was approx. 20 µl of a spore suspension with 5 x10^4 urediniospores/ml. Inoculated plants were incubated 24 h in a mist chamber at 25ºC in the dark and then transferred to a growth chamber at 22ºC with 12-h photoperiod, at 40µmol photons/s/m^2 of light intensity. Twelve days later, plants were evaluated for the presence of rust pustules. Those plants showing negative results (i.e. no pustules) were evaluated again 21 days post-inoculation to confirm the absence of pustules. DNA was extracted from urediniospores present on pustules from inoculated plants, sequenced as described above, and compared with the inoculated specimen-sequence to confirm that contamination had not occurred. Pathogenicity tests were replicated once.

RESULTS

Symptoms and morphology

Similar symptoms were observed on different hosts infected with *P. psidii*. Lesions were primarily observed on young tissues such as actively growing leaves and shoots (Fig. 1.1). Bright orange pustules with orange-yellow urediniospores were present on all evaluated hosts, but dark orange-brown teliospores were observed only on *E. globulus* and *E. grandis*. Grey discoloration of old lesions was observed on *E. grandis,* and shoot tips were dead on *E. grandis* and *Myrr. atropurpureum* var. *octandrum* (Fig. 1.1). Teliospores were similar to those reported by Walker (1983), roughly ellipsoidal to cylindrical to broadly clavate, one-septate, constricted at the central septum, 26-42 x 15-22 µm, with the upper cell generally slightly wider and shorter than the lower, wall pale golden yellow, pore apical in the upper cell and just below the septum in the lower cell, pedicels either deciduous or short (up to 15 µm long). However, in sample UY895 teliospores had pedicels of up to 25 µm long (Fig. 1.2. A). Germinated teliospores produced a four-celled promycelium with four basidiospores (Fig. 1.2.B).

Urediniospores examined from each specimen showed high levels of similarity in spore size, spine density and distribution on the spore surface. Urediniospores 19-26 x 15-22 µm, yellow, unicellular, spherical to elliptical, base truncate, finely and uniformly echinulate with spines up to 1 µm long, 0.5-1.5 µm apart, were observed in all collected samples. In some urediniospores, a bald patch without spines was observed (Fig. 1.2 C-F).

Phylogenetic analysis

DNA fragments of approximately 1,000 bp were amplified with the selected primers for all specimens. The ITS dataset consisted of 43 ingroup sequences plus *Helicobasidium purpureum* that was considered an outgroup taxon based on Aime (2006). Aligned DNA sequences of 623 total characters included the complete ITS region (ITS1, 5.8 and ITS2), of which 181 characters were constant, 62 variable characters were parsimony-uninformative and 380 were parsimony informative. Neighbor-joining, maximun parsimony and Bayesian analyses resulted in trees of similar topology. The heuristic search analysis of the data resulted in 24 most parsimony trees (TL = 1598 steps; CI = 0.558; RI = 0.807; HI = 0.442). The distance tree obtained from the neighbor joining analysis is shown in Figure 1.4.

Minor variation in the ITS sequences (1 change) was observed among the sequences of the three samples collected on *Eucalyptus* spp. In contrast, the two samples collected on the native Myrtaceous trees displayed most variation (4 changes) in the ITS2 region. The parsimony analysis showed a high level of similarity among these five samples and they grouped together with ITS sequences of *P. psidii* obtained from GenBank while being clearly separated from the most closely related species for which sequences are published (30 June 2008). In addition, the two sequences obtained from specimens from native trees (i.e. UY220 and UY221) had a "G" at position 414 of the sequence whereas all the other *P. psidii* specimens collected on *Eucalyptus* spp. have an "A" at this position.

Pathogenicity tests

The two rust samples collected from native Myrtaceae trees (UY220 and UY 221) that were used in the pathogenicity tests, were able to infect and produce new uredinial pustules on the different clones of *E. globulus* tested. However, UY220 was able to sporulate only on *E. grandis* clones "D" and "F", and no infection was observed on *E. grandis* clone "E". *Syzygium jambos* showed no signs of infection by either isolate used in the inoculations (Table 1.3).

Although severity of infection was not specifically assessed, clear differences in number and size of pustules were observed between rust samples on different clones of *E. globulus*: "A" was just slightly infected by both rust samples, "C" was more intensively infected by UY220 but slightly infected by UY221 and "B" was slightly infected by UY220 and moderately infected by UY221.

DISCUSSION

Results of this study have led to the discovery of two previously unknown native Myrtaceae hosts of *P. psidii* in Uruguay. They further provide conclusive evidence based on DNA sequence comparisons, that the rust fungus occurs on native Myrtaceae in the country and that it is the same fungus that is found on non-native *Eucalyptus* spp. in plantations. DNA-based evidence for these findings are supported by morphological characteristics of the fungus. The results reported here also have shown for the first time that isolates from native trees can infect *Eucalyptus* spp.

Previous reports of *P. psidii* in Uruguay were from *Psidium brasiliensis* (Koch de Brotos *et al*., 1981) and *E. globulus* (Balmelli *et al.,* 2004; Telechea *et al.,* 2003). In this study, although not abundant, the fungus was found on *Myrci. pungens, Myrr. atropurpureum* var. *octandrum, E. globulus* and *E. grandis*. Both the findings on native trees and the scarcity of the infections observed on them suggest that *P. psidii* is native in Uruguay and if so, it would be under strong ecological homeostasis within native trees. Uruguay has 35 native species of Myrtaceae (Brussa and Grela, 2007) and it is expected that many of these trees could be host to this unusual rust that has a wide host range (Simpson *et al*., 2006) and has undergone significant host shifts to non-native trees such as *Eucalyptus* (Coutinho *et al*., 1998; Slippers *et al.,* 2005) and *Metrosideros* (Uchida *et al*., 2006). To the best of our knowledge, this is also the first report of *P. psidii* on *M. atropurpureum* var. *octandrum.* This is also the first report of *P. psidii* on these two native Myrtaceous hosts in Uruguay confirmed by morphological characteristics and phylogenetic comparisons. *Puccinia psidii* has been previously reported on *M. pungens* in Brazil (Hennen *et al*., 2005), but has not been previously reported on this host in Uruguay.

Symptoms on both *Eucalyptus* spp. and native Myrtaceae trees, were consistent with those described previously (Alfenas *et al.,* 2004; Old *et al.,* 2003). The profuse formation of teliospores observed in this study under field conditions suggests that *P. psidii* is a heteroecious macrocyclic rust for which the alternate aecial host is unknown. This view was also proposed by Simpson *et al.* (2006). However, it is possible that *P. psidii* is apomictic, since aecia and aeciospores have been observed after inoculations with basidiospores on *S. jambos* and *Eucalyptus* (Ferreira, 1989; Figueiredo *et al.,* 1984).

Even though the number of nuclei present in each basidiospore produced from germinated teliospores was not examined examine, four basidiospores were produced from each teliospore and it is expected that these would give rise to monokaryotic basidiospores. Alfenas *et al.* (2004) made a similar observation and it raises a question regarding the rust life cycle and when the fungus undergoes dikaryotization. Pycnia have never been observed in *P. psidii* and the stage where dikaryon formation takes place has yet to be discovered.

Results of this study provide strong preliminary evidence to show that *P. psidii* is genetically diverse in Uruguay. Although the sample size was relatively small, DNA sequence data showed that isolates are genetically different. Furthermore, pathogenicity tests with different *P. psidii* isolates also suggested differences in the susceptibility of *Eucalyptus* hosts. Genetic variation based on much larger collections of isolates is justified to better understand the differences in resistance and susceptibility in *Eucalyptus* clones observed.

An interesting observation in this study was that *S. jambos* was not infected in pathogenicity tests. This tree is one of the hosts most susceptible to *P. psidii* elsewhere in the world (Junghans *et al.,* 2003). Physiological variability is known in *P. psidii* and characterization of different physiological groups (or biotypes) based on cross inoculations have been described (Aparecido *et al.,* 2003; Coelho *et al.,* 2001; De Castro *et al*., 1983). Lack of susceptibility to isolates of *P. psidii* in *S. jambos* emphasizes the fact that the pathogen is physiologically variable in Uruguay and that it is most likely native to the area in which it was discovered in this study. This is likely to complicate *Eucalyptus* forestry in Uruguay and it will mean that screening of clones will need to include the breadth of variability of the rust. It will also be important to understand the population structure of the rust to allow the development of effective breeding programs that will minimize the economic impact of *P. psidii* in Uruguay.

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To the financial and logistic support of Colonvade, Forestal Oriental, Rivermol and Stora Enso as well as Eufores and Forestal Oriental for supplying clones for inoculation. Also thank Andrés Berrutti, Brett Arenz, Charles Barnes, Gustavo Balmelli, Jason Smith, Kim Nguyen and Les Szabo for their assistance and discussions on various aspects of these investigations.

Table 1.1: List of species, both native Myrtaceae and exotic *Eucalyptus*, that were sampled in this study.

Myrtaceae species native to Uruguay*	***Eucalyptus* species**
Acca sellowiana	*E. camaldulensis*
Agariota eucalyptides	*E. cinerea*
Blepharocalyx salicifolius	*E. dunnii*
Calyptranthes concinna	*E. ficifolia*
Eugenia involucrata	***E. globulus***
E. mansonii	***E. grandis***
E. repanda	*E. maidenii*
E. uniflora	*E. robusta*
E. uruguayensis	*E. tereticornis*
Gomidesia palustris	*E. viminalis*
Hexachlamis edulis	
Myrceugenia euosma	
Myrce. glaucescens	
Myrcianthes cisplatensis	
Myrci. pungens	
Myrciaria tenella	
Myrrhinium atropurpureum var. octandrum	
Psidium luridum	
P. incanum	
P. pubifolium	

(*) Those species where rust infections were observed are in bold.

Table 1.2: List of sequences used in this study, including those for which sequences were obtained from GenBank.

Collection ID#	Rust	Host species	Location[a]	GenBank accession no.	Reference
UY217[b]	***Puccinia psidii***	***Eucalyptus grandis***	**Tacuarembo, Uruguay**	**EU348742**	**This study**
UY220	***P. psidii***	***Myrrhinium atropurpureum* var. *octandrum***	**Tacuarembo, Uruguay**	**EU439920**	**This study**
UY221	***P. psidii***	***Myrcianthes pungens***	**Tacuarembo, Uruguay**	**EU439921**	**This study**
UY894	***P. psidii***	***E. globulus***	**Maldonado, Uruguay**	**EU348743**	**This study**
UY895	***P. psidii***	***E. globulus***	**Maldonado, Uruguay**	**EU348744**	**This study**
MG 8	*P. psidii*	*Psidium guajava*	Minas Gerais, Brazil	EF210141	Langrell *et al.*, 2008
MG 9	*P. psidii*	*Syzygium jambos*	Minas Gerais, Brazil	EF210142	Langrell *et al.*, 2008
UFV-12	*P. psidii*	n/a	Brazil	EF210143	Langrell and Glen, GenBank
UFV-12b	*P. psidii*	n/a	Brazil	EF210144	Langrell and Glen, GenBank
n/a	*P. psidii*	*Melaleuca quinquenervia*	Florida, USA	EF599767	Uchida *et al.*, 2006
n/a	*P. psidii*	*Metrosideros polymorpha*	Hawaii, USA	EF599768	Uchida *et al.*, 2006
11487	*Aecidium* sp.	n/a	-	AY956563	Abbasi *et al.*, 2004
CrKor-1	*Cronartium ribicola*	*Pinus koraiensis*	-	L76497	Vogler and Bruns, 1998
AFTOL-ID 712	*Gymnosporangium juniperi-virginianae*	n/a	-	DQ267127	Matheny *et al.*, GenBank
TUB 011550	*Helicobasidium purpureum*	*Ranunculus* sp.	-	AY292455	Lutz *et al.*, 2004
AFTOL-ID 987	*Kuehneola uredinis*	n/a	-	DQ911604	Matheny and Hibbett, GenBank
HSZ0341	*Puccinia allii*	*Allium schoenoprasum*	-	AF511080	Aniskter *et al.*, 2004
HSZ0344	*P. allii*	*A. sativum*	-	AF511078	Aniskter *et al.*, 2004
HSZ0219	*P. andropogonis*	n/a	-	DQ344517	Szabo, 2006
HSZ0027	*P. andropogonis*	n/a	-	DQ344518	Szabo, 2006
RN-9	*P. cerinthes-agropyrina*	*Elytrigia sp.*	-	DQ460720	Jafary *et al.*, 2006
HSZ0928	*P. graminis f.sp. dactylis*	*Dactylis glomerata*	-	DQ417390	Barnes and Szabo, 2007
HSZ0929	*P. graminis f.sp. poae*	*Poa pratensis*	-	DQ417389	Barnes and Szabo, 2007
IBA8759	*P. hemerocallidis*	n/a	-	AB232547	Chatasiri *et al.*, 2006
IBA8749	*P. hemerocallidis*	n/a	-	AB232546	Chatasiri *et al.*, 2006
HSZ0681	*P. holcina*	*Holcus lanatus*	-	DQ512999	Jafary *et al.,* 2006
HSZ0891	*P. holcina*	*Hol. mollis*	-	DQ513000	Jafary *et al.,* 2006

Collection ID#	Rust	Host species	Location[a]	GenBank accession no.	Reference
CDL22/81	*P. hordei*	n/a	-	AY511086	Aniskter *et al*., 2004
CDL64-2B	*P. hordei*	n/a	-	AY187089	Aniskter *et al*., 2004
11511 F	*P. persistens*	n/a	-	AY956561	Abbasi *et al*., 2004
RN-8	*P. persistens*	*Elytrigia repens*	-	DQ460721	Jafary *et al.,* 2006
11506 F	*P. recondita*	n/a	-	AY956553	Abbasi *et al*., 2004
ANK77081	*P. recondita*	*Triticum turgidum*	-	AF511082	Barnes and Szabo, 2007
HSZ0711	*P. striiformis* f. sp. *hordei*	*Hordeum vulgare*	-	DQ417402	Barnes and Szabo, 2007
PSH13	*P. striiformis* f. sp. *hordei*	*Hor. vulgare*	-	DQ417408	Barnes and Szabo, 2007
HSZ0722	*P. striiformis* f. sp. *tritici*	*T. aestivum*	-	DQ417405	Barnes and Szabo, 2007
HSZ0724	*P. striiformis* f. sp. *tritici*	*T. aestivum*	-	DQ417406	Barnes and Szabo, 2007
11491 F	*P. taeniatheri*	n/a	-	AY956557	Abbasi *et al*., 2004
HSZ0741	*P. triticina*	*T. aestivum*	-	DQ417409	Barnes and Szabo, 2007
HSZ0741	*P. triticina*	*T. aestivum*	-	DQ417411	Barnes and Szabo, 2007
Brazil-1	*Phakopsora pachyrhizi*	*Glycine max*	-	EU523736	Silva *et al*., 2008
MUT Zimbabwe	*Phakopsora pachyrhizi*	*Glycine max*	-	AF333499	Frederick *et al*., 2002
H94638	*Uromyces appendiculatus*	n/a	-	AB115738	Chung *et al*., 2004
AFTOL-ID 976	*U. appendiculatus*	n/a	-	DQ411530	Matheny *et al*., GenBank

([a]) Location only indicated for *P. psidii* collections

([b]) Specimens sequenced in this study are shown in bold.

Table 1.3: Results of pathogenicity tests. Three clones of *E. globulus*, three of *E. grandis* and *S. jambos* were inoculated with the two rust samples collected from native Myrtaceae trees (UY220 and UY 221).

	Eucalyptus globulus			***Eucalyptus grandis***			***Syzygium jambos***
Rust ID	**"A"[a]**	**"B"**	**"C"**	**"D"**	**"E"**	**"F"**	**NN[b]**
UY220	+	+	+	+	-	+	-
UY221	+	+	+	-	-	-	-

([a]) code of the clone

([b]) *No name*

+: Indicates presence of pustules 21 days post-inoculation

-: Indicates no pustules observed 21 days post-inoculation.

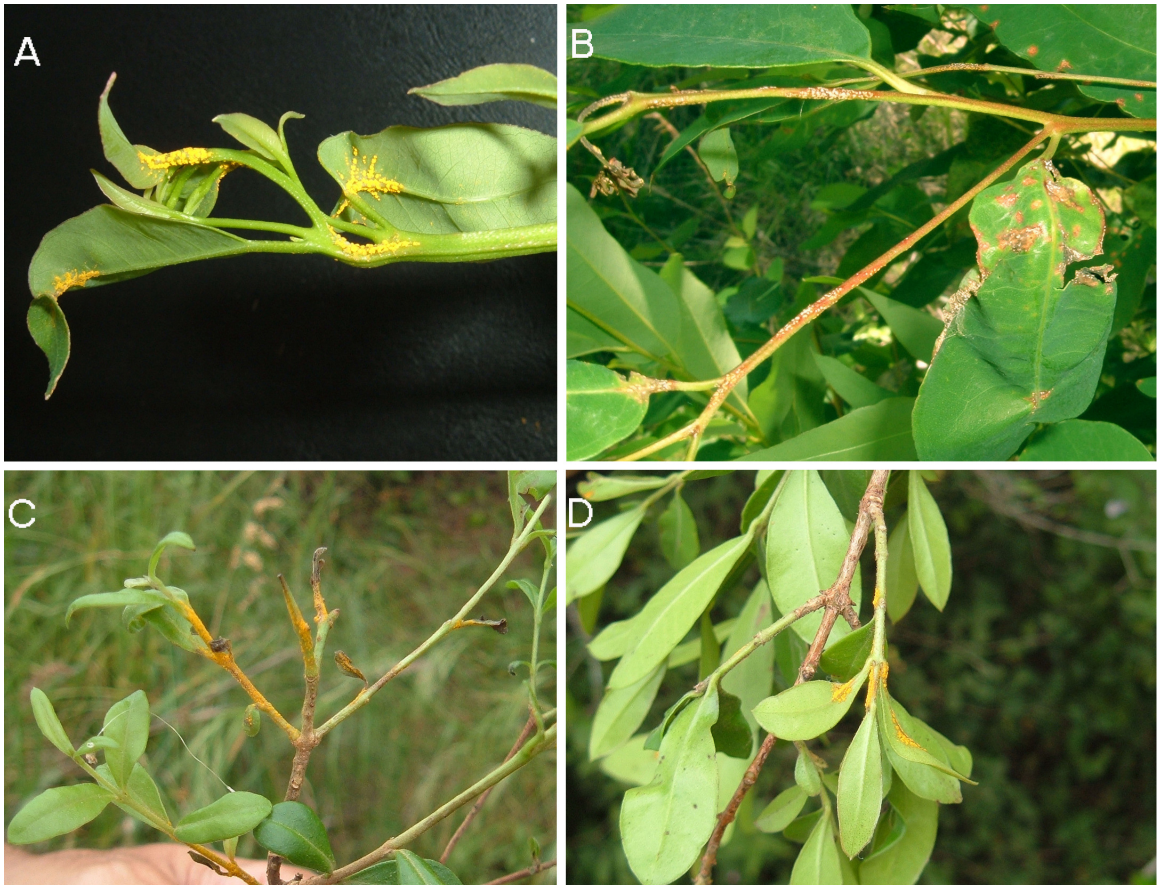

Fig. 1.1: Symptoms of *Eucalyptus* rust on different hosts. **A.** Young lesions on *E. grandis*, the pustules are bright orange on young tissue. **B.** Old lesions on *E. grandis*, grey discoloration on leaves and twigs and dead shoot tip. **C-D.** Pustules on twigs, leaves and petioles of *Myrrhinium atropurpureum* var. *octandrum* appear bright orange. Trees also have dead shoot tips. Arrows show areas of dying shoot tips and location of orange urediniospores on infected branches.

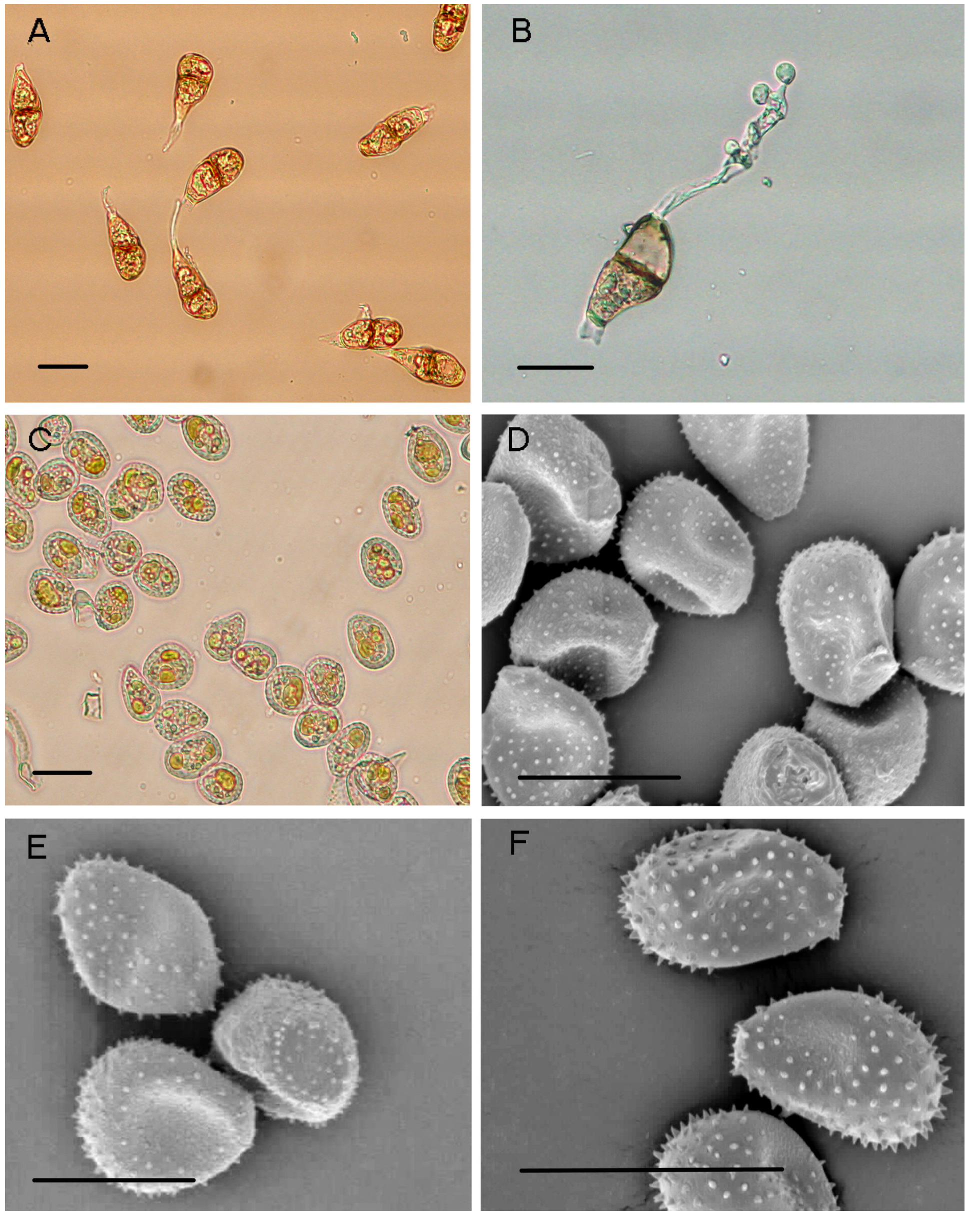

Fig. 1.2: A, B, and C: Light micrographs of teliospores and urediniospores of *P. psidii*. (A) Teliospores observed in sample UY895 with most characteristics as previously described by Walker (1983). However, some spores display a pedicel up to 25 µm long; (B) Germinated teliospore. Black arrows indicate each basidiospore and the white arrow indicates the location where the fourth basidiospore had been ejected; (C) Urediniospores from sample UY217. **D, E, and F:** Scanning electron micrographs of gold-coated urediniospores of *P. psidii* collected on: (D) *E. grandis* (UY217), (E) *Myrrhinium atropurpureum* var. *octandrum* (UY220), and (F) *Myrcianthes pungens* (221). Bars = 20 µm.

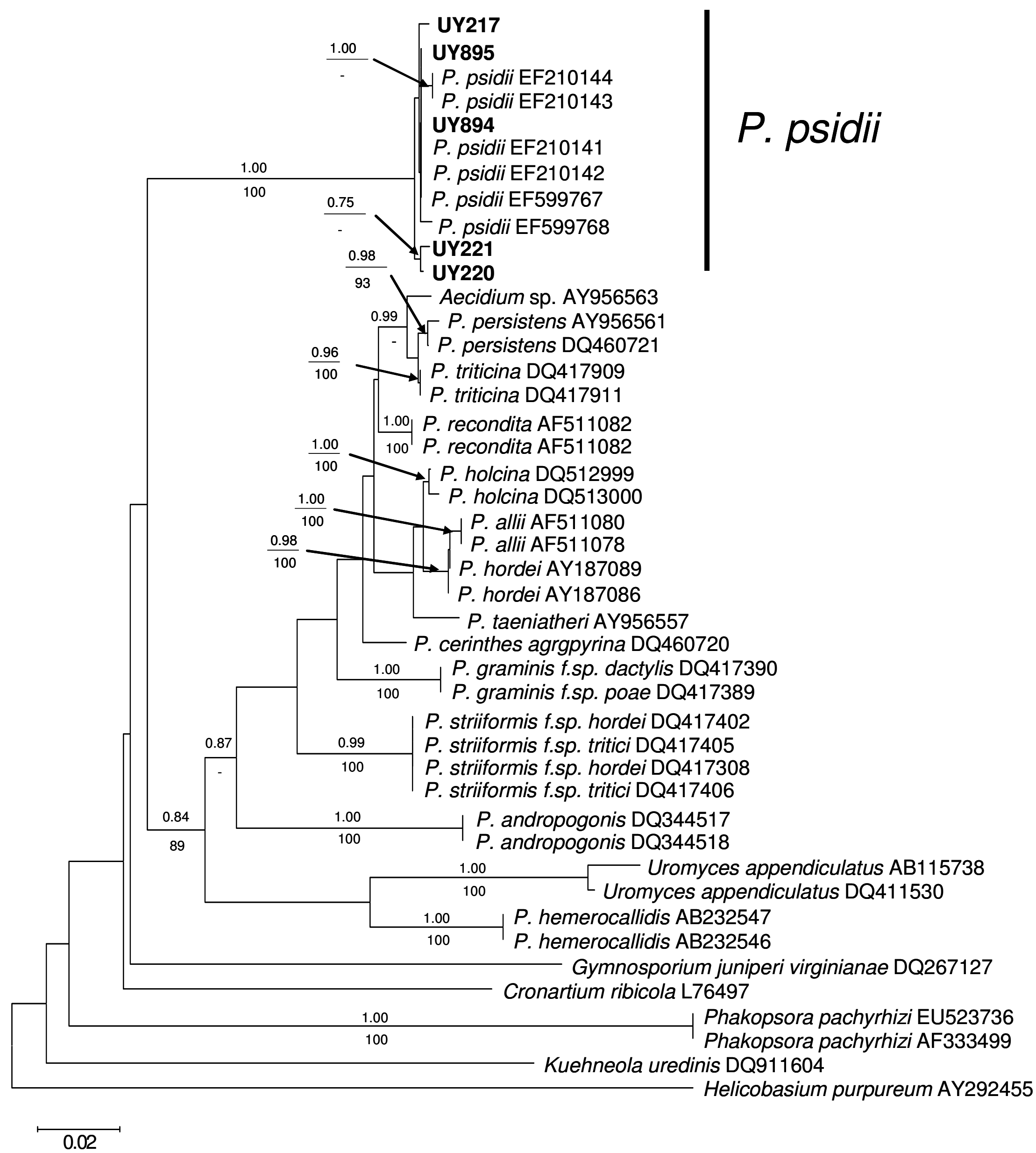

Fig. 1.3: Distance tree obtained from the neighbor-joining analysis based on the ITS region indicates the phylogenetic relationship among rust sequences obtained from rust on *Eucalyptus* and native Myrtaceae trees in Uruguay (labeled "UY" and in bold), *P. psidii,* and other related rusts. Bootstrap values of 1,000 replicates of maximum parsimony (>75%) and posteriori probabilities are shown below and above branches, respectively. The tree was rooted with *Helicobasidium purpureum.*

Chapter 2: Discovery of the eucalypt pathogen, *Quambalaria eucalypti* infecting a non-*Eucalyptus* host in Uruguay

ABSTRACT

Quambalaria eucalypti, a serious pathogen of *Eucalyptus,* is believed to be native to Australia and introduced into various southern hemisphere countries including Uruguay. In this study, the discovery of *Q. eucalypti* causing leaf lesions on *Myrceugenia glaucescens,* a native tree to Uruguay, is recorded. The identity of the pathogen was confirmed using DNA sequence comparisons of the internal transcribed spacer (ITS) region of the rDNA operon as well as morphological characteristics. This is the first record of the pathogen on a host other than *Eucalyptus*. It clearly indicates a disturbing example of an alien invasive pathogen having undergone a host-shift from non-native *Eucalyptus* to a native tree in Uruguay.

INTRODUCTION

The eucalypt pathogen *Quambalaria eucalypti* (Wingf., Crous & Swart) Simpson (Simpson, 2000) was first reported from nurseries in South Africa where it caused shoot lesions and leaf spots on commercially propagated *Eucalyptus grandis* Hill: Maid. clones (Wingfield *et al*., 1993). It was considered of minor importance in South Africa until 2005, when it was observed causing serious stem disease in 1-year-old *E. nitens* Maiden plantations (Roux *et al.,* 2006). In South America, the pathogen was first reported in Uruguay infecting twigs of *E. globulus* ssp. *globulus* Labill (hereafter referred to as *E. globulus*) in 1999 (Bettucci *et al.,* 1999), but severe damage has not been observed in the country. *Quambalaria eucalypti* was first found in Brazil in 2000 causing shoot and leaf lesions on *Eucalyptus* spp. (Alfenas *et al.,* 2001), and it is currently responsible for significant disease problems during clonal propagation of *Eucalyptus* (Alfenas *et al.,* 2004; Andrade *et al.,* 2005).

The genus *Quambalaria* is well known in Australia, particularly due to the damage that *Q. pitereka* and *Q. coyrecup* cause on species of *Corymbia* (Paap *et al.,* 2008; Pegg *et al.,* 2008; Simpson, 2000). These fungi are confined to the eucalypts, most of which are native to Australia, and it is intriguing that *Q. eucalypti* has not been found in Australia until very recently. The discovery of the fungus causing leaf spots and stem lesions on *Eucalyptus* spp. in Queensland and New South Wales by Pegg *et al.* (2008) adds credence to the view that *Quambalaria* spp. are pathogens native to Australia (de Beer *et al.,* 2006; Paap *et al.,* 2008; Pegg *et al.,* 2008; Roux *et al*., 2006; Wingfield *et al*., 1993).

In 2007, disease surveys were conducted in native forests in Uruguay and leaf lesions resembling symptoms caused by *Quambalaria* were observed on *Myrceugenia glaucescens* (Camb.) Legrand et Kausel, a Myrtaceae tree native to the country. The discovery of a possible *Quambalaria* sp. on a host other than *Eucalyptus* is of concern. The aim of this study was to identify the causal agent of the disease and to consider the possibility that a host jump from exotic *Eucalyptus* to a native tree might have occurred.

METHODS

Sampling, symptom description and fungal morphology

In June 2007, infected leaves were collected from native *Myrceugenia glaucescens* in the province of Tacuarembo, Uruguay. In the laboratory, lesions were described, excised, surface-disinfested in 70% ethyl alcohol for 30 sec, rinsed twice in sterile distilled water, blotted dry on sterile filter paper, and plated onto 2% malt extract agar (MEA) (2% malt extract, 1.5% agar; Oxoid, Basingstoke, England) in Petri plates. Plates were incubated at room temperature (~20ºC) for one week. Three single hyphal-tip cultures were obtained from emerging colonies. For morphological characterization, mycelium, conidiophores and conidia were mounted in lactic acid on microscope slides and examined under a Nikon Eclipse E600 light microscope and photographed with a Nikon Digital Camera DXM1200F (Nikon Inc., Melville, NY). A set of 50 measurements were made of all taxonomically relevant structures.

Because the three cultures showed identical colony and conidial morphology a single isolate (UY1718) was selected for phylogenetic analyses. For comparative purposes, an isolate of *Q. eucalypti* from a leaf lesion on *E. globulus* growing in a plantation located in the province of Durazno in Uruguay (UY1036) was included in the study.

DNA extraction, PCR and sequencing

For DNA extractions from isolates UY1036 and UY1718, cultures were grown on 2% MEA at room temperature for 15 days. Mycelium was scrapped directly from the surface of the colonies and transferred to microfuge (1.5 ml) with 3-mm glass beads and extraction buffer of the Qiagen Plant DNeasy Mini Kit (Qiagen Inc., Valencia, CA). These were vigorously shaken using a vortex mixer and placed in a water bath at 60ºC for 1 hr. DNA extraction from the mycelial slurry was performed using the Qiagen Plant DNeasy Mini Kit following the manufacturer's instructions. Polymerase Chain Reaction (PCR) amplifications were conducted using primers ITS1 and ITS4 (White *et al*. 1990) to amplify the internal transcribed spacer (ITS) region of the ribosomal DNA operon. PCR was performed in a 25 µl reaction mixture of 1.0 µl of 0.05% casein, 12.5 µl of Amplitaq Gold PCR Master-Mix (Applied Biosystems, Foster City, CA), 1.0 µl of 10 mM ITS1, 1.0 µl of 10 mM ITS4, 8.5 µl of ddH_2O and 1.0 µl of DNA template of 10 ng.μl^{-1}. PCR amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) with the following parameters: initial denaturation for 5 min at 94ºC, then 35 cycles of 1 min at 94ºC, 1 min at 50ºC, 1 min at 72ºC, followed by a final elongation step of 5 min at 72ºC, and hold at 10ºC.

PCR products were stained with SYBR Green nucleic acid dye (MBL International, Woburn, MA) and visualized on 1.5% under UV light. Amplified DNA was purified and prepared for sequencing using ExoSAP-IT PCR clean-up kit (USB Corp., Cleveland, OH) following the manufacturer's instructions. Sequencing reactions were performed using the same primers. Sequences obtained in this study were deposited in GenBank (accessions EU439922 and EU439923 for isolates UY1036 and UY1718, respectively).

Sequences were subjected to BLAST searches in NCBI Genbank (http:www.ncbi.nlm.nih.gov/blast/Blast.cgi, verified 20 March 2008), and published sequences of related species were downloaded. Sequences were aligned online using the E-INS-i strategy in MAFFT v. 6 (http:align.bmr.kyushu-u.ac.jp/mafft/online/server/, verified 20 March 2008) (Katoh *et al*., 2005), and the aligned data set deposited in TreeBASE (SN3789).

Phylogenetic analyses

Neighbor-joining and maximum parsimony analyses were performed using PAUP v. 4.0b10. (Swofford, 2002). The best substitution model for neighbor-joining analysis was determined using Modeltest v. 3.7 (Posada and Crandall, 1998). Gaps were treated as missing data and all characters were treated as unordered and of equal weight. Maximum parsimony analysis was performed using the heuristic search option with simple taxa additions 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 replicas. Tree length (TL), consistency index (CI), retention index (RI), and homoplasy index (HI) were calculated.

RESULTS

Symptom description

Lesions observed on *M. glaucescens* leaves were characterized by round, brown-reddish spots of variable size between 2-4 mm (Fig. 2.1a). The margins of the lesions were generally round and well defined in the adaxial leaf surface and more diffuse in the abaxial surface (Fig. 2.1b and 1c, respectively). Fungal structures were not observed on the lesions and association with wounds was not evident.

Fungal morphology

The morphology of the three pure cultures obtained from the leaf lesions were similar to those described for *Q. eucalypti.* Colonies on MEA were white and finely floccose with aerial hyphae raised up to 1mm above the colony surface (Fig. 2.1d). Conidiophores arose directly from the mycelium bearing a cluster of conidia. Primary conidia were hyaline, globose to ovoid, and 4-8 x 2-3 µm. Secondary conidia, 2-4 x 1-2 µm, were also observed (Fig. 2.1e).

Phylogenetic analyses

The ITS sequences of the isolates obtained from *M. glaucescens* and *Eucalyptus globulus* (i.e. UY1718 and UY1036, respectively) were identical to each other and grouped consistently with ITS sequences of other *Q. eucalypti* isolates included in the analyses. The two phylogenetic analyses yielded trees with similar topology. From a total of 640 characters, 441 were constant, 11 variable characters were parsimony-uninformative and 188 were parsimony informative. Heuristic search analysis of the data resulted in one tree (TL = 235 steps; CI = 0.932; RI = 0.968; HI = 0.068). The neighbor-joining tree was chosen for presentation (Fig. 2.2), displaying bootstrap values for neighbor-joining and maximum parsimony analyses.

In both analyses, the two sequences obtained in this study grouped with *Q. eucalypti* sequences from Australia and South Africa (Fig. 2.2), including the sequence from the ex-type isolate of the species. The three other known species of *Quambalaria* also formed distinct, well-supported lineages.

DISCUSSION

Results from this study confirmed the presence of *Q. eucalypti* on the native *M. glaucescens* in Uruguay. This is the first report of *Q. eucalypti* infecting a host residing outside of the genus *Eucalyptus*. The pathogen has been known on *Eucalyptus* in Uruguay for some time (Bettucci *et al.,* 1999) and the results of this study suggest that it has undergone a host shift to a native tree.

Symptoms observed on *M. glaucescens* were similar to those described by Wingfield *et al.* (1993) on *Eucalyptus*. However, whitish mycelial growth with masses of spores characteristic of *Q. eucalypti* infections was not observed on the lesions. Further collecting will determine whether the absence of white pustules is characteristic for this host or whether this difference was due to environmental conditions occurring prior to the sampling.

The discovery of *Q. eucalypti* on a native Uruguayan tree is disturbing because the fungus is a virulent primary pathogen that has the capacity to cause severe disease (Alfenas *et al.,* 2004; Andrade *et al.,* 2005; Roux *et al.,* 2006; Wingfield, 1993). Host shifts such as the one that appears to have occurred with *Q. eucalypti* are relatively well known amongst tree pathogens (Woolhouse *et al.,* 2005; Slippers *et al*., 2005) and many have lead to serious disease epidemics. In the case of the Myrtaceae, the *Eucalyptus* rust caused by *Puccinia psidii* provides a remarkable example of host jump. This pathogen is native to South America, and has undergone a host jump from the native *Psidium pomiferum* (syn. *Psidium guajava*) to the introduced *Eucalyptus* species in Brazil (Coutinho *et al*., 1998; Glen *et al*., 2007).

Host jumps such as the one that has apparently occurred with *Q. eucalypti* are often mediated by close genetic relationships among hosts (Slippers *et al*., 2005). In this case, *E. globulus* and *M. glaucescens* reside respectively in the closely related tribes Eucalypteae and Myrteae, in the Myrtaceae (Wilson *et al.,* 2005). However, cases of host jumps occurring more widely in the Myrtales and, for example, between the Melastomataceae and the Myrtaceae are emerging (Wingfield, 2003) and they raise serious concern for the biosecurity of the Myrtaceae, worldwide.

Geographical proximity and opportunities of cross-species transmission are mostly responsible for the appearance of new host-parasite combinations (Roy, 2001). Thus, species growing adjacent to infected plants are exposed to increased inoculum pressure that increases the probabilities of eventual infection. In Uruguay, *Eucalyptus* plantations are geographically located close to native Myrtaceae trees, and biotic exchange can easily occur (see Chapter 1 of this Thesis). Thus, pathogens introduced with germplasm of *Eucalyptu*s to promote a growing paper and pulp industry, could threaten the native flora of Uruguay and probably other parts of South America in the future.

During the past three years, several surveys were conducted extensivly country-wide in Uruguay and have consequently sampled a large number of native Myrtaceae trees. Only a single tree was found with infections of *Q. eucalypti*. At the present time, there is no evidence that an epidemic is emerging but continued monitoring is needed in Uruguay to assess the importance of *Q. eucalypti* in Uruguay.

ACKNOWLEDGMENTS

Thanks for the financial and logistic support of Colonvade, Forestal Oriental, Rivermol and Stora Enso. Also thanks to Andrés Berrutti for his assistance in the identification of native Myrtaceae trees.

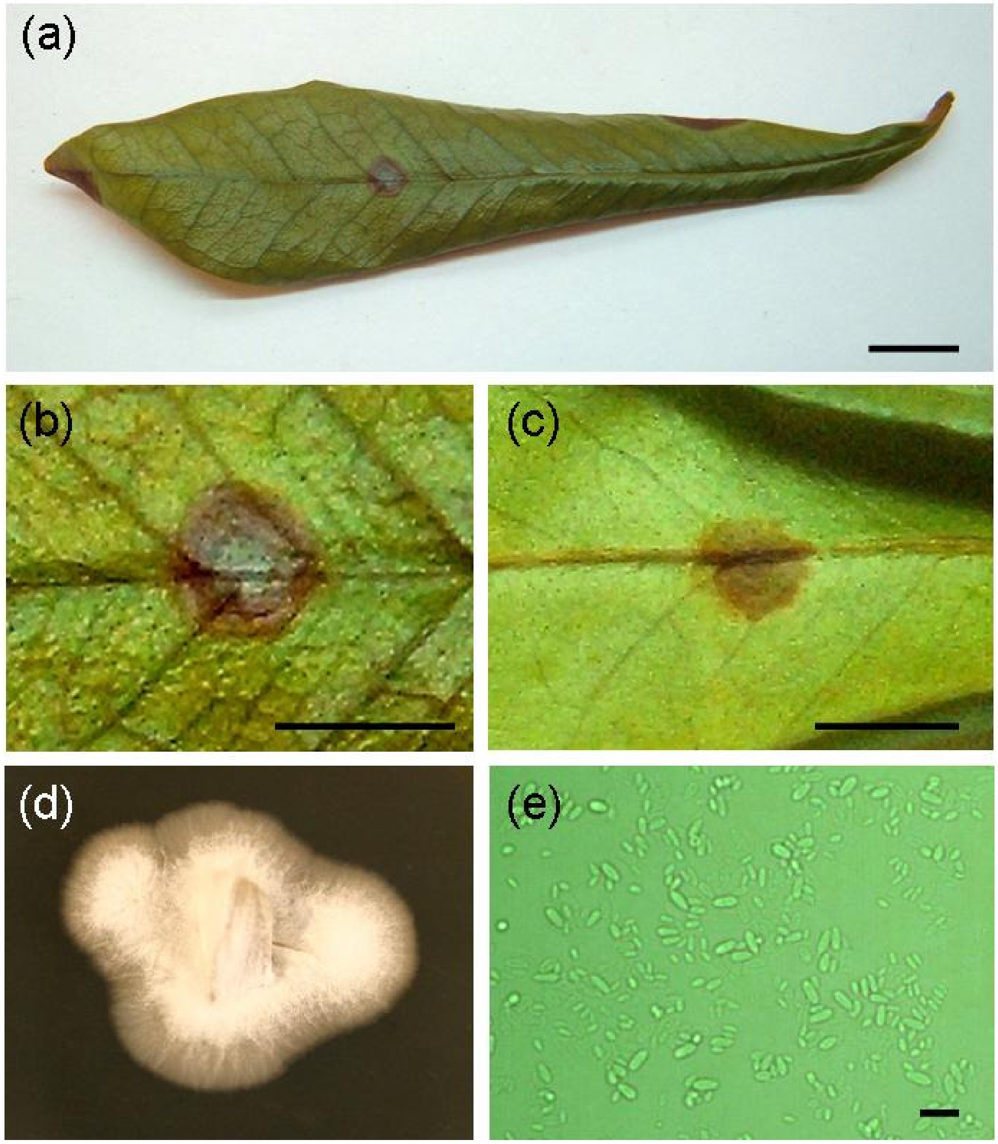

Fig. 2.1: (a) *Quambalaria eucalypti* symptoms on *Myrceugenia glaucescens*, scale bar = 10 mm; (b) spot on the adaxial and (c) abaxial leaf surface, scale bar = 5 mm; (d) white, floccose colony of *Q. eucalypti* isolate UY1718; (e) primary and secondary conidia of isolate UY1718, scale bar = 10 µm.

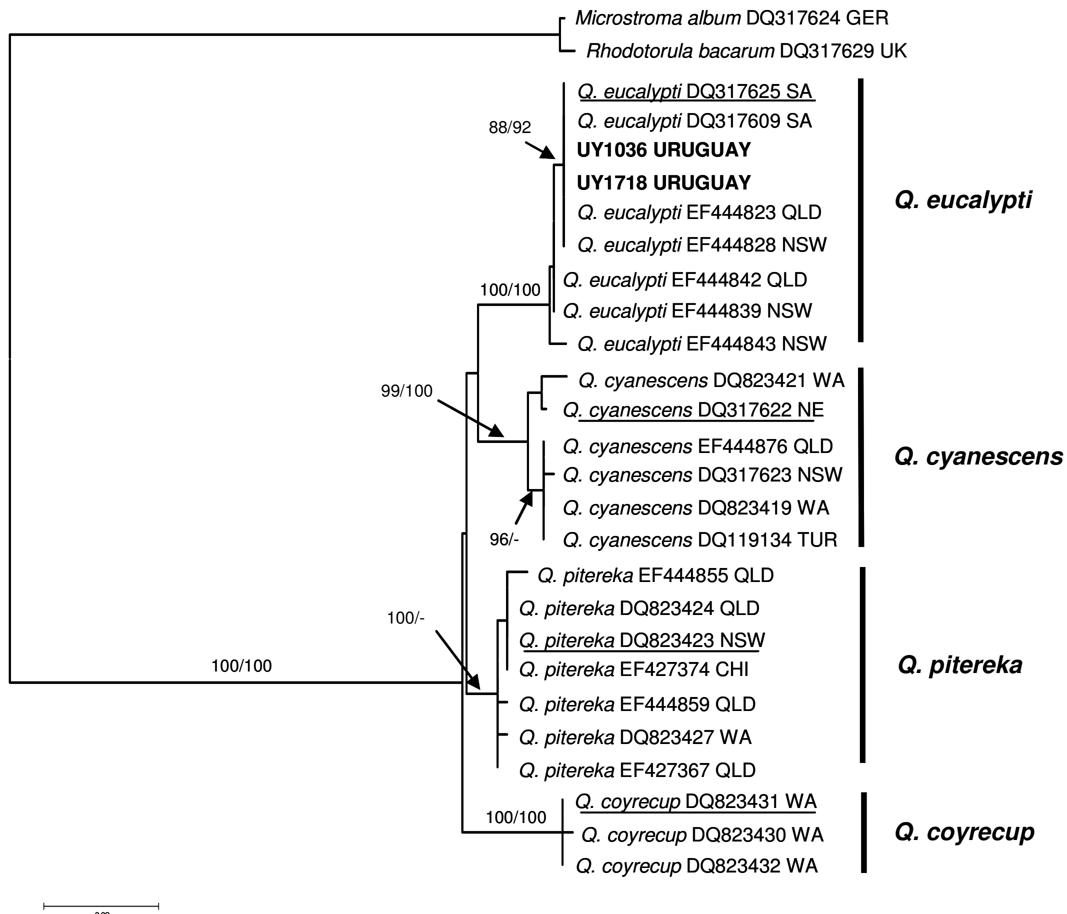

Fig. 2.2: Neighbor-joining phylogenetic tree from the ITS sequence data obtained using TVM+G model. GenBank accession number and country of origin is shown for each sequence, CHI: China; GER: Germany; NET: Netherlands; NSW: New South Wales, Australia; QLD: Queensland, Australia; SA: South Africa; TUR: Turkey; UK: United Kingdom; WA: Western Australia. Bootstrap values of 1000 replicates of neighbor-joining and maximum parsimony analyses are shown at the branches, respectively. Only bootstrap values higher than 75% are shown. *Microstroma album* and *Rhodotorula bacarum* were used as outgroup taxa. The two isolates obtained in this study are in bold and the ex-type cultures are shown underlined. Branch lengths are scaled and scale bar is 0.02 nucleotide substitutions per site.

Chapter 3: Mycosphaerellaceae associated with *Eucalyptus* leaf diseases and stem cankers in Uruguay

ABSTRACT

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 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 ITS region of the rDNA. A total of ten Mycosphaerellaceae 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 the country. 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 *K. 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 occurring in Uruguay is disturbing and raises concern regarding the introduction of new pathogens that could threaten not only *Eucalyptus* plantations but also native forests.

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, 2003; 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 residing in *Mycosphaerella*, *Teratosphaeria*, and several anamorph genera where the teleomorph is unknown (Crous *et al.*, 2007a) have been recorded on *Eucalyptus* (Andjic *et al*., 2007; Burgess *et al.*, 2007; Cortinas *et al.*, 2006b; Crous *et al.*, 2004a; Crous *et al*., 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). Thus, Carnegie *et al.* (1994) found a negative correlation between tree height and diameter and severity of MLD in *Eucalyptus globulus* plantations. Likewise, Carnegie *et al.* (1998) reported that even a 10% infection resulted in a 17% reduction in height of *E. globulus* plantations, whereas Lundquist and Purnell (1987) found a significant reduction in growth rate when more than 25% of the juvenile foliage of *E. nitens* was lost due to MLD.

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 work 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 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 species occurring on *Eucalyptus* plantations in the country.

MATERIALS AND METHODS

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. 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 hours. Leaf samples 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). Petri dishes were incubated in the dark at 17-18ºC. After 24-48 hours, 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 and mounted on a slide and observed under a light microscope for germination patterns record 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-disinfested in 70% ethyl alcohol for 30 sec, 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 liter. 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 min, the conidial suspension was spread onto the surface of 2% MEA. After 24-36 hours, germinating conidia were transferred to a 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.

DNA extraction, PCR, sequencing and phylogenetic analyses

Genomic DNA was extracted from 29 isolates representing the different morphological forms emerging from the survey (Table 3.1). Cultures were grown on 2% MEA at 25 C for 30 days. Mycelium scrapped directly from the colonies was transferred to microfuge (1.5 ml) with 3-mm glass beads and extraction buffer of the Qiagen Plant DNeasy Mini Kit (Qiagen Inc., Valencia, CA), vigorously shaked using a vortex mixer and placed in a water bath at 60ºC for 1 hr. 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 amplified 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 1.0 µl of 0.05% casein, 12.5 µl of Amplitaq Gold PCR Master-Mix (Applied Biosystems, Foster City, CA), 1.0 µl of 10 mM of each primer, 8.5 µl of ddH_2O and 1.0 µl of DNA template. PCR amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) using the following PCR cycling conditions: initial denaturation for 5 min at 94ºC; 1 min at 94ºC; 1 min at 50ºC; 1 min at 72ºC; cycle to step 2, 35 times; followed by a final elongation step of 5 min at 72ºC.

PCR products stained with SYBR Green nucleic acid dye (MBL International, Woburn, MA) were visualized on 1.5% agarose gels under UV light. ExoSAP-IT PCR clean-up kit (USB Corp., Cleveland, OH) was used to purify amplicons for sequencing following manufacturer's instructions. Sequencing reactions were performed using the same primers with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) 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).

BLAST searches in NCBI Genbank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, verified 26 June 2008), were conducted with the sequences obtained in this study. 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* (Table 3.1). 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 26 June 2008) (Katoh et al., 2005).

Phylogenetic analysis was performed using PAUP Version 4.0b10 (Swofford, 2002) for neighbor-joining (NJ) and maximum parsimony (MP) analyses, and Mr. Bayes v3.1.2 (Ronquist and Huelsenbeck, 2003) for Bayesian analysis. Best model for neighbor-joining analysis was determined in Modeltest version 3.7 (Posada and Crandall, 1998) where the GTR+I+G model was selected from the Akaike information criterion (AIC). 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 (TBR) 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).

The best nucleotide substitution model for the Bayesian analysis was selected using MrModeltest v2.2 (Nylander, 2004) 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 using AIC. Four 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.

RESULTS

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 3.1).

DNA comparisons and phylogenetic inference

DNA 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 3.1. The 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). Neighbor-joining, maximum parsimony and Bayesian analyses resulted in trees of similar topology. The NJ tree is shown in Figure 3.1 with bootstrap values of the NJ and MP analyses and the posteriori probabilities obtained in the Bayesian analysis shown on the branches. The aligned sequence data was deposited in TreeBASE (accession SN3972).

Ten distinct species residing in the Mycosphaerellaceae emerged from these analyses (Fig. 3.1). 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, Mycosphaerella aurantia, M. heimii, M. lateralis, M. marksii, M. scytalidii, Pseudocercospora norchienses, Teratosphaeria molleriana, T. ohnowa* and *T. pluritubularis* (Fig. 3.1).

Mycosphaerellaceae species were found to occur in most of the major areas where *Eucalyptus* is gown (Fig. 3.2). Five Mycosphaerellaceae species were associated with a single *Eucalyptus* species and these were *M. aurantia, M. heimii, M. lateralis, T. ohnowa* and *T. pluritubularis*. *Mycosphaerella 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 Florida, Lavalleja and Río Negro. *Teratosphaeria molleriana* was isolated from diseased *E. globulus* and *E. maidenii* leaves in Florida and Lavalleja. *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 Lavalleja, 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 Florida, and Tacuarembó and Rivera, respectively.

DISCUSSION

Results of this study reveal ten species of Mycosphaerellaceae 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 represents a taxonomically complex group and many species have yet to be identified (Crous *et al.*, 2006, Crous *et al.*, 2007b). The 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 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 Mycosphaerellaceae species. That is the case of *M. scytalidii, P. norchiensis, T. ohnowa* and *T. pluritubularis* which have been very recently described and for which nothing is known regarding etiology. Further investigation to determine the economic importance of these fungi, along with their geographical distribution in the country is warranted.

Teratosphaeria molleriana and *M. aurantia* are considered primary pathogens in other 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. *Mycosphaerella heimii* is also considered a primary pathogen affecting 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). This species is particularly interesting because it was recently reported as causing leaf disease on *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. This fungus was found 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; Kularatne *et al.,* 2004) 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 a diverse *Eucalyptus* spp. over a broad geographical distribution in Uruguay. *Kirramyces 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 only been known 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 of these species 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. All the species found have been previously reported in other countries and it is likely that most species have been introduced to 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 (see Chapter 2 of this Thesis) .

Continued monitoring is needed to provide an ongoing view of which Mycosphaerellaceae species are infecting *Eucalyptus* plantations. This research has provided the first comprehensive information regarding the Mycosphaerellaceae associated with *Mycosphaerella* Leaf Diseases 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. Epidemiologic 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 investigation should concentrate on understanding the pathology and ecology of the species collected in this study.

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Table 3.1: List of sequences used in the phylogenetic analysis including those obtained from species isolated during this study and reference sequences obtained from Genbank. Cultures from Uruguay sequenced in this study are in bold indicated with the prefix "UY".

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

Culture ID	Teleomorph	Anamorph	Host	Location of collection	GenBank accession no.	Reference
UY1199	Unknown	*K. gauchensis*	*E. maidenii*	Florida	EU851933	This study
UY1240	*T. ohnowa*	Unknown	*E. viminalis*	Lavalleja	EU851934	This study
UY1522	Unknown	*K. gauchensis*	*E. tereticornis*	Rivera	EU851935	This study
UY1528	Unknown	*Ps. norchiensis*	*E. dunnii*	Rivera	EU851936	This study
UY1530	Unknown	*K. gauchensis*	*E. tereticornis*	Rivera	EU851937	This study
UY1561	Unknown	*Ps. norchiensis*	*E. grandis*	Rivera	EU851938	This study
CBS120740	*M. acaciigena*	Unknown	*Eucalyptus* sp.	-	EF394822	Crous *et al.*, 2007b
CPC13350	*M. acaciigena*	Unknown	*E. camaldulensis x E.*	-	EF394823	Crous *et al.*, 2007b
CBS110500 [T]	*M. aurantia*	Unknown	*E. globulus*	-	AY725531	Crous *et al.*, 2004a
MURU151	*M. aurantia*	Unknown	*E. globulus*	-	AY150331	Maxwell *et al.*, 2003
MURU152	*M. aurantia*	Unknown	*E. globulus*	-	AY509742	Maxwell *et al.*, 2005
CBS110969	*M. colombiensis*	*Ps. colombiensis*	*E. urophylla*	-	AY752149	Crous *et al.*, 2004b
CBS114238	*M. comunis*	*Dissoconium commune*	*E. globulus*	-	AY725541	Crous *et al.*, 2004a
CBS112890	*M. comunis*	*D. commune*	*E. nitens*	-	AY725540	Crous *et al.*, 2004a
CBS681.95 [T]	*M. crystallina*	*Ps. crystallina*	*E. bicostata*	-	AY490757	Verkley *et al.*, 2004
CMW3042	*M. crystallina*	*Ps. crystallina*	*Eucalyptus* sp.	-	AF309611	Crous *et al.*, 2001
CMW5166	*M. ellipsoidea*	Unknown	*Eucalyptus* sp.	-	AF309593	Crous *et al.*, 2001
CMW4934	*M. ellipsoidea*	Unknown	*Eucalyptus* sp.	-	AF309592	Crous *et al.*, 2001
CBS111519 [T]	*M. endophytica*	*Pseudocercosporella endophytica*	*Eucalyptus* sp.	-	DQ267579	Hunter *et al.*, 2006
CBS114662	*M. endophytica*	*Pseudocercosporella endophytica*	*Eucalyptus* sp.	-	DQ302953	Crous *et al.*, 2006
CBS110501	*M. gregaria*	Unknown	*E. globulus*	-	DQ267585	Hunter *et al.*, 2006
MURU237	*M. gregaria*	Unknown	*E. globulus*	-	AY509755	Maxwell *et al.*, 2005
CBS110682 [T]	*M. heimii*	*Ps. heimii*	*Eucalyptus* sp.	-	DQ239992	Cortinas *et al.*, 2006a
CBS120743	*M. heimii*	*Ps. heimii*	*E. urophylla*	-	EF394838	Crous *et al.*, 2007b
CMW5719	*M. heimii*	*Ps. heimii*	*Eucalyptus* sp.	-	AF452516	Crous *et al.*, 2006
CPC13371	*M. heimii*	*Ps. heimii*	*E. urophylla*	-	EF394840	Crous *et al.*, 2007b
CBS111190	*M. heimioides*	*Ps. heimioides*	*Eucalyptus* sp.	-	AF309609	Crous *et al.*, 2001

Culture ID	Teleomorph	Anamorph	Host	Location of collection	GenBank accession no.	Reference
CBS111364	*M. heimioides*	*Ps. heimioides*	*Eucalyptus* sp.	-	DQ267586	Hunter *et al*., 2006
CBS114774	*M. irregulariramosa*	*Ps. irregulariramosa*	*E. saligna*	-	AF309607	Crous *et al*., 2001
CMW5223	*M. irregulariramosa*	*Ps. irregulariramosa*	*E. saligna*	-	AF309608	Crous *et al*., 2001
CBS111001 [T]	*M. keniensis*	*Unknown*	*E. grandis*	-	AF309601	Crous *et al*., 2001
STE-U2123	*M. konae*	*Pseudocercospora* sp.	*Leucadendron* sp.	-	AY260086	Taylor *et al*., 2003
STE-U2125	*M. konae*	*Pseudocercospora* sp.	*Leucadendron* sp.	-	AY260085	Taylor *et al*., 2003
CBS110748	*M. lateralis*	*D. dekkeri*	*Eucalyptus* sp.	-	AF309624	Crous *et al*., 2001
CBS111169	*M. lateralis*	*D. dekkeri*	*E. globulus*	-	AY725550	Crous *et al*., 2004a
CBS112895	*M. madeirae*	*Pseudocercospora* sp.	*E. globulus*	-	AY725553	Crous *et al*., 2004a
CBS682.95 [T]	*M. marksii*	*Ps. epispemogoniana*	*E. grandis*	-	DQ267587	Hunter *et al*., 2006
CBS110920	*M. marksii*	*Ps. epispemogoniana*	*E. botryoides*	-	AF309588	Crous *et al*., 2001
CMW3358 [T]	*M. parkii*	*Stenella parkii*	*Eucalyptus* sp.	-	AF309590	Crous *et al*., 2001
CBS118493 [T]	*M. scytalidii*	Unknown	*Eucalyptus* sp.	-	DQ303016	Crous *et al*., 2006
CBS516.93	*M. scytalidii*	Unknown	*E. globulus*	-	DQ303014	Crous *et al*., 2006
CBS118909 [T]	*M. stramenti*	Unknown	*Eucalyptus* sp.	-	DQ303042	Crous *et al*., 2006
CPC10547 [T]	*M. thailandica*	*Ps. thailandica*	*Acacia mangium*	-	AY752156	Crous *et al*., 2004a
CBS119974 [T]	*M. vietnamensis*	Unknown	*E. grandis* hybrid	-	DQ632675	Burgess *et al*., 2007
STE-U2769	*M. walkeri*	*Sorderhenia eucalypticola*	*Eucalyptus* sp.	-	AF309616	Crous *et al*., 2001
CBS680.95 [T]	*T. africana*	Unknown	*E. viminalis*	-	AF309602	Crous *et al*., 2001
CMW3025	*T. africana*	Unknown	*E. viminalis*	-	AF283690	Smith *et al*., 2001
CBS110975	*T. cryptica*	*C. nubilosum*	*E. globulus*	-	AF309623	Crous *et al*., 2001
CBS111012 [T]	*T. flexuosa*	Unknown	*E. globulus*	-	AF309603	Crous *et al*., 2001
CBS111163	*T. flexuosa*	Unknown	*E. grandis*	-	DQ302957	Crous *et al*., 2006
CBS118495	*T. gamsii*	Unknown	*Eucalyptus* sp.	-	DQ302959	Crous *et al*., 2006
CBS120146	*T. molleriana*	*C. molleriana*	*Eucalyptus* sp.	-	EF394844	Crous *et al*., 2007b
CBS111164	*T. molleriana*	*C. molleriana*	*E. globulus*	-	AF309620	Crous *et al*., 2001
CBS116005	*T. nubilosa*	*Uwebraunia juvenis*	*E. globulus*	-	AF309618	Crous *et al*., 2001

Culture ID	Teleomorph	Anamorph	Host	Location of collection	GenBank accession no.	Reference
CBS110949 [T]	*T. ohnowa*	Unknown	*E. grandis*	-	AY725575	Crous *et al*., 2004a
CBS120745	*T. ohnowa*	Unknown	*E. dunnii*	-	EF394845	Crous *et al*., 2007b
CBS118508 [T]	*T. pluritubularis*	Unknown	*E. globulus*	-	DQ303007	Crous *et al*., 2006
CBS118504 [T]	*T. pseudocryptica*	*Colletogloeopsis* sp.	*Eucalyptus* sp.	-	DQ303010	Crous *et al*., 2006
CBS118507 [T]	*T. secundaria*	Unknown	*Eucalyptus* sp.	-	DQ303020	Crous *et al*., 2006
CBS111002	*T. secundaria*	Unknown	*E. grandis*	-	DQ303017	Crous *et al*., 2006
CBS118506 [T]	*T. stramenticola*	Unknown	*Eucalyptus sp.*	-	DQ303043	Crous *et al*., 2006
CBS120086	Unknown	*C. dimorpha*	*Eucalyptus sp.*	-	DQ923528	Summerell *et al*.,
CBS120085	Unknown	*C. dimorpha*	*Eucalyptus sp.*	-	DQ923529	Summerell *et al*.,
CBS120729	Unknown	*D. australiensis*	*E. platyphylla*	-	EF394854	Crous *et al*., 2007b
CBS120039	Unknown	*D. eucalypti*	*E. tereticornis*	-	EF394855	Crous *et al*., 2007b
CBS111369	Unknown	*K. destructans*	*E. grandis*	-	AF309614	Crous *et al.,* 2001
CBS120303	Unknown	*K. gauchensis*	*E. grandis*	-	EU019290	Crous *et al*., 2007a
CMW17326	Unknown	*K. gauchensis*	*Eucalyptus* sp.	-	DQ303068	Crous *et al*., 2006
CBS120301	Unknown	*K. zuluensis*	*E. grandis*	-	DQ240207	Cortinas *et al*., 2006b
CBS120302	Unknown	*K. zuluensis*	*E. grandis*	-	DQ240214	Cortinas *et al*., 2006b
CBS120029	Unknown	*Passalora schizolobii*	*Schizolobium parahybum*	-	DQ885903	Wingfield *et al*., 2006
CMW5148 [T]	Unknown	*Pseudocercospora basiramifera*	*E. pellita*	-	AF309595	Crous *et al.,* 2001
CBS111280	Unknown	*Ps. basitruncata*	*E. grandis*	-	DQ267601	Hunter *et al*., 2006
CBS114664	Unknown	*Ps. basitruncata*	*E. grandis*	-	DQ267600	Hunter *et al*., 2006
CMW13586 [T]	Unknown	*Ps. flavomarginata*	*E. camaldulensis*	-	DQ155657	Hunter *et al*., 2007
CBS111069 [T]	Unknown	*Ps. natalensis*	*Eucalyptus* sp.	-	DQ303077	Crous *et al*., 2006
CBS120738	Unknown	*Ps. norchiensis*	*Eucalyptus* sp.	-	EF394859	Crous *et al*., 2007b
CBS111286	Unknown	*Ps. paraguayensis*	*E. nitens*	-	DQ267602	Hunter *et al*., 2006
CBS114242 [T]	Unknown	*Ps. pseudoeucalyptorum*	*E. globulus*	-	AY725526	Crous *et al*., 2004a
CBS111175 [T]	Unknown	*Ps. robusta*	*E. robusta*	-	AF309597	Crous *et al.,* 2001
CBS121101 [T]	Unknown	*Stenella eucalypti*	*E. tereticornis*	-	EF394865	Crous *et al*., 2007b

Culture ID	Teleomorph	Anamorph	Host	Location of collection	GenBank accession no.	Reference
CMW7773	*'Botryosphaeria' ribis*	*Neofusiccocum ribis*	*Ribes* sp.	-	AY236936	Slippers *et al*., 2004

[T]: ex-type cultures

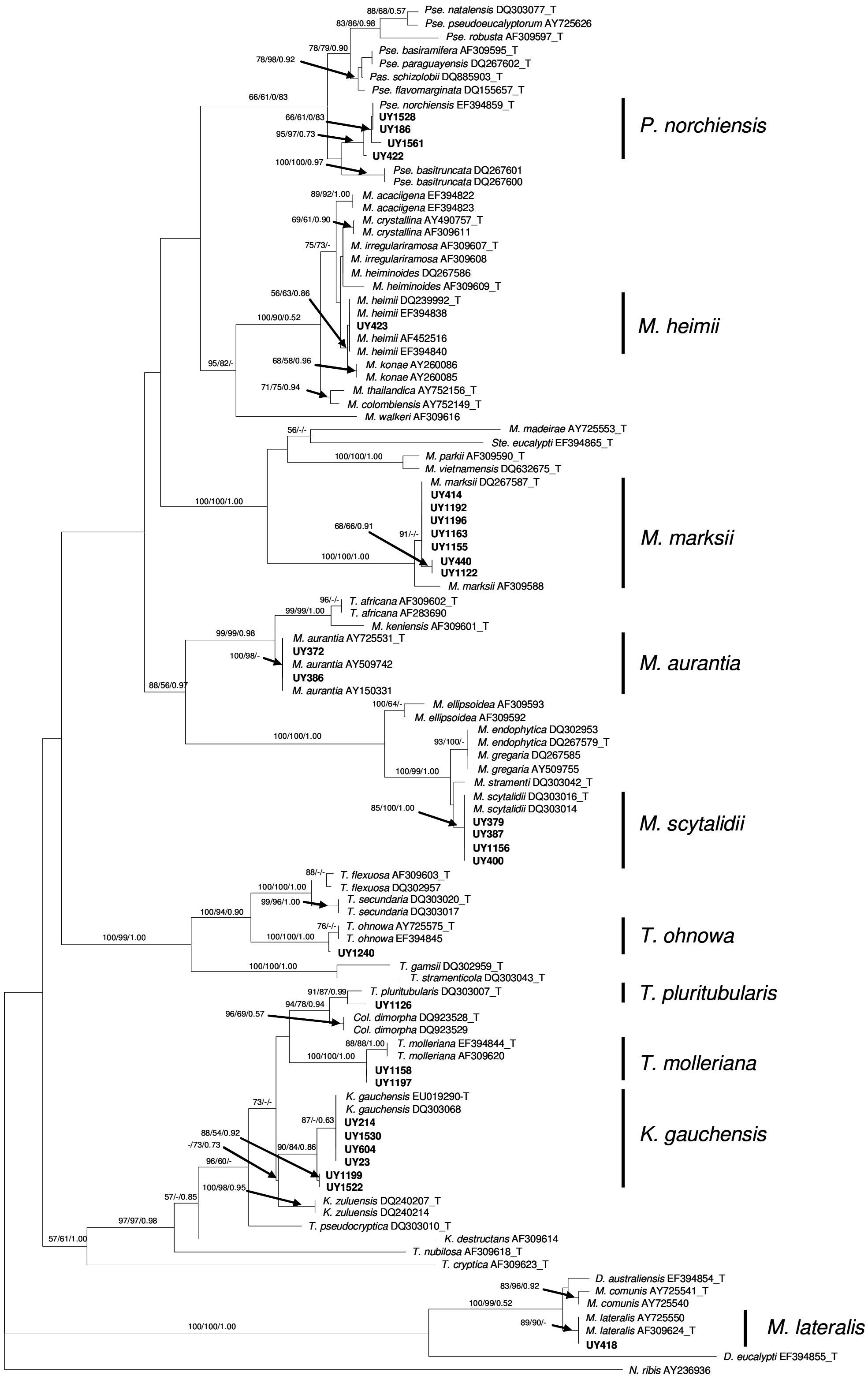
88/68/0.57
Pse. natalensis DQ303077_T
83/86/0.98
Pse. pseudoeucalyptorum AY725626
Pse. robusta AF309597_T
78/79/0.90
Pse. basiramifera AF309595_T
78/98/0.92
Pse. paraguayensis DQ267602_T
Pas. schizolobii DQ885903_T
Pse. flavomarginata DQ155657_T
66/61/0/83
Pse. norchiensis EF394859_T
66/61/0/83
UY1528
UY186
95/97/0.73
UY1561
UY422
P. norchiensis
100/100/0.97
Pse. basitruncata DQ267601
Pse. basitruncata DQ267600
89/92/1.00
M. acaciigena EF394822
M. acaciigena EF394823
69/61/0.90
M. crystallina AY490757_T
M. crystallina AF309611
75/73/-
M. irregulariramosa AF309607_T
M. irregulariramosa AF309608
M. heiminoides DQ267586
M. heiminoides AF309609_T
56/63/0.86
M. heimii DQ239992_T
M. heimii EF394838
100/90/0.52
UY423
M. heimii AF452516
M. heimii EF394840
M. heimii
68/58/0.96
M. konae AY260086
95/82/-
M. konae AY260085
71/75/0.94
M. thailandica AY752156_T
M. colombiensis AY752149_T
M. walkeri AF309616
56/-/-
M. madeirae AY725553_T
Ste. eucalypti EF394865_T
100/100/1.00
M. parkii AF309590_T
M. vietnamensis DQ632675_T
M. marksii DQ267587_T
UY414
100/100/1.00
UY1192
UY1196
68/66/0.91
91/-/-
UY1163
UY1155
M. marksii
100/100/1.00
UY440
UY1122
M. marksii AF309588
96/-/-
T. africana AF309602_T
99/99/1.00
T. africana AF283690
M. keniensis AF309601_T
99/99/0.98
M. aurantia AY725531_T
UY372
100/98/-
M. aurantia AY509742
M. aurantia
UY386
M. aurantia AY150331
88/56/0.97
100/64/-
M. ellipsoidea AF309593
M. ellipsoidea AF309592
M. endophytica DQ302953
100/100/1.00
93/100/-
M. endophytica DQ267579_T
M. gregaria DQ267585
100/99/1.00
M. gregaria AY509755
M. stramenti DQ303042_T
M. scytalidii DQ303016_T
85/100/1.00
M. scytalidii DQ303014
UY379
M. scytalidii
UY387
UY1156
UY400
88/-/-
T. flexuosa AF309603_T
100/100/1.00
T. flexuosa DQ302957
99/96/1.00
T. secundaria DQ303020_T
100/94/0.90
T. secundaria DQ303017
76/-/-
T. ohnowa AY725575_T
100/100/1.00
T. ohnowa EF394845
T. ohnowa
100/99/1.00
UY1240
100/100/1.00
T. gamsii DQ302959_T
T. stramenticola DQ303043_T
91/87/0.99
T. pluritubularis DQ303007_T
T. pluritubularis
94/78/0.94
UY1126
96/69/0.57
Col. dimorpha DQ923528_T
Col. dimorpha DQ923529
88/88/1.00
T. molleriana EF394844_T
100/100/1.00
T. molleriana AF309620
T. molleriana
UY1158
UY1197
73/-/-
K. gauchensis EU019290-T
K. gauchensis DQ303068
87/-/0.63
UY214
UY1530
K. gauchensis
88/54/0.92
90/84/0.86
UY604
-/73/0.73
UY23
96/60/-
UY1199
UY1522
100/98/0.95
57/-/0.85
K. zuluensis DQ240207_T
K. zuluensis DQ240214
97/97/0.98
T. pseudocryptica DQ303010_T
57/61/1.00
K. destructans AF309614
T. nubilosa AF309618_T
T. cryptica AF309623_T
D. australiensis EF394854_T
83/96/0.92
M. comunis AY725541_T
100/99/0.52
M. comunis AY725540
89/90/-
M. lateralis AY725550
100/100/1.00
M. lateralis AF309624_T
M. lateralis
UY418
D. eucalypti EF394855_T
N. ribis AY236936
0.02

Fig. 3.1: Neighbor-joining phylogenetic tree from the ITS sequence data. Species name and GenBank accession number is shown for each sequence. Sequences labeled with a "T" at the end correspond to the ex-type culture. Bootstrap values of 1000 replicates of neighbor-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. Branch lengths are scaled and scale bar is 0.02 nucleotide substitutions per site.

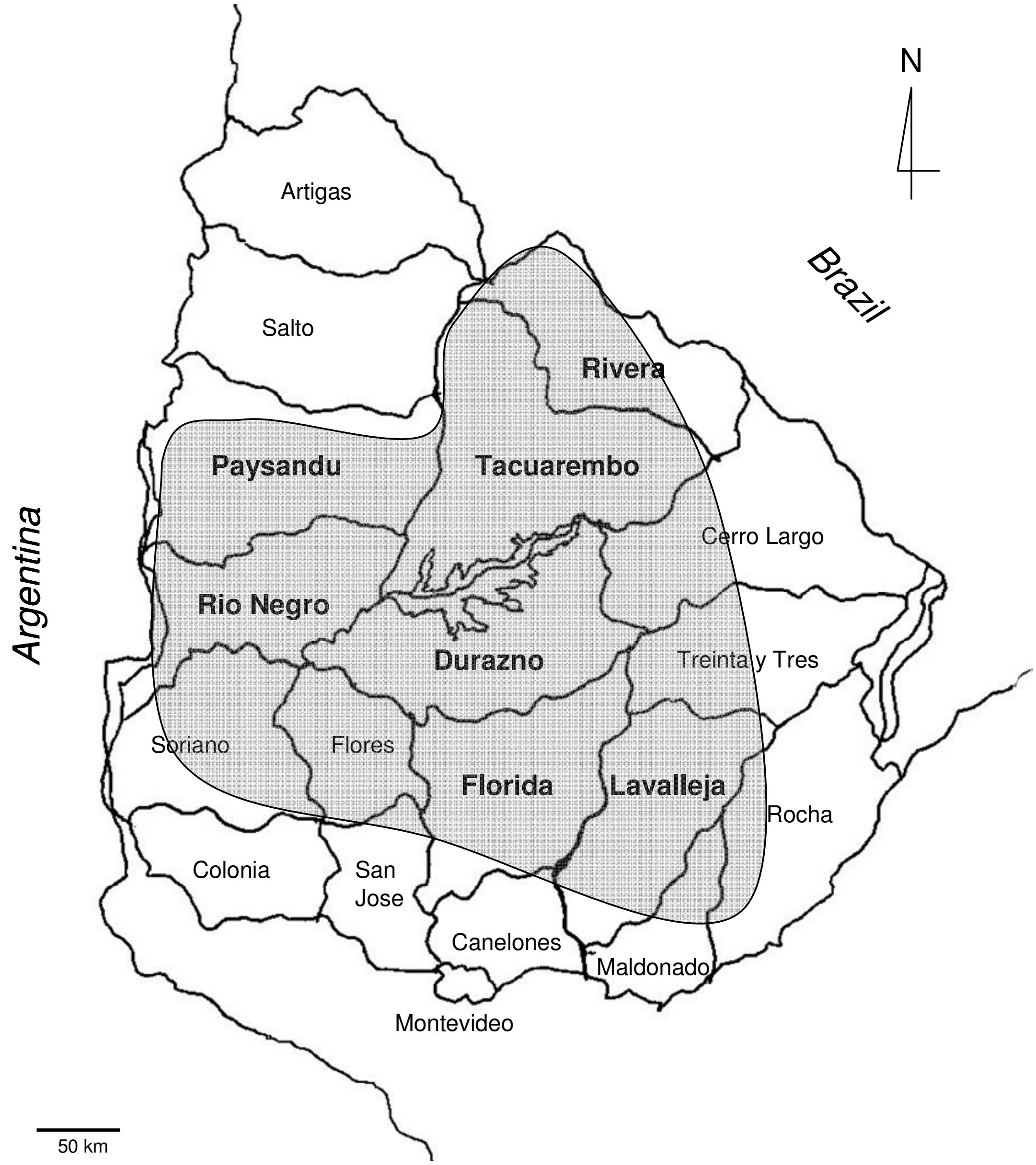

Fig. 3.2: Map of Uruguay showing in bold those provinces where Mycosphaerellaceae species were found infecting *Eucalyptus*. Shaded area indicates major *Eucalyptus* growing areas.

Chapter 4: Mycosphaerellaceae species on native Myrtaceae in Uruguay: Evidence of fungal host jumps

ABSTRACT

Mycosphaerella spp. are well-known causal agents of leaf diseases on an important number of plant species. In Uruguay, a relatively large number of Mycosphaerellaceae are found on *Eucalyptus* but nothing is known regarding these fungi on native Myrtaceae. The aim of this study was to identify Mycosphaerellaceae associated with leaf diseases on native Myrtaceae in Uruguay and to consider whether host jumps might have occurred. Several native forests throughout the country were surveyed with special attention to those located close to *Eucalyptus* plantations. Six species of Mycosphaerellaceae are reported on native Myrtaceous trees and four of these were previously reported on *Eucalyptus* in Uruguay. Those occurring both on *Eucalyptus* and native Myrtaceae included *Mycosphaerella aurantia, M. heimii, M. marksii* and *Pseudocercospora norchiensis.* In addition, *M. yunnanensis,* a species known to occur on *Eucalyptus* but to date has not been found 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 seems likely 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 has been associated with *Mycosphaerella* leaf diseases (MLD), which are considered particularly important in *Eucalyptus* plantations worldwide (Cortinas *et al.*, 2006; Crous *et al.*, 2004; Crous *et al.*, 2006; Maxwell *et al.*, 2003; Hunter *et al.,* 2006; Park *et al.*, 2000; Summerell *et al.*, 2006). These fungi cause leaf spots, leaf blotches, or petiole and stem cankers that often result in stressed and stunted trees adversely affecting commercial plantations (Carnegie *et al.,* 1994; Carnegie *et al.,* 1998; Lundquist and Purnell, 1987; Park *et al.,* 2000).

Although most studies on MLD have focused primarily on *Eucalyptus*, species of Mycosphaerellaceae have also been found infecting Myrtaceae species other than those residing in the *Eucalyptus* genus and at least 23 species have been found to occur on non-*Eucalyptus* Myrtaceae species worldwide (Carnegie *et al.*, 2007; Crous, 1999; Sivanesan and Shivas, 2002). However, despite the intensive effort to identify species occurring on *Eucalyptus* and other Myrtaceae in the last decade, there is no report of the same species of Mycosphaerellaceae occurring on both *Eucalyptus* and non-*Eucalyptus* Myrtaceae.

Most *Eucalyptus* species are native to Australia and have been vastly moved worldwide. Where *Eucalyptus* are grown as non-natives, they have largely been separated from their natural enemies (Burgess and 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.

Eucalypts are not only threatened 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* that is native on Myrtaceae in South and Central America and that 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 (Gryzenhout *et al.,* 2006; Hodges *et al.*, 1986; Myburg *et al*., 2003; Rodas *et al*., 2005).

Where new pathogens have been introduced into new areas, they have the potential to cause serious diseases of related native plants. It is for this reason that *P. psidii* is one of the most highly feared pathogens of Eucalypts and other native Myrtaceae in Australia (Glen *et al.*, 2007; Grgurinovic *et al.*, 2006). Thus, the recent appearance of the pathogen in Hawaii

(Uchida *et al*., 2006), where it is severely damaging native *Meterosideros,* might be considered the start of many serious disease problems emerging from the movement of *Eucalyptus* spp. to new areas.

Eucalyptus is widely planted in Uruguay and has already been seriously affected by diseases thought to have been introduced from other areas where these trees are being planted. Yet very little is known regarding the pathogens of native Myrtaceae in Uruguay or whether these trees might be threatened by *Eucalyptus* pathogens or vice versa. Uruguay has a large resource of native Myrtaceae (Brussa and Grela, 2007) and the aim of this study was to identify Mycosphaerellaceae species associated with MLD on native Myrtaceae species and examine their relationship with those currently affecting *Eucalyptus* plantations in Uruguay.

MATERIALS AND METHODS

Samples and isolations

Trees belonging to the Myrtaceae family were surveyed in native forests throughout Uruguay with special attention to those located close to *Eucalyptus* plantations. Leaves showing symptoms resembling those caused by species of Mycosphaerellaceae 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 family (Table 4.1). Sampled trees were distributed over the main areas where *Eucalyptus* is planted (Fig. 4.1).

Lesions on leaves bearing pseudothecia were processed for isolation following the procedure described by Crous (1998). Pieces of lesion with mature pseudothecia were soaked in sterile water for two hours. The leaf pieces were then dried on sterilized paper and adhered with adhesive tape to the undersides of a 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 17-18ºC in dark for 24-48 hours. Ascospores that had been ejected onto the media and had germinated were observed under a microscope to record the germination patterns 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 sec, 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 liter to minimize bacterial contamination. Plates were then incubated at room temperature and emerging colonies were subcultured onto fresh 2% MEA plates. Only those cultures with colony morphology resembling species of Mycosphaerellaceae were included in further study. For these isolates, pure cultures were made by transferring hyphal tips to clean culture and thus ensuring that isolates represent a single genotype. Cultures were grouped based on ascospore germination pattern, conidial and ascospores morphology, and colony morphology. These morphological characteristics were then used to confirm groupings emerging from the phylogenetic analyses.

DNA extraction, PCR, sequencing and phylogenetic analyses

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

The entire ribosomal DNA internal transcribed spacer regions (ITS1 and ITS2) plus the 5.8S gene of the rDNA were amplified using the primers ITS1 and ITS4 (White *et al.*, 1990). PCR amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) with the following conditions: initial denaturation for 5 min at 94ºC, then 35 cycles of 1 min at 94ºC, 1 min at 50ºC, 1 min at 72ºC, followed by a final elongation step of 5 min at 72ºC, and held at 10ºC. A 25-µl reaction mixture comprised of 1.0 µl of 0.05% casein, 12.5 µl of Amplitaq Gold PCR Master-Mix (Applied Biosystems, Foster City, CA), 1.0 µl of 10 mM ITS1, 1.0 µl of 10 mM ITS4, 8.5 µl of ddH_2O and 1.0 µl of DNA template.

PCR products were stained with SYBR Green nucleic acid dye (MBL International, Woburn, MA) 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) following manufacturer's instructions. For sequencing reactions the same pair of primers was used in separate reactions with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) 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). Sequences were deposited in GenBank and accession numbers are shown in Table 4.2.

Sequences were subjected to BLAST searches in NCBI Genbank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, verified 26 June 2008), and sequences of the closest match species were downloaded from GenBank. Where available sequences that represented the ex-type cultures of the closely matching species were used as well as all species of Mycosphaerellaceae previously reported from *Eucalyptus.* Following a first preliminary phylogenetic analysis, the alignment was trimmed, discarding those species less phylogenetically related to the sequences under investigation and populating the remainder of the data set with at least two sequences per taxon when possible (Table 4.2). In addition, sequences of Mycosphaerellaceae species obtained from *Eucalyptus* spp. in Uruguay (see Chapter 3 of this Thesis) were included into the alignment for comparisons. 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 26 June 2008) (Katoh et al., 2005). Aligned sequence data and phylogenetic trees were deposited in TreeBASE (accession SN3973).

Neighbor-joining and Maximum parsimony analyses were performed using PAUP v. 4.0b10. (Swofford 2002). The best substitution model for neighbor-joining analysis was determined using Modeltest v. 3.7 (Posada and 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). Gaps were treated as missing data and all characters were treated as unordered and of equal weight. Maximum parsimony 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 and Bull, 1993) and tree length (TL), consistency index (CI), retention index (RI), and homoplasy index (HI) were calculated.

Bayesian analysis was performed using Mr. Bayes v3.1.2 (Ronquist and Huelsenbeck, 2003). The best nucleotide substitution model for this analysis was selected using MrModeltest v2.2 (Nylander, 2004) from which the model GTR+I+G was selected using AIC. Four MCMC chains starting from random tree topology were run over 10 million generations. Trees were sampled every 100th generation and burn-in value was set at 156 since the likelihood values were stationary after 15,600 generations. To obtain the estimates for the posterior probabilities, a 50% majority rule consensus of the remaining 99,778 trees was computed from a total of 199,623 sampled trees.

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 seven species, namely *Acca sellowiana, Blepharocalyx salicifolius, Eugenia uruguayensis, Hexachlamis edulis, Myrceugenia glaucescens, Myrcianthes cisplatensis* and *Myrrhinium atropurpureum* var. *octandrum*. 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 14 groups for further investigation using DNA sequence comparison.

DNA comparison and phylogenetic analyzes

Sequences were generated in both directions and DNA amplicons of ~ 550 nucleotides were obtained after assembly. Following BLAST searches, the 14 sequences obtained in this study were aligned with closest taxa available in GenBank or other species of Mycosphaerellaceae that have been previously reported on *Eucalyptus*. The alignment consisted of 87 ingroup sequences and *Neofusicoccum ribis* as the outgroup taxon.

Sequence alignment resulted in a total of 706 characters of which 232 were constant, 104 variable characters were parsimony-uninformative and 370 were parsimony informative. Neighbor joining (NJ), maximun parsimony (MP) and Bayesian analyses resulted in trees of similar topology. The heuristic search analysis of the data resulted in six most parsimony trees (TL=1267 steps; CI=0.611; RI=0.866; HI=0.389). Strict consensus tree of the six most parsimonious trees is shown (Fig. 4.2) with the bootstrap values of 1000 replicates of NJ and MP analyses and the posteriori probabilities obtained from the Bayesian analysis displayed on the branches.

Species identified

A diverse group of Mycosphaerellaceae was found to occur on diseased leaves of native Myrtaceae in this study. Phylogenetic analyses revealed a total of six distinct species residing in the Mycosphaerellaceae. These included *Mycosphaerella aurantia, M. heimii, M. marksii, M. yunnanensis*, *Passalora loranthi* and *Pseudocercospora norchiensis*. In addition, two groups of isolates did not cluster with any known species and are considered to represent undescribed *Mycosphaerella* species.

Mycosphaerella aurantia was the most widely occurring species infecting a diverse group of native trees. It was found associated with leaf spots on *H. edulis, Myrce. glaucescens* and *Myrci. cisplatensis* in the western region of the country (i.e. provinces of Paysandú and Río Negro) and on *B. salicifolius,* in the northern region (i.e. province of Rivera).

Mycosphaerella heimii was associated with leaf spots only on *Myrce. glaucescens* in Rio Negro, and the sequence of this isolate was identical to the isolate obtained from *E. dunnii* in Uruguay. Also *M. marksii* was isolated from samples collected in the province of Rio Negro, but at this location the fungus was from *Eug. uruguayensis.* Although the DNA sequence for this isolate was different from the sequence of the ex-type culture by one base of the ITS1 region, it was identical to two sequences of *M .marksii* obtained from *E. globulus* and *E. grandis* in Uruguay presented in Chapter 3 of this Thesis.

Mycosphaerella yunnanensis was found on leaf lesions on *B. salicifolius* and *Myrr. atropurpureum* var. *octandrum* in Rivera. Phylogenetic grouping was strongly supported by the three analyses and sequences differed only at one base from the ex-type sequence. *Passalora loranthi* was found on leaf spots on *Acca sellowiana* in the province of Rivera and *Pse. norchiensis* was found on leaves of two native tree species, *A. sellowiana* and *B. salicifolius*, also in the province of Rivera.

In addition to those isolates that could be identified with some confidence, there were three isolates from *B. salicifolius* in the province of Rio Negro and Rivera, which were phylogenetically distant from known species of Mycosphaerellaceae. Thus, isolate UY497 was phylogenetically close to *M. areola,* but the ITS sequence is clearly distinct from this species. Similarly, isolates UY1481 and UY1383 formed a separate group closely related to *M. acaciigena* and to the *M. heimii* complex. These fungi most probably represent undescribed species and for the purpose of this study, they were treated as *Mycosphaerella* sp.

DISCUSSION

Results of this study clearly show that there is a relatively diverse group of species belonging to the Mycosphaerellaceae associated with leaf spots on native Myrtaceae in Uruguay. Four species, namely *M. aurantia, M. heimii, M. marksii* and *Pse. norchiensis,* are well known *Eucalyptus* leaf spot associated fungi, previously reported to infect *Eucalyptus* in Uruguay (Balmelli *et al.,* 2004; Crous *et al.*, 2006; Chapter 3 of this Thesis).

A fascinating aspect of this study lies in the fact that it provides 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 to occur on *Eucalyptus* leaves in countries other than

Uruguay and it seems most likely that they were introduced into Uruguay 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 *Q. eucalypti* (see Chapter 2 of this Thesis) and *Neofusicoccum eucalyptorum* (see Chapter 5 of this Thesis). This is, however, the first evidence of species of Mycosphaerellaceae undergoing such host shifts.

Mycosphaerella aurantia was found associated with leaf spots on four native Myrtaceae species widespread in Uruguay. 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*. DNA sequence comparisons in this study showed that the isolates obtained in this study were grouped with *M. aurantia* but were strongly separated from *T. africana* and *M. keniensis*.

Mycosphaerella heimii was found associated with leaf lesions on *Myrce. glaucescens*. Although Hunter *et al.* (2006) considered *M. heimii* to represent a member of a species complex due to the difficulty differentiating this species from *M. heiminoides*, *M. crystallina* or *M. irregulariramosa,* the isolate UY322 consistently grouped with *M. 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 *E. dunnii* in Uruguay (see Chapter 3 of this Thesis). *Mycosphaerella heimii* is known from Australia, Brazil, Madagascar, Portugal, Thailand, Uruguay and Venezuela (Crous *et al.*, 2006; Crous *et al.*, 2007b; Hunter *et al*., 2004; Chapter 3 of this Thesis) where it has only been found on *Eucalyptus*. Our results suggest that it is able to cross host boundaries and all indications are that in Uruguay, it has moved from *Eucalyptus* onto native Myrtaceae. *Mycosphaerella yunnanensis* found on native Myrtaceae in this study has recently been described by Burgess *et al.* (2007) from *E. urophylla* in China. We found this species associated with leaf spots on the native *B. salicifolius* and *Myrr. 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 *Pas. loranthi* associated with leaf disease on *A. sellowiana*. This finding adds a Myrtaceae to the list of hosts attacked by this fungus.

Pseudocercospora norchiensis, found on *A. sellowiana* and *B. salicifolius* in this study, was very recently described by Crous *et al.* (2007b) on leaves of *Eucalyptus* collected in Italy. Very little is known regarding this fungus but it was recently found on *E. dunnii, E. globulus* and *E. grandis* in the northern region of Uruguay (see Chapter 3 of this Thesis).

Although *Pse. luzardii* was grouped closely with *Pse. norchiensis*, the similarity in morphological features of isolates UY1436 and UY1484 with those described for *Pse. norchiensis* as well as the molecular data (100% similarity with the ex-type sequence of *Pse. norchiensis*) support the identification of these isolates as *Pse. norchiensis*. In addition, the reference sequence of *Pse. luzardii* (AF362057) in GenBank differed from *Pse. norchiensis* (EF394859) at three nucleotides in the ITS2 region. The former species has been reported only on *Hancornia speciosa* (Apocynaceae) in Brazil (Furnaletto and Dianese, 1999) and it probably represents a distinct species.

In this study, three isolates that apparently represent two undescribed species of Mycosphaerellaceae were encountered. One of the isolates (UY497) has close phylogenetic relationship to *M. areola* but it showed only 95% similarity in the 458 nucleotides compared. The other two isolates (UY1481 and UY1383) grouped together in the three phylogenetic analyses and they were closely related to species in the *M. heimii*-complex. It was interesting that the three unidentified isolates were all from leaf lesions on *B. salicifolius.* Additional isolates from this tree are being obtained to assemble sufficient material to describe the fungi as new.

To the best of our knowledge, this study represents the first to broadly consider the *Mycosphaerella* spp. on native Myrtaceae growing in association with non-native *Eucalyptus* plantations. Various *Mycosphaerella* species, previously only known from *Eucalyptus* were encountered on native Myrtaceae. This intriguing result suggests that these fungi are moving from non-native *Eucalyptus* to native trees. It has also been shown that fungi previously thought to be specific to *Eucalyptus* have a wider host range. Almost nothing is known regarding the importance and pathogenicity of these species but they are known to be associated with leaf spots. 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 disease problems more serious than those observed on *Eucalyptus* must be considered.

While there are growing numbers of examples of pathogens of native Myrtaceae moving to *Eucalyptus* where these trees are grown as exotics, there are far fewer examples of movement of apparently introduced *Eucalyptus* pathogens to native plants. Results of this study provide the worrying evidence that this movement is far more common than has been expected. Although the consequences have yet to be realized, the results illustrate the danger of moving crop plants between countries together with fungi that are poorly understood.

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Table 4.1: List of Myrtaceae species native to Uruguay that were sampled in this study. Tree species in bold had Mycosphaerellaceae species isolated from them.

Tree Species
Acca sellowiana
Agariota eucalyptides
Blepharocalyx salicifolius
Calyptranthes concinna
Eugenia involucrata
E. mansonii
E. repanda
E. uniflora
E. uruguayensis
Gomidesia palustris
Hexachlamis edulis
Myrceugenia euosma
Myrce. glaucescens
Myrcianthes cisplatensis
Myrci. pungens
Myrciaria tenella
Myrrhinium atropurpureum* var. *octandrum
Psidium luridum
P. incanum
P. pubifolium

Table 4.2: 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	Teleomorph	Anamorph	Host	Location of collection	GenBank accession no.
UY322	*Mycosphaerella heimii*	*Pseudocercospora heimii*	*Myrceugenia glaucescens*	Río Negro	EU853466
UY372	*M. aurantia*	Unknown	*Eucalyptus grandis*	Río Negro	EU851913
UY386	*M. aurantia*	Unknown	*Euc. grandis*	Río Negro	EU851915
UY423	*M. heimii*	*Pse. heimii*	*Euc. dunnii*	Río Negro	EU851921
UY440	*M. marksii*	Unknown	*Euc. grandis*	Río Negro	EU851922
UY454	*M. marksii*	Unknown	*Eugenia uruguayensis*	Río Negro	EU853467
UY483	*M. aurantia*	Unknown	*Myrcianthes cisplatensis*	Río Negro	EU853468
UY497	*Mycosphaerella* sp.	Unknown	*Blepharocalyx salicifolius*	Río Negro	EU853469
UY523	*M. aurantia*	Unknown	*Myrce. glaucescens*	Río Negro	EU853470
UY657	*M. aurantia*	Unknown	*Hexachlamis edulis*	Paysandú	EU853471
UY1122	*M. marksii*	Unknown	*Euc. globulus*	Florida	EU851924
UY1163	*M. marksii*	Unknown	*Euc. dunnii*	Florida	EU851929
UY1192	*M. marksii*	Unknown	*Euc. globulus*	Lavalleja	EU851930
UY1382	*M. aurantia*	Unknown	*B. salicifolius*	Rivera	EU853472
UY1383	*Mycosphaerella* sp.	Unknown	*B. salicifolius*	Rivera	EU853473
UY1436	Unknown	*Pse. norchiensis*	*Acca sellowiana*	Rivera	EU853474
UY1462	*M. yunnanensis*	Unknown	*Myrrhinium atropurpureum* var. *octandrum*	Rivera	EU853475
UY1481	*Mycosphaerella* sp.	Unknown	*B. salicifolius*	Rivera	EU853476
UY1483	*M. yunnanensis*	Unknown	*B. salicifolius*	Rivera	EU853477
UY1484	Unknown	*Pse. norchiensis*	*B. salicifolius*	Rivera	EU853478
UY1506	Unknown	*Passalora loranthi*	*Acca sellowiana*	Rivera	EU853479
UY1528	Unknown	*Pse. norchiensis*	*Euc. dunnii*	Rivera	EU851936
UY1561	Unknown	*Pse. norchiensis*	*Euc. grandis*	Rivera	EU851938

Culture ID	Teleomorph	Anamorph	Host	Location of collection	GenBank accession no.
CPC3837 [T]	*M. acaciigena*	*Pse. acaciigena*	*Acacia mangium*	-	AY752143
CBS120740	*M. acaciigena*	*Pse. acaciigena*	*Eucalyptus* sp.	-	EF394822
CPC13350	*M.acaciigena*	*Pse. acaciigena*	*E. camaldulensis x E. urophylla*	-	EF394823
CICR-3	*M. areola*	*Ramularia areola*	*Gossypium arboreum*	-	DQ459081
CICR-1	*M. areola*	*R. areola*	*G. herbaceum*	-	DQ459082
CBS110500 [T]	*M. aurantia*	Unknown	*Euc. globulus*	-	AY725531
MURU151	*M. aurantia*	Unknown	*Euc. globulus*	-	AY150331
MURU152	*M. aurantia*	Unknown	*Euc. globulus*	-	AY509742
MURU222	*M. aurantia*	Unknown	*Euc. globulus*	-	AY509744
CBS110969 [T]	*M. colombiensis*	*Pse. colombiensis*	*Euc. urophylla*	-	AY752149
CBS114238 [T]	*M. comunis*	*Dissoconium commune*	*Euc. globulus*	-	AY725541
CBS681.95 [T]	*M. crystallina*	*Pse. crystallina*	*Euc. bicostata*	-	AY490757
CMW3042	*M. crystallina*	*Pse. crystallina*	*Eucalyptus* sp.	-	AF309611
CBS120735 [T]	*M. elongata*	Unknown	*E. camaldulensis x E. urophylla*	-	EF394833
CBS111519 [T]	*M. endophytica*	*Pseudocercosporella endophytica*	*Eucalyptus* sp.	-	DQ267579
CBS110682 [T]	*M. heimii*	*Pse. heimii*	*Eucalyptus* sp.	-	DQ239992
CBS120743	*M. heimii*	*Pse. heimii*	*Euc. urophylla*	-	EF394838
CMW5719	*M. heimii*	*Pse. heimii*	*Eucalyptus* sp.	-	AF452516
CPC13371	*M. heimii*	*Pse. heimii*	*Euc. urophylla*	-	EF394840
CBS111190 [T]	*M. heimioides*	*Pse. heimioides*	*Eucalyptus* sp.	-	AF309609
CBS111364	*M. heimioides*	*Pse. heimioides*	*Eucalyptus* sp.	-	DQ267586
CBS114774 [T]	*M. irregulariramosa*	*Pse. irregulariramosa*	*Euc. saligna*	-	AF309607
CMW5223	*M. irregulariramosa*	*Pse. irregulariramosa*	*Euc. saligna*	-	AF309608
CBS111001 [T]	*M. keniensis*	*Unknown*	*Euc. grandis*	-	AF309601
STE-U2123	*M. konae*	*Pseudocercospora* sp.	*Leucadendron* sp.	-	AY260086
STE-U2125	*M. konae*	*Pseudocercospora* sp.	*Leucadendron* sp.	-	AY260085

Culture ID	Teleomorph	Anamorph	Host	Location of collection	GenBank accession no.
CBS326.52	*M. laricina*	*Pseudocercospora* sp.	*Larix decidua*	-	AY152590
Lari01.03	*M. laricina*	*Pseudocercospora* sp.	n/a	-	DQ019342
CBS110748 [T]	*M. lateralis*	*D. dekkeri*	*Eucalyptus* sp.	-	AF309624
CBS112895 [T]	*M. madeirae*	*Pseudocercospora* sp.	*Euc. globulus*	-	AY725553
STE-U348	*M. marasasii*	*Stenella marasasii*	*Syzygium* sp.	-	AF309591
CBS682.95 [T]	*M. marksii*	*Pse. epispemogoniana*	*Euc. grandis*	-	DQ267587
CBS110920	*M. marksii*	*Pse. epispemogoniana*	*Euc. botryoides*	-	AF309588
CBS110981	*M. marksii*	*Pse. epispemogoniana*	*Eucalyptus* sp.	-	DQ302977
CBS111670	*M. marksii*	*Pse. epispemogoniana*	*Euc. globulus*	-	DQ302978
ctil0102	*M. microsora*	*Passalora microsora*	*Tilia americana*	-	DQ019352
CMW3358 [T]	*M. parkii*	*Stenella parkii*	*Eucalyptus* sp.	-	AF309590
CBS118493 [T]	*M. scytalidii*	Unknown	*Eucalyptus* sp.	-	DQ303016
CBS118909 [T]	*M. stramenti*	Unknown	*Eucalyptus* sp.	-	DQ303042
CPC10547 [T]	*M. thailandica*	*Pse. thailandica*	*Acacia mangium*	-	AY752156
CBS119974 [T]	*M. vietnamensis*	Unknown	*Euc. grandis hybrid*	-	DQ632675
STE-U2769	*M. walkeri*	*Sorderhenia eucalypticola*	*Eucalyptus* sp.	-	AF309616
CBS119975 [T]	*M. yunnanensis*	Unknown	*Eucalyptus* sp.	-	DQ632686
CBS119976	*M. yunnanensis*	Unknown	*Eucalyptus* sp.	-	DQ632687
CMW23445	*M. yunnanensis*	Unknown	*Eucalyptus* sp.	-	DQ632688
CBS680.95 [T]	*Teratosphaeria africana*	Unknown	*Euc. viminalis*	-	AF309602
CMW3025	*T. africana*	Unknown	*Euc. viminalis*	-	AF283690
CBS116005 [T]	*T. nubilosa*	*Uwebraunia juvenis*	*Euc. globulus*	-	AF309618
CBS110949 [T]	*T. ohnowa*	Unknown	*Euc. grandis*	-	AY725575
CBS118508 [T]	*T. pluritubularis*	Unknown	*Euc. globulus*	-	DQ303007
CBS120303 [T]	Unknown	*Kirramyces gauchensis*	*Euc. grandis*	-	EU019290
n/a	Unknown	*Pas. loranthi*	n/a	-	AY348311

Culture ID	Teleomorph	Anamorph	Host	Location of collection	GenBank accession no.
CBS120029 [T]	Unknown	*Pas. schizolobii*	*Schizolobium parahybum*	-	DQ885903
CMW5148 [T]	Unknown	*Pse. basiramifera*	*Euc. pellita*	-	AF309595
CBS111280	Unknown	*Pse. basitruncata*	*Euc. grandis*	-	DQ267601
CBS114664	Unknown	*Pse. basitruncata*	*Euc. grandis*	-	DQ267600
CBS110777 [T]	Unknown	*Pse. eucalyptorum*	*Eucalyptus* sp.	-	AF309598
CMW13586 [T]	Unknown	*Pse. flavomarginata*	*Euc. camaldulensis*	-	DQ155657
STE-U2556	Unknown	*Pse. luzardii*	*Hancornia speciosa*	-	AF362057
CBS111069 [T]	Unknown	*Pse. natalensis*	*Eucalyptus* sp.	-	DQ303077
CBS120738 [T]	Unknown	*Pse. norchiensis*	*Eucalyptus* sp.	-	EF394859
STE-U1458	Unknown	*Pse. paraguayensis*	*Eucalyptus* sp.	-	AF309596
CBS121101 [T]	Unknown	*Stenella eucalypti*	*Euc. tereticornis*	-	EF394865
CMW7773	*Botryosphaeria' ribis*	*Neofusiccocum ribis*	*Ribes* sp.	-	AY236936

[T]: ex-type cultures

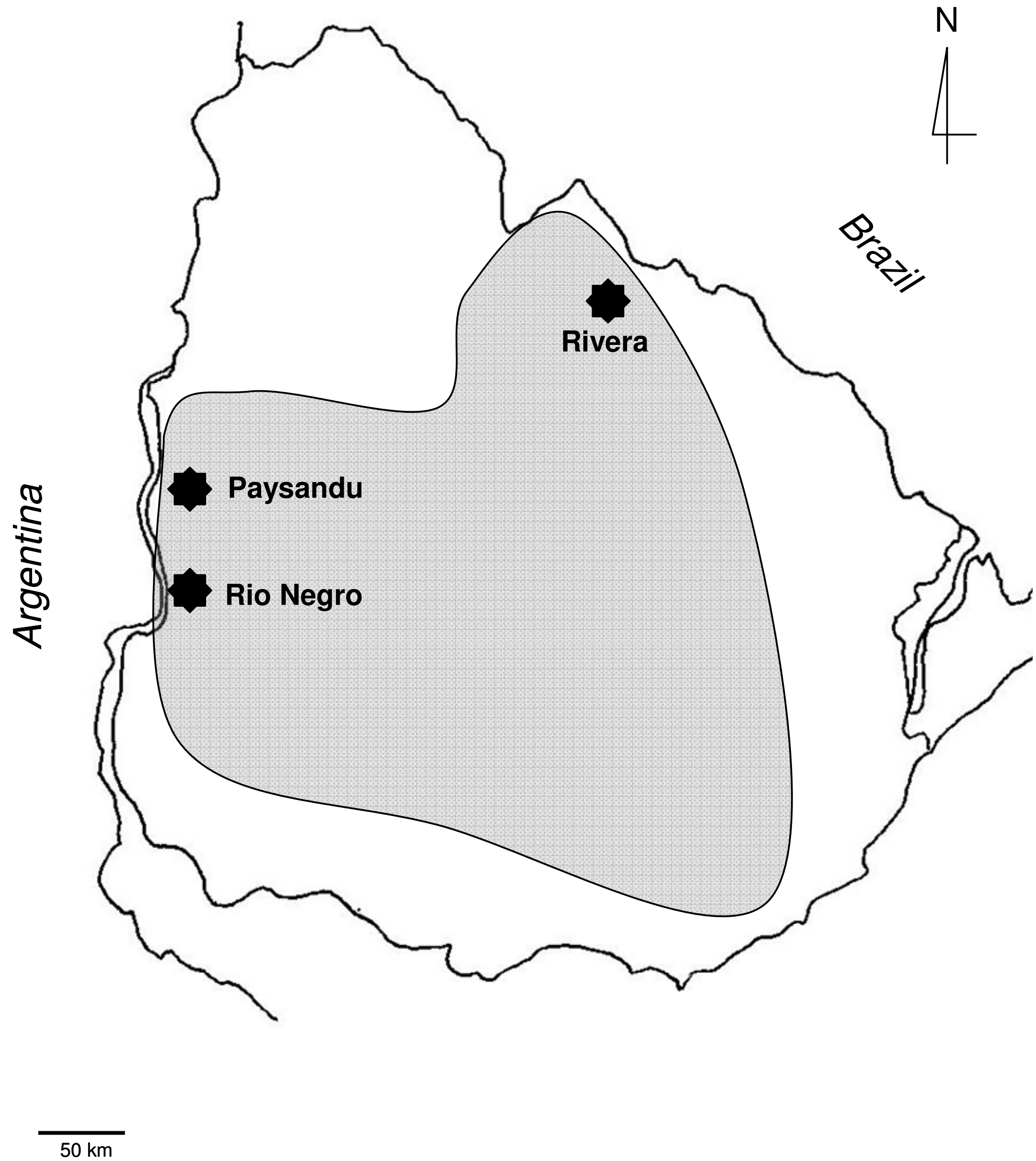

Fig. 4.1: Map of Uruguay. The shaded area indicates the geographic distribution of *Eucalyptus* plantations in the country. Stars indicate those locations where Mycosphaerellaceae species were found occurring on native trees.

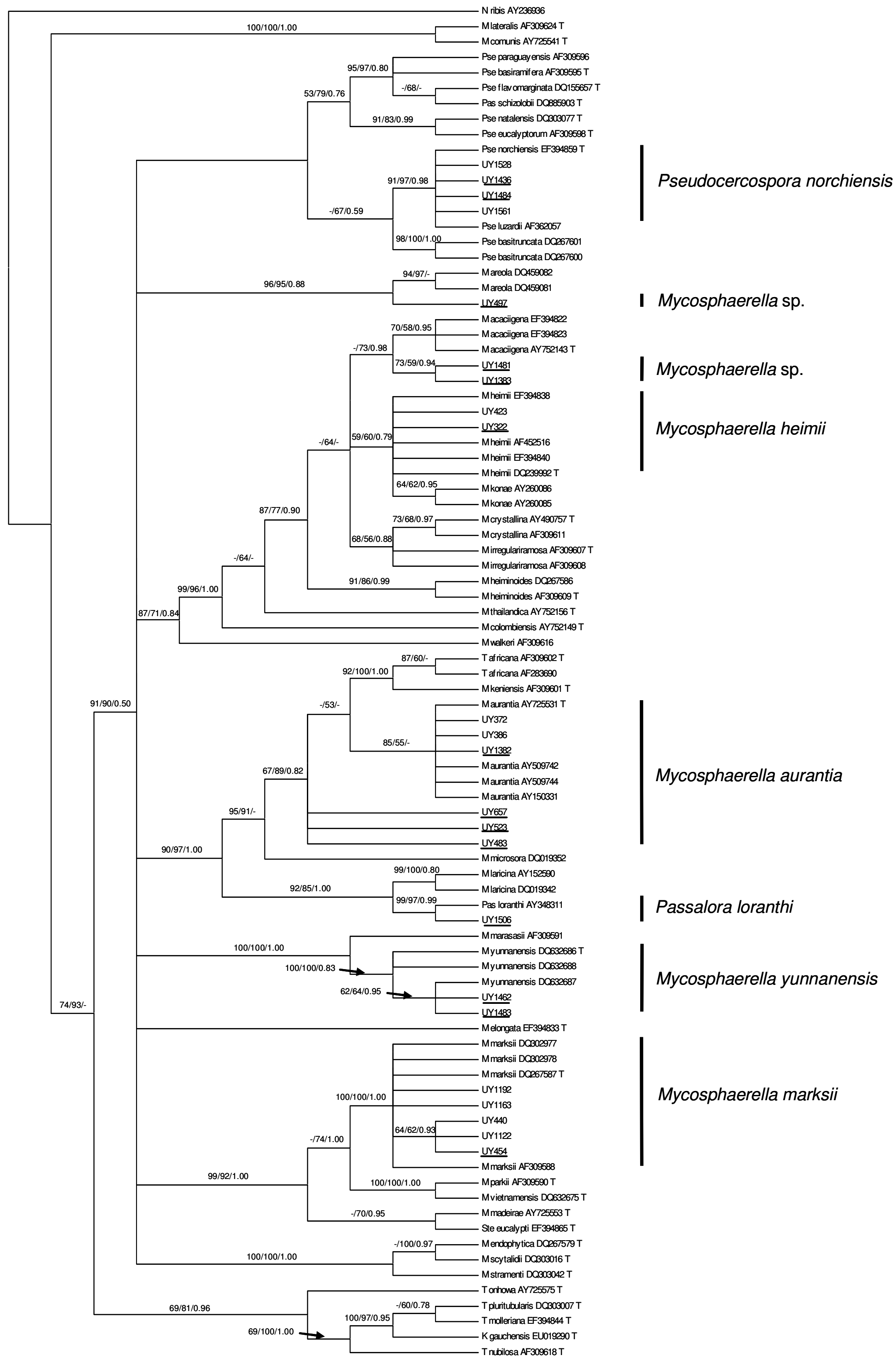
N ribis AY236936
100/100/1.00
M lateralis AF309624 T
M comunis AY725541 T
95/97/0.80
Pse paraguayensis AF309596
Pse basiramifera AF309595 T
-/68/-
Pse flavomarginata DQ155657 T
Pas schizolobii DQ885903 T
53/79/0.76
91/83/0.99
Pse natalensis DQ303077 T
Pse eucalyptorum AF309598 T
Pse norchiensis EF394859 T
UY1528
91/97/0.98
UY1436
UY1484
-/67/0.59
UY1561
Pse luzardii AF362057
98/100/1.00
Pse basitruncata DQ267601
Pse basitruncata DQ267600
94/97/-
M areola DQ459082
96/95/0.88
M areola DQ459081
UY497
M acaciigena EF394822
70/58/0.95
M acaciigena EF394823
M acaciigena AY752143 T
-/73/0.98
73/59/0.94
UY1481
UY1383
M heimii EF394838
UY423
UY322
-/64/-
59/60/0.79
M heimii AF452516
M heimii EF394840
M heimii DQ239992 T
64/62/0.95
M konae AY260086
M konae AY260085
87/77/0.90
73/68/0.97
M crystallina AY490757 T
M crystallina AF309611
68/56/0.88
M irregulariramosa AF309607 T
M irregulariramosa AF309608
-/64/-
91/86/0.99
M heimioides DQ267586
99/96/1.00
M heimioides AF309609 T
87/71/0.84
M thailandica AY752156 T
M colombiensis AY752149 T
M walkeri AF309616
87/60/-
T africana AF309602 T
92/100/1.00
T africana AF283690
M keniensis AF309601 T
91/90/0.50
-/53/-
M aurantia AY725531 T
UY372
UY386
85/55/-
UY1382
M aurantia AY509742
M aurantia AY509744
67/89/0.82
M aurantia AY150331
UY657
95/91/-
UY523
UY483
90/97/1.00
M microsora DQ019352
99/100/0.80
M laricina AY152590
M laricina DQ019342
92/85/1.00
99/97/0.99
Pas loranthi AY348311
UY1506
M marasasii AF309591
100/100/1.00
M yunnanensis DQ632686 T
100/100/0.83
M yunnanensis DQ632688
M yunnanensis DQ632687
62/64/0.95
UY1462
UY1483
74/93/-
M elongata EF394833 T
M marksii DQ302977
M marksii DQ302978
M marksii DQ267587 T
UY1192
100/100/1.00
UY1163
UY440
64/62/0.93
UY1122
-/74/1.00
UY454
M marksii AF309588
99/92/1.00
100/100/1.00
M parkii AF309590 T
M vietnamensis DQ632675 T
-/70/0.95
M madeirae AY725553 T
Ste eucalypti EF394865 T
-/100/0.97
M endophytica DQ267579 T
100/100/1.00
M scytalidii DQ303016 T
M stramenti DQ303042 T
T onhowa AY725575 T
69/81/0.96
-/60/0.78
T pluritubularis DQ303007 T
100/97/0.95
T molleriana EF394844 T
69/100/1.00
K gauchensis EU019290 T
T nubilosa AF309618 T
Pseudocercospora norchiensis
Mycosphaerella sp.
Mycosphaerella sp.
Mycosphaerella heimii
Mycosphaerella aurantia
Passalora loranthi
Mycosphaerella yunnanensis
Mycosphaerella marksii

Fig. 4.2: Cladogram of Mycosphaerellaceae species found on native Myrtaceae trees in Uruguay based on the ITS region of the rDNA operon. Strict consensus of the 6 most parsimonious trees inferred from heuristic searches using PAUP. Species name and GenBank accession number are shown for each sequence. Sequences labeled with a "T" at the end correspond to the ex-type culture. Bootstrap values of 1,000 replicates of neighbor-joining and maximum parsimony analyses and posteriori probabilities of the Bayesian analysis of 10 millions generations are shown at the branches, respectively. Only bootstrap values higher than 50% 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 underlined.

Chapter 5: *Neofusicoccum eucalyptorum,* an introduced *Eucalyptus* pathogen, occurring on native Myrtaceae in Uruguay

ABSTRACT

Neofusicoccum eucalyptorum is a canker-associated fungus that has thus far been thought to be highly specialized on *Eucalyptus*. However, when surveying the microbial population inhabiting native Myrtaceae trees in Uruguay, fungal cultures resembling *N. eucalyptorum* were isolated. This intriguing occurrence of *N. eucalyptorum* in hosts other than *Eucalyptus* called for further investigation to confirm the identity of these cultures and to have a preliminary comparison of genetic and phenotypic variation of these isolates to those obtained from *Eucalyptus* species. Several surveys were conducted throughout Uruguay to obtain samples from native forest, focusing primarily on those species residing in the Myrtaceae family. Fungal identification was based on morphology and confirmed with molecular techniques by sequencing the internal transcribed spacer (ITS) of the rDNA operon. Genetic diversity was also assessed using inter simple sequence repeats (ISSR) markers whereas phenotypic characterization was performed by inoculating seedlings of an *E. grandis* clone. Morphology and molecular identification confirmed the occurrence of *N. eucalyptorum* on *Blepharocalyx salicifolius, Myrceugenia glaucescens* and *Myrrhinium atropurpureum* var. *octandrum*. This is the first report of *N. eucalyptorum* occurring in hosts other than *Eucalyptus*. Genetic characterization indicates genetic variability among isolates originating from native trees and introduced *Eucalyptus*. Pathogenicity tests confirmed the ability of this species to produce cankers and showed that cross pathogenicity among hosts takes place and phenotypic variability is present among isolates. Despite the limited number of isolates available, results showed that *N. eucalyptorum* is not clonal in Uruguay and genetic variation was observed amongst isolates from both groups of hosts. This study provides information that will assist breeding programs in attempts to obtain disease resistant *Eucalyptus* plantations and also establishes new concerns for the threat of this pathogen to native trees.

INTRODUCTION

Neofusicoccum eucalyptorum (teleomorph *'Botryosphaeria' eucalyptorum*) is a serious pathogen on *Eucalyptus*. It was first described by Smith *et al*. (2001) in South Africa as a canker pathogen of *Eucalyptus* trees. It was later found in Australia as the dominant Botryosphaeriaceae species isolated from cankers on native and planted *Eucalyptus* (Slippers *et al.*, 2004b), and as an endophyte in *E. globulus* (Burgess *et al.*, 2006). Several Botryosphaeriaceae species are common endophytes that cause disease after the onset of stress, with drought being the most commonly cited agent of stress associated with the disease (Old *et al.*, 1990; Pusey, 1989; Wene and Schoeneweiss, 1980; Slippers and Wingfield, 2007). Although the impact of opportunistic endophytes is difficult to assess, Smith *et al.* (2001) analyzed the pathogenicity of several isolates of *N. eucalyptorum,* and concluded that the species was pathogenic to eucalypts, even though isolates of *N. eucalyptorum* were less virulent than those of *B. dothidea*.

The host specialization observed for *N. eucalyptorum,* which has been reported only from *Eucalyptus* spp. (Smith *et al*., 2001; Slippers *et al.*, 2004b; Burgess *et al*., 2006), and its abundance and wide distribution in Eastern Australia suggest that this pathogen likely originated in Australia and was introduced with planting stock or seeds into other countries where *Eucalyptus* were planted (Slippers *et al.*, 2004b). In Uruguay, *N. eucalyptorum* seems to be commonly present in *Eucalyptus* plantations and has been found endophytically infecting *Eucalyptus* and also sporulating on woody debris of *E. maidenii* left over from pruning (Alonso, 2004; see Chapter 6 of this Thesis).

The area planted to *Eucalyptus* in Uruguay has nearly tripled in the 10–year period 1995 to 2005 from 175,000 ha to ca. 500,000 ha (MGAP, 2005) and this explosive increase has also been associated with increased disease problems. Nevertheless, limited work has been done on *Eucalyptus* pathogens in the country and very little is known about the biology and epidemiology of *N. eucalyptorum*. In addition, the biotic interaction between introduced *Eucalyptus* and native Myrtaceae trees is also of great concern. Uruguay has a rich diversity of native Myrtaceae trees with a total of 35 species reported by Brussa and Grela (2007) and the exchange of pathogens between introduced *Eucalyptus* and native trees could result in negative economical impact, as well as ecological disturbance or even catastrophic damage (Anderson *et al*., 2004; Desprez-Loustau *et al*., 2007; Pavlic *et al*., 2007; Slippers *et al.*, 2005; Woolhouse *et al.*, 2005). For this reason, several surveys were conducted over the main forest regions to obtain a better understanding of the Botryosphaeriaceae species occurring on introduced and native hosts in Uruguay (see Chapter 6 of this Thesis). During those surveys a group of isolates displaying morphological characters resembling *N. eucalyptorum* were isolated from native Myrtaceae species. The intriguing occurrence of *N. eucalyptorum* in hosts other than *Eucalyptus* called for further investigation. The objectives of this study were therefore to identify the isolates obtained from native Myrtaceae species, and to compare the genetic and phenotypic variation among isolates obtained from native hosts and those from *Eucalyptus* species. The identification of genetic variation among isolates of *N. eucalyptorum* along with the characterization of phenotypic reactions obtained from inoculation tests are of importance to breeding programs focused on selection for durable genetic resistance to this pathogen.

MATERIALS AND METHODS

Fungal isolates

Symptomatic and asymptomatic material was collected between 2005 and 2008 from several native forests with special attention to those located close to *Eucalyptus* plantations (less than 500 m away) throughout the country. Myrtaceae species within the native forests were prioritized for study due to their close phylogenetic relationship with the genus *Eucalyptus* (Wilson *et al.,* 2005). Samples were also collected from *Eucalyptus* plantations for comparison. Endophytic isolates were obtained from asymptomatic fresh material. Leaf, petiole and twig sections were sequentially surface-disinfested in 70% ethyl alcohol for 1 min, immersed in 0.4% sodium hypochlorite for 2 min, then rinsed twice in sterile distilled water and blotted dry on sterile filter paper. Disinfested plant tissue was placed on 2% malt extract agar (MEA) (2% malt extract, 1.5% agar; Oxoid, Basingstoke, England). Plates were incubated at room temperature (~20ºC) for one week. Colonies resembling Botryosphaeriaceae were selected for this study, and maintained in 2% MEA at 8ºC. To verify the efficacy of the surface disinfested and to assure the growth of only endophytic microorganisms, imprints of sample surfaces were made on MEA plates and observed for one week to confirm that fungi did not grow. One specimen was obtained from a stem canker. In this case the isolation was done from wood tissue at the advancing zone of the lesion, which was surface-disinfested in 70% ethyl alcohol for 30 sec, rinsed twice in sterile distilled water and blotted dry on sterile filter paper. Disinfested tissue was placed on 2% MEA and incubated at room temperature (~20ºC) for one week. Colonies resembling Botryosphaeriaceae were sub-cultured to a fresh 2% MEA plate for further investigation.

Morphological characterization

Isolates were grown on 1.5% water agar (WA) (Sigma Chemicals, St. Louis, MO) with sterilized pine needles placed onto the medium surface to stimulate the production of fruiting structures (pycnidia) and conidia. Plates were incubated at 22ºC under continuous black light until pycnidia were observed on the pine needles (approx. 3 weeks after plating). Monosporic cultures were generated by plating a spore suspension taken from two pycnidia, suspended in 300 µl of sterile water on WA. Germinating conidia were lifted from the agar plates and transferred to fresh 2% MEA.

Pycnidia and conidia produced on pine needles were mounted on microscope slides, examined under a standard light microscope Nikon Eclipse E600 and photographed with a Nikon Digital Camera DXM1200F (Nikon Inc., Melville, NY). Five isolates with structures resembling *N. eucalyptorum* obtained from different hosts were further analyzed using molecular techniques. Isolates of *N. eucalyptorum* obtained from *Eucalyptus* hosts were included in the analysis for comparison.

DNA extraction and genetic analysis

The five isolates from native Myrtaceae trees plus those obtained from *Eucalyptus* hosts were grown in 2% MEA plates at room temperature for a week. Mycelia were scrapped and transferred to microfuge (1.5 ml) with 1-mm glass beads and extraction buffer of the Qiagen Plant DNeasy Mini Kit (Qiagen Inc., Valencia, CA). Subsequently, the tubes were vigorously shaken using a vortex mixer for 1 min and placed in a water bath at 60ºC for 1 hr. DNA was extracted using the Qiagen Plant DNeasy Mini Kit following manufacturer's instructions. The internal transcribed spacer region of the ribosomal DNA operon was amplified, sequenced and analyzed for single nucleotide polymorphisms to identify the isolates. Additionally, inter simple sequence repeat primers were used to compare genome fingerprinting variability among isolates.

Internal Transcribed Spacer (ITS) analysis

The ITS region of the ribosomal DNA operon (ITS) was amplified using primers ITS1 (5' TTC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al.*, 1990). A 25-µl reaction mixture of 1.0 µl of 0.05% casein, 12.5 µl of Amplitaq Gold PCR Master-Mix (Applied Biosystems, Foster City, CA), 1.0 µl of 10 mM ITS1, 1.0 µl of 10 mM ITS4, 8.5 µl of ddH_2O and 1.0 µl of DNA template was used for

Polymerase Chain Reactions (PCR). Amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) with the following parameters: 5 min at 94ºC; 1 min at 94ºC; 1 min at 50ºC; 1 min at 72ºC; cycle to step 2, 35 times; 5 min at 72ºC; hold at 10ºC.

PCR products were visualized on 1.5% agarose gels, purified and prepared for sequencing using ExoSAP-IT PCR clean-up kit (USB Corp., Cleveland, OH) following the manufacturer's instructions. The same primers were used for sequencing reactions with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 377 automated DNA sequencer. ChromasPro software version 1.33 (Technelysium Pty. Ltd., Eden Prairie, MN) was used for the assemblage of the forward and reverse sequences. Sequences obtained in this study were deposited in GenBank and accession numbers are given in Table 5.1. All ITS sequences were subjected to BLAST searches in NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, verified 28 June 2008), and these sequences of the ex-type cultures of closest match species were downloaded from GenBank. 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 28 June 2008) (Katoh *et al.*, 2005).

Phylogenetic analysis was performed using PAUP Version 4.0b10 (Swofford, 2002). For neighbor-joining analysis the model TrN + I was selected using Modeltest v. 3.7 (Posada and Crandall, 1998). Gaps were treated as missing data and all characters were treated as unordered and of equal weight. Heuristic search option with simple taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm was selected for maximum parsimony analysis. Support for the nodes of the shortest trees was determined by analysis of 1000 bootstrap replicates (Hillis and Bull, 1993) and tree length (TL), consistency index (CI), retention index (RI), and homoplasy index (HI) were calculated. Alignment was deposited in TreeBASE (SN3974).

Inter Simple Sequence Repeat (ISSR) analysis

The small number of isolates obtained from native trees did not allow a full assessment of the genetic structure of the populations occurring on native trees and *Eucalyptus*. Nevertheless, inter simple sequence repeat (ISSR) markers were used to carryout a first estimation of the genetic relationship between both populations with the aim of having a preliminary assessment of the genetic variability among isolates and to analyze the occurrence of fungal genotype-host species association.

Eleven isolates including four from native hosts and seven from *Eucalyptus* were selected for genetic comparison using ISSR primers. ISSR marker analysis is a PCR-based technique used to amplified sequences between adjacent, inversely orientated microsatellites regions using simple sequence repeat primers. Thus, this technique allows the detection of polymorphism in microsatellites and inter-microsatellite loci without knowledge of DNA sequence (Zietkiewicz *et al.*, 1994). ISSR markers are similar to RAPD but with the advantage that they are highly reproducible. This technique has been widely used to investigate genetic diversity and population genetic structure primarily in plants and insects (Reddy and Nagaraju, 1999; Reddy *et al.*, 2002), and less commonly used with fungi (Menzies *et al*., 2003). To our knowledge ISSR markers have not been previously used to study the genetic diversity of *N. eucalyptorum*. Therefore a total of 28 ISSR primers including anchored and unanchored dinucleotide, trinucleotide and tetranucleotide repeats were tested with a set of three *N. eucalyptorum* isolates. The simple sequence repeat primer M13 was also tested because this core sequence has been widely used to generate fingerprints in eukaryotes and proved to amplify certain Botryosphaeriaceae species (Zhou *et al*., 2001). Although twelve primers showed clear PCR products, the four primers listed in Table 5.2 were selected for obtaining clear, reproducible and polymorphic fragments among isolates.

PCR amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) in a 25-µl reaction volume containing 1.2 x buffer with $MgCl_2$ (Roche Molecular Biomedicals, Alameda, CA), 200 µm of each dNTP, 600 nm of primer, 3.5 U of Taq DNA polymerase and 10 ng of genomic DNA. PCR parameters were initial denatured for 2 min at 95ºC, followed by 40 cycles of 30 sec at 95 ºC, annealing temperature of 48 ºC for 45 sec, 2 min at 72 ºC for extension and a final extension step of 10 min at 72ºC. PCR products were stained with SYBR Green nucleic acid dye (MBL International, Woburn, MA), separated in 2% agarose gel and photographed on an UV transilluminator. Each ISSR amplification was repeated once to confirm band patterns. Each isolate was scored for each amplification product as present (1) or absent (0), regardless of the strength of the band. Dendrograms were produced by cluster analysis of the similarity coefficients using the unweighted pair-group method using arithmetic average (UPGMA) using POPGENE v 1.32 (Yeh *et al*., 1999).

The resolving power (R_p) of the primers was calculated by the formula $R_p = \sum I_b$ according with Prevost and Wilkinson (1999), where I_b is the band informativeness calculated as $1 - (2 \text{ x}[0.5 - p])$, where p is the proportion of the 11 analyzed genotypes containing the band. Even though the number of analyzed genotypes was too small for a population analysis, Nei's gene diversity (*H*) and Shannon's information index (*I*) were calculated with the objective of having a preliminary estimation of genetic variability.

Pathogenicity tests

The pathogenicity and aggressiveness of the isolates obtained from native Myrtaceae and introduced *Eucalyptus* trees were tested using an adaptation of the method described by Simeto *et al.* (2007) using the mycelial plug technique. Briefly, the region of the stem to be wounded was previously surface disinfected with 70% ethyl alcohol. A wound was made on the stem of 4-month-old seedlings of a clone of *E. grandis* at approximately 10 cm above the soil and between two nodes using a cork borer of 5 mm diameter to remove the bark and expose the cambium. Mycelial plugs from pure cultures grown for a week on 2% MEA at room temperature were taken using the same cork borer size and placed into the wound with the mycelial surface facing the cambium. A piece of sterile cotton soaked in sterile water was attached to the inoculated wound with Ready Por Nº 545 tape (Sagrin S.A., Montevideo, Uruguay) to prevent desiccation of the plug. Each isolate was inoculated into the stems of three seedlings. Plugs of sterile MEA were inoculated into stems of three trees as controls. Inoculated trees were maintained outside under a structure with a plastic roof and open sides with temperature ranging from 15 to 25 ºC. Stem diameter at the site of the inoculation and lesion length were measured one and three weeks post inoculation and photographed for records. Data were subjected to analysis of variance (ANOVA) using the Generalized Linear Model procedure (PROC GLM) of SAS (release 9.1; SAS Institute, Inc., Cary, NC). When the F test was significant ($P<0.05$) the treatment means were compared using Tukey's studentized range (HSD) test at $P=0.05$.

To complete Koch's postulates, one inoculated stem per isolate was randomly selected for re-isolation of the inoculated fungus. Thus, pieces of wood from the edges of the lesions were surface-disinfested in 70% ethyl alcohol for 1 min, immersed in 0.4% sodium hypochlorite for 2 min, then rinsed twice in sterile distilled water and blotted dry on sterile filter paper. Disinfested plant tissue was placed on 2% MEA and incubated at room temperature (~20ºC) for one week. Fungal identification was based on colony and conidial morphology.

RESULTS

Sampling and fungal isolates

A total of 216 trees representing 20 distinct species residing in the Myrtaceae were surveyed. Five isolates resembling *N. eucalyptorum* were obtained from plant tissue from five different trees representing three different host species and four different locations (Table 5.3). A large number of isolates resembling *N. eucalyptorum* were obtained from *Eucalyptus* samples and a total of nine isolates were randomly selected for genetic and phenotypic comparisons, including at least one from each of the six *Eucalyptus* species (Table 5.1).

Morphology and ITS sequence comparisons

The five isolates obtained from native Myrtaceae plus the nine from *Eucalyptus* showed identical colony and conidial morphology to each other and had morphological characteristics that were similar to those described by Smith *et al*. (2001) for *N. eucalyptorum*. Pycnidia were observed after two weeks of incubation on sterile pine needle-WA plates and they produced hyaline, granular, ovoid to slightly clavate conidia of 18-25 µm long by 7-12 µm wide. Ascostromata and other teleomorph structures were not observed.

ITS sequence comparisons showed that the five isolates obtained from native Myrtaceae trees and those isolates obtained from *Eucalyptus* spp. from Uruguay were identical in the 518 bp of the analyzed ITS amplicon. When compared with the ex-type culture of *N. eucalyptorum* (CMW10126), the latter showed a mutation in position 25 of the alignment with a thiamine instead of a cytosine and a deletion in position 488 where the other isolates have a cytosine. However, the isolates obtained in this study showed 100% similarity to isolate CMW10125 that was also included in the *N. eucalyptorum* description by Smith *et al.* (2001).

The alignment contained 20 ingroup taxa including five isolates obtained from native Myrtaceous hosts, nine isolates obtained from *Eucalyptus* spp. in Uruguay, and other Botryosphaeriaceae species closely related to *N. eucalyptorum*. *Botryosphaeria dothidea* was the outgroup taxon. The accession numbers of those sequences obtained in this study as well as those obtained from GenBank are listed in Table 5.1. Out of 518 total characters, 461 were constant, 36 variable characters were parsimony-uninformative and 21 were parsimony informative. Heuristic search analysis of the data resulted in one most parsimonious tree (TL = 60 steps; CI = 0.983; RI = 0.971; HI = 0.017). Identical tree topology was obtained with the neighbor-joining analysis and the tree is shown in Figure 5.1.

Inter simple sequence repeat (ISSR) analysis

The overall amplified PCR fragment size ranged from 250 to 2100 bp. A total of 38 ISSR loci were analyzed and 28 (78.7%) yielded polymorphic band patterns. The resolving power of the primers ranged from 1.091 to 3.818 (Table 5.2). To analyze the genetic diversity of the eleven isolates, including four obtained from native Myrtaceous hosts and seven from *Eucalyptus,* two different analyses were conducted, one considering both groups as a single population and the other considering each group as a distinct population. The genetic diversity found in the 11 analyzed isolates is shown in Table 5.4. Overall results indicate the presence of genetic variability (H = 0.2010; I = 0.3215) with the isolates obtained from native trees showing a higher diversity than those obtained from *Eucalyptus* (H = 0.2664 and 0.1332, respectively). The genetic identity between the isolates from native trees and those from *Eucalyptus* was 0.9827 based on the unbiased estimation described by Nei (1978). The grouping observed in the dendogram constructed by UPGMA indicates that the set of tested isolates from native trees and *Eucalyptus* grouped randomly with no clear association with hosts species (Figure 5.2).

Pathogenicity tests

All of the tested isolates obtained from Myrtaceous hosts were pathogenic on *E. grandis,* with lesion development and necrotic tissue advancing from the inoculated wound within a week of inoculation. No lesions were observed on seedlings inoculated with sterile MEA plugs that served as controls. In addition, statistical analyses indicated that no differences (P>0.05) were found among treatments for stem diameter, indicating that all the seedlings were of similar size at the inoculation time. However, treatments differed in lesion length measured one week after inoculation and also when evaluated two weeks later (i.e. three weeks after inoculation). With the exception of isolate UY1070 that was obtained from *E. maidenii*, all the isolates resulted in lesion length significantly different from the control treatment (P<0.05; Figure 5.3). Also significant differences (P>0.05) in aggressiveness were observed among certain isolates. *Neofusicoccum eucalyptorum* was re-isolated from all the inoculated stems selected for re-isolations**.**

DISCUSSION

This study presents the first report of the *Eucalyptus* pathogen, *N. eucalyptorum,* infecting hosts outside of the genus *Eucalyptus*. These findings raise concerns about previous assumptions that the pathogen had a narrow host range and was highly specialized in

Eucalyptus (Slippers and Wingfield, 2007). Pathogenicity tests indicate that all isolates obtained from native Myrtaceae were able to infect and produce stem cankers on *E. grandis*. Additionally, the preliminary assessment of isolate population structure showed the presence of genetic and phenotypic variation among collected isolates.

The fact that *N. eucalyptorum* was found occurring on three different species, namely *B. salicifolius, Myrc. glaucescens* and *Myrr. atropurpureum* var. *octandrum,* provides evidence of a remarkably wider host range than previously thought (Slippers and Wingfield, 2007). *Neofusicoccum eucalyptorum* was previously found occurring on several *Eucalyptus* species in Australia, Chile, South Africa and Uruguay (Ahumada, 2003; Alonso, 2004; Burgess *et al*., 2006; Smith *et al*., 2001), but not on non-*Eucalyptus* hosts. These results strongly suggest that further investigations on the biology, ecology and epidemiology of this fungus are warranted and researchers should be alerted to the possibility of this pathogen affecting other non-*Eucalyptus* hosts in other countries.

Although this fungus was found on three distinct host species, the small number of trees from which *N. eucalyptorum* was isolated (i.e. five trees) suggest that it is still not extensively distributed in trees native to Uruguay. However, the occurrence on native trees in four different provinces of Uruguay is alarming and indicates that interactions among *Eucalyptu*s, native trees and this pathogen are likely occurring countrywide. Continued investigations are needed to monitor disease progression of this pathogen and to obtain a better understanding of the importance that this disease will have on native trees in Uruguay.

The host specialization previously observed for *N. eucalyptorum,* on only *Eucalyptus,* and its abundance and wide distribution in Eastern Australia (Smith *et al*., 2001; Slippers *et al*., 2004b; Burgess *et al*., 2006) suggest that this pathogen likely originated in Australia and that it was introduced with *Eucalyptus* germplasm to Uruguay where it moved to native Myrtaceae. Historically, anthropogenic pathogen introduction has been considered the major driver of devastating host jump experiences (Slippers *et al.,* 2005; Woolhouse *et al*., 2005). Although the inoculum pressure may not be a determinant for some species (Ficetola *et al*., 2008), it is of general consensus that high propagule pressure along with geographical proximity are mostly responsible for the appearance of new host-parasite combinations (Altizer *et al*., 2003; Lockwood *et al*., 2005). Therefore, species growing adjacent to infected plants are exposed to inoculum that increases the probabilities of eventual infections. In Uruguay, *Eucalyptus* plantations are geographically located close to native Myrtaceae trees, and *N. eucalyptorum* has been very commonly found on *Eucalyptus* plantations all over the main planted areas (see Chapter 6 of this Thesis). This suggests that both factors, high inoculum pressure and geographic proximity, may be responsible for the occurrence of *N. eucalyptorum* on native Myrtaceous hosts.

Our results indicate that the ITS region of this fungal species is highly conserved as no polymorphism was observed among isolates collected in this study. To assess the genetic diversity present in the isolates obtained, we first employed the simple sequence repeat (SSR) markers developed by Slippers *et al.* (2004a). However, this approach was discarded because only two pairs of SSR primers worked properly with the set of isolates tested (data not shown). Therefore, the use of ISSR markers was tested and results obtained in this study indicate that ISSR markers were very useful in detecting genetic variability among isolates.

The results based on the 38 analyzed loci showed genetic variability among isolates and the UPGMA indicates no clear association with hosts species because isolates obtained from native Myrtaceae hosts grouped with other isolates obtained from *Eucalyptus.* The higher genetic variability observed among isolates obtained from native trees when compared with those from *Eucalyptus* should be considered with caution since the limited number of isolates included into this study might not represent the population present in either *Eucalyptus* or native trees. Nevertheless, results indicate that *N. eucalyptorum* is not clonal in Uruguay and genetic variation is present both in isolates obtained from native trees and those from introduced hosts. Additional investigation using a larger number of isolates from both groups of hosts will provide better genetic characterization and help to elucidate the putative gene flow among populations.

The pathogenicity tests confirmed the ability of *N. eucalyptorum* to infect *E. grandis* and cause stem cankers. Smith *et al.* (2001) reported pathogenicity of this fungus by inoculating five isolates obtained from *E. grandis* and *E. nitens* onto a clone of *E. grandis* (ZG14), although they found no significant differences in lesion length among isolates. In our study, lesion lengths were different and variability among isolates in aggressiveness may exist. Additionally, isolates obtained from native hosts were not only pathogenic to the clone of *E. grandis* tested, but also produced the largest lesions. The pathogenicity observed on *E. grandis* for those isolates obtained from native trees indicates that this pathogen has the ability to move from one host species to another. Results observed one week after the inoculation on clonal seedlings were consistent with those observed two weeks later (Figure 5.3). This provides the possibility of having a precise phenotypic characterization of isolate aggressiveness in just one week post inoculation. Furthermore, the coefficient of variation observed was 14.1% and 13.5% respectively (data not shown). Therefore, the use of clonal 4-month-old seedlings and the short period of time needed to have a consistent reaction (1 week) make this method appropriate for a quick phenotypic characterization of *N. eucalyptorum* isolates.

Several studies have provided evidence that introduced *Eucalyptus* species and native Myrtaceae trees can share pathogens (Coutinho *et al.*, 1998; Pavlic *et al*., 2007). Additionally, Burgess *et al.* (2006) demonstrated that there is no restriction to the movement of *N. australe* between *E. globulus* plantations and native forest in Australia. In Uruguay, recent studies have confirmed this relationship for *Puccinia psidii* (see Chapter 1 of this Thesis) and *Quambalaria eucalypti* (see Chapter 2 of this Thesis). Our study adds *N. eucalyptorum* to the list and raises additional concerns regarding this pathogen not only in Uruguay but in other regions where *Eucalyptus* has been introduced. The negative impact of host jump events in plant pathology has been well documented and many examples have been repeatedly cited in the literature (Anderson *et al*., 2004; Desprez-Loustau *et al*., 2007; Slippers *et al.*, 2005; Woolhouse *et al.*, 2005). Host jumps have occurred in both directions, from native hosts to introduced plant species and vice versa (Coutinho *et al.*, 1998; Milgroom *et al.*, 1996) and biotic exchanges between both hosts are expected to increase as the planted area and age of plantations increase (Strauss, 2001). Further investigation is needed to have a better understanding of the economical and ecological impact of *N. eucalyptorum* attack on both native and introduced Myrtaceae.

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Table 5.1: List of *Neofusicoccum eucalyptorum* isolates and related species included in this study. Isolates obtained from native Myrtaceae trees are shown in bold.

Culture ID	Species	Host	GenBank Accession number
UY77	*Neofusicoccum eucalyptorum*	*Eucalyptus grandis*	EU860370
UY185	*N. eucalyptorum*	*E. maidenii*	EU860371
UY336	***N. eucalyptorum***	***Myrceugenia glaucescens***	**EU860372**
UY394	*N. eucalyptorum*	*E. dunnii*	EU080919
UY587	*N. eucalyptorum*	*E. tereticornis*	EU080921
UY966	***N. eucalyptorum***	***Blepharocalyx salicifolius***	**EU860373**
UY1070	*N. eucalyptorum*	*E. maidenii*	EU080929
UY1074	*N. eucalyptorum*	*E. grandis*	EU860374
UY1149	*N. eucalyptorum*	*E. dunnii*	EU860375
UY1177	***N. eucalyptorum***	***Blepharocalyx salicifolius***	**EU860376**
UY1190	*N. eucalyptorum*	*E. globulus*	EU080930
UY1233	*N. eucalyptorum*	*E. viminalis*	EU080932
UY1298	***N. eucalyptorum***	***Myrrhinium atropurpureum var. octandrum***	**EU080934**
UY1314	***N. eucalyptorum***	***Myrrhinium atropurpureum var. octandrum***	**EU860377**
CMW10125	*N. eucalyptorum*	*E. grandis*	AF283686
CMW10126 [T]	*N. eucalyptorum*	*E. grandis*	AF283687
CBS115679	*N. eucalypticola*	*E. grandis*	AY615141
CBS115767	*N. eucalypticola*	*E. rossii*	AY615143
CBS118531	*N. mangiferae*	*Mangifera indica*	AY615185
CMW13998	*N. mangiferae*	*Syzygium cordatum*	DQ316081
CBS115476 [T]	*Botryosphaeria dothidea*	*Prunus* sp.	AY236949

[T]: ex-type cultures, UY = isolates obtained in this study and in bold those isolates obtained from native Myrtaceae hosts.

Table 5.2: List of primers used for ISSR amplification, total number of analyzed loci, number and percentage of polymorphic loci, and resolving power.

Primer*	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci	Amplified fragment size range (bp)	Resolving power
5' DDB$(CCA)_5$	10	10	100	700-1800	3.818
5' HVH$(GTG)_5$	9	4	44.4	680-1450	1.091
5' $(CAG)_5$	10	7	70	570-2100	2.182
5' BDB$(ACA)_5$	9	7	77.8	250-1800	2.727

(*) Single letters abbreviations for mixed bases positions D= A, G or T not C; B= C, G or T not A; H= A, C or T not G; V= A, C or G not T.

Table 5.3: Isolates of *Neofusicoccum eucalyptorum* obtained from native Myrtaceae trees in Uruguay.

Isolate ID	Host	Isolated from	Location
UY336	*Myrceugenia glaucescens*	Stem canker	Rio Negro (32º 53' S; 57º 59' W)
UY966	*Blepharocalyx salicifolius*	Healthy leaf	Durazno (33º 19' S; 56º 17' W)
UY1177	*Blepharocalyx salicifolius*	Healthy twig	Lavalleja (34º 11' S; 55º 16' W)
UY1298	*Myrrhinium atropurpureum var. octandrum*	Healthy petiole	Maldonado (34º 17' S; 54º 41' W)
UY1314	*Myrrhinium atropurpureum var. octandrum*	Healthy petiole	Maldonado (34º 20' S; 54º 35' W)

Table 5.4: Genetic diversity of *N. eucalyptorum* isolates obtained from native Myrtaceae hosts and *Eucalyptus*.

Population	Number of samples	Number of polymorphic loci	Percentage of polymorphic loci	*H**	*I**
Native	4	25	65.8	0.2664	0.3906
Eucalyptus	7	16	42.1	0.1332	0.2073
Total	11	28	73.7	0.2010	0.3215

* *H*: Nei's genetic diversity; *I:* Shannon's information index

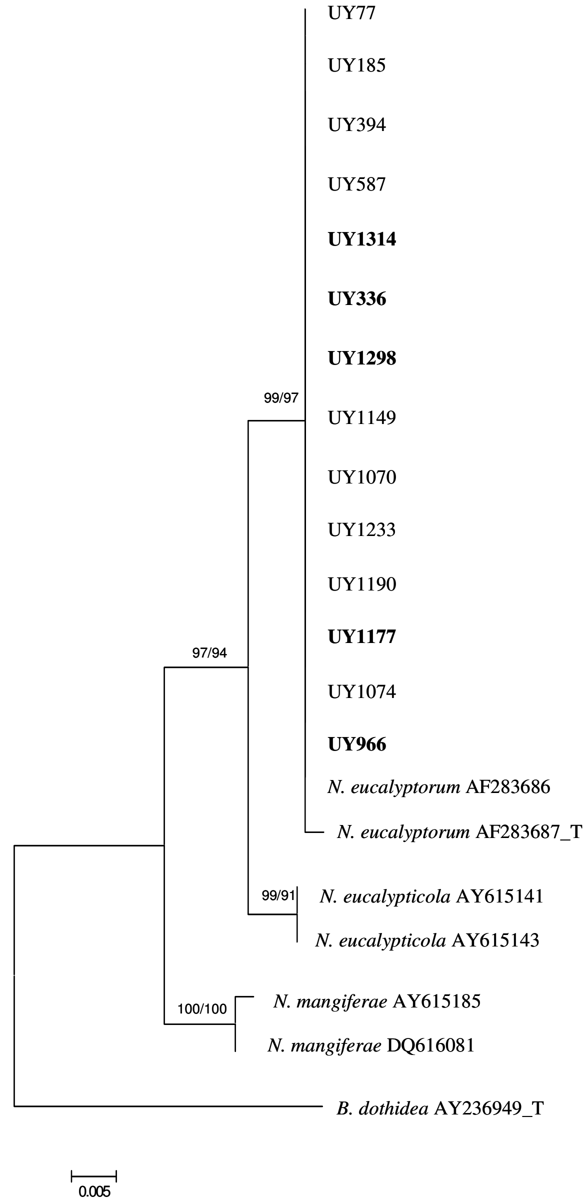

Figure 5.1: Distance tree based on the neighbor-joining analysis of the ITS region using the TrN+I model. Bootstrap values of 1,000 replications for neighbor-joining and maximum parsimony analyses are shown at the nodes, respectively. The tree was rooted with *Botryosphaeria dothidea*. Sequences obtained in this study are indicated with a prefix "UY", isolates obtained from native Myrtaceae are in bold and ex-type cultures are labeled with a "T" at the end. Scale bar indicates 0.005 substitutions per site.

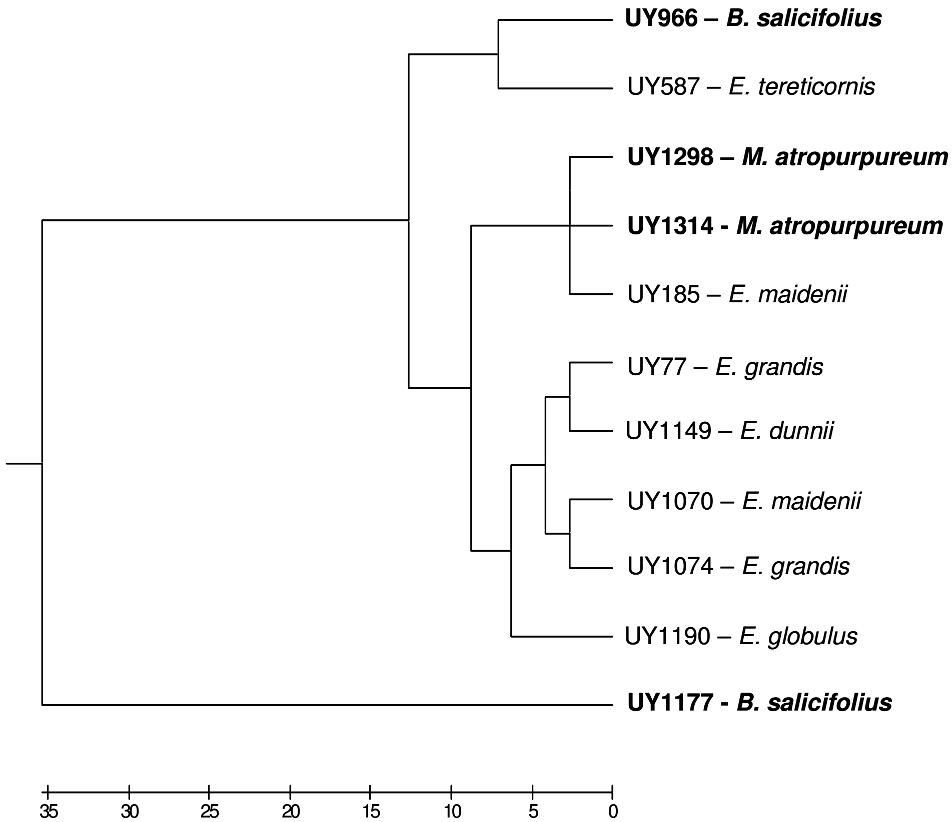

Figure 5.2: Dendogram of the *N. eucalyptorum* isolates obtained from native Myrtaceae (in bold) and *Eucalyptus* in Uruguay constructed by unweighted pair group with arithmetic average based on the 38 loci obtained with four ISSR primers.

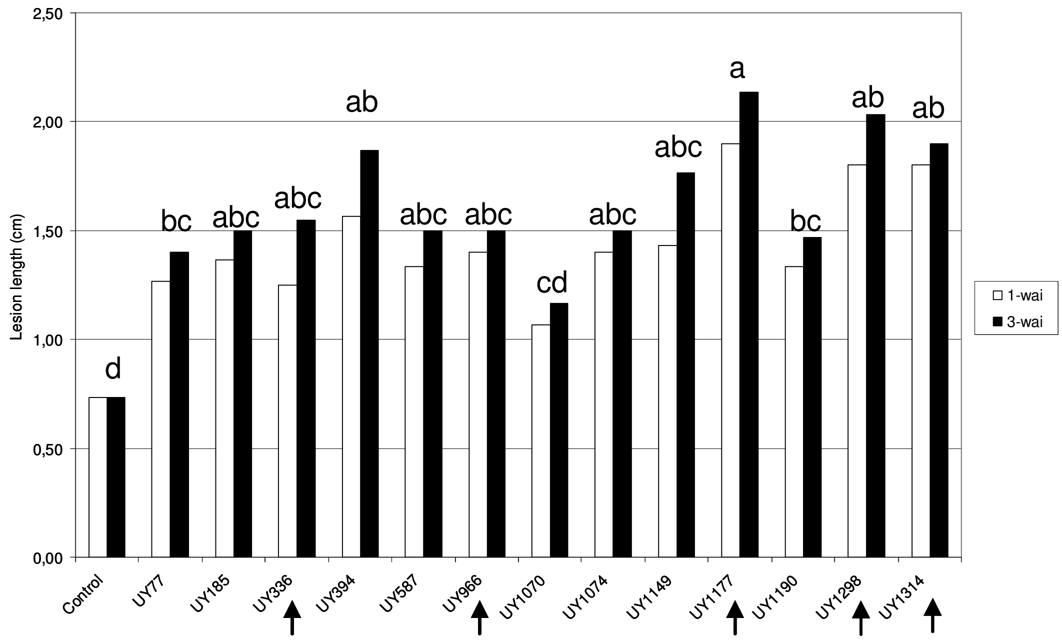

Figure 5.3: Mean lesion length (cm) of three replicates for each *N. eucalyptorum* isolate and the control one and three weeks after inoculation, respectively. Letters indicate mean separation based on Tukey's test ($P<0.05$) shown only for lesion length at 3-wai. Isolates obtained from native Myrtaceous hosts are indicated with an arrow

Chapter 6: Endophytic and canker-associated Botryosphaeriaceae occurring on non-native *Eucalyptus* and native Myrtaceae trees in Uruguay

ABSTRACT

Species of the Botryosphaeriaceae are important pathogens causing cankers and die-back on many woody plants. In Uruguay, *Neofusicoccum eucalyptorum*, *N. ribis* and *B. dothidea* have previously been associated with stem cankers on plantation grown *Eucalyptus globulus*. However, very little is known about the occurrence and species diversity of Botryosphaeriaceae in native Myrtaceae forests or what their relationship is to those species infecting *Eucalyptus* plantations. The objectives of this research were to identify the Botryosphaeriaceae species present as endophytes or associated with cankers in both introduced and native tree hosts in Uruguay, and to test the pathogenicity of selected isolates obtained from native trees on *Eucalyptus*. Symptomatic and asymptomatic material was collected countrywide from *Eucalyptus* plantations and native Myrtaceae trees. Monosporic cultures were identified based on conidial morphology and comparisons of DNA sequences of the ITS, EF1-α, and RPB2 regions. Six Botryosphaeriaceae species were identified. *Botryosphaeria dothidea, N. eucalyptorum* and *N. parvum-N. ribis* complex were isolated from both introduced *Eucalyptus* and native Myrtaceae trees whereas *Lasiodiplodia pseudotheobromae* was found only on *Myrcianthes pungens. Diplodia* sp.1 and *Dothiorella* sp.1 are novel species found only on native Myrtaceous hosts. Pathogenicity tests indicate that isolates obtained from native trees and identified as *L. pseudotheobromae*, *N. eucalyptorum* and *N. parvum-N.ribis* complex are pathogenic to *E. grandis*. *Lasiodiplodia pseudotheobromae* has not been found on *Eucalyptus* in Uruguay and represent a serious threat to this host. The results emphasize the importance of considering native hosts for early detection of potential threats and when assessing the population structure of known *Eucalyptus* pathogens and potentially new pathogens that could affect *Eucalyptus* plantations.

INTRODUCTION

The Botryosphaeriaceae is a very diverse group that includes endophytes and plant pathogens of trees. It is well known that certain endophytic fungi may become pathogenic when trees become stressed (Old *et al.*, 1990; Pusey, 1989; Wene and Schoeneweiss, 1980). Diseases caused by Botryosphaeriaceae are almost exclusively associated with some type of stress and drought stress is one of the most commonly cited factors associated with these fungi (Slippers and Wingfield, 2007). Botryosphaeriaceae have been reported to cause serious diseases on *Eucalyptus* worldwide. Stem cankers and die-back of *Eucalyptus* spp. have for a long time been associated with *Botryosphaeria dothidea* (Barnard *et al.*, 1987; Old and Davison, 2000; Smith *et al.*, 1994; Yuan and Mohammed, 1999), but in recent years a number of other species of the Botryosphaeriaceae have also been associated with diseases on this host (Slippers *et al*., 2004a; Slippers *et al.*, 2007). Severe *Botryosphaeria* cankers have also been observed on *Eucalyptus* in Uruguay causing growth losses, tree mortality, and coppice failure (Balmelli and Resquin, 2005). Additionally, due to the explosive increase in the area planted with introduced species, the biotic interaction between introduced *Eucalyptus* and native Myrtaceae trees has provided an intriguing situation to study.

Uruguay has a rich diversity of native Myrtaceae trees with a total of 35 species reported by Brussa and Grela (2007). It is of general concern that biotic exchange of pathogens may result between introduced *Eucalyptus* and native trees, which could result in negative economical impact as well as an ecological disturbance or catastrophe. Endophytic *B. dothidea*, *Neofusicoccum eucalyptorum* (=’*Botryosphaeria’ eucalyptorum*) and *N. ribis* (=’*B’. ribis*) were found in some *Eucalyptus* spp. (Alonso, 2004; Bettucci and Alonso, 1997), while *Myrceugenia glaucescens* is the only native Myrtaceae host where a species of Botryosphaeriaceae, *B. dothidea,* has been found (Bettucci *et al.,* 2004).

Eucalyptus spp. are exotic in Uruguay and pathogens affecting these trees could have been introduced as well. However, native trees could also serve as an important source of fungi pathogenic to *Eucalyptus*, as is being found in other parts of the world (Wingfield, 2003). Burgess *et al.* (2006) have demonstrated that there is no restriction to the movement of *N. australe* between native forests and plantations in Australia and it has been demonstrated repeatedly that Myrtaceae are hosts of many pathogens that can infect *Eucalyptus* spp. (Coutinho *et al.,* 1998; Seixas *et al.*, 2004; Wingfield *et al.*, 2001; Wingfield, 2003). Since little is known about the Botryosphaeriaceae species occurring on introduced and native Myrtaceae hosts in Uruguay, the aim of this research was to obtain a more comprehensive understanding of the species that are endophytes and those that are associated with cankers, and to test the pathogenicity of the isolates obtained from native trees on *Eucalyptus*.

MATERIALS AND METHODS

Sampling and fungal isolates

Several surveys were conducted throughout Uruguay with the aim of isolating and identifying fungi present on native Myrtaceae and non-native *Eucalyptus* species. Symptomatic and asymptomatic material was collected from *Eucalyptus* plantations and nearby native forest (less than 500 m between them). Endophytic microorganisms were isolated from asymptomatic fresh material. Leaf, petiole and twig sections were sequentially surface-disinfested in 70% ethyl alcohol for 1 min, immersed in 0.4% sodium hypochlorite for 2 min, then rinsed twice in sterile distilled water and blotted dry on sterile filter paper. Disinfested plant tissue was placed on 2% malt extract agar (MEA) (2% malt extract, 1.5% agar; Oxoid, Basingstoke, England). Plates were incubated at room temperature (~20ºC) for one week. Colonies resembling Botryosphaeriaceae were selected for this study, and maintained on 2% MEA at 8ºC. To verify the efficacy of the surface disinfested and to assure the growth of only endophytic microorganisms, imprints of sample surfaces were made on MEA plates and observed for one week to confirm that fungi did not grow.

Isolation from cankers was done from wood tissue at the advancing zone of the lesion, which was surface-disinfested in 70% ethyl alcohol for 30 sec, rinsed twice in sterile distilled water and blotted dry on sterile filter paper. Disinfested tissue was placed on 2% MEA and incubated at room temperature (~20ºC) for one week. Colonies resembling Botryosphaeriaceae were subcultured to a fresh 2% MEA plate for further investigation.

Morphological characterization

To stimulate isolates to produce fruiting structures (pycnidia) and conidia, they were grown on 1.5% water agar (WA) (Sigma Chemicals, St. Louis, MO) with sterilized pine needles placed onto the medium surface. Plates were incubated at 22ºC under continuous black light until pycnidia were observed on the pine needles (approx. 3 weeks after plating). Monoconidial cultures were obtained by plating a conidial suspension taken from two pycnidia, suspended in 300 µl of sterile water on WA. Germinating conidia were lifted from the agar plates and transferred to fresh 2% MEA.

For morphological characterization, pycnidia and conidia produced on pine needles were mounted on microscope slides, and examined under a standard light microscope Motic DMBA200-B (Motic®, British Columbia, Canada). Isolates were grouped by conidial morphology and host, and at least two specimens per group were further analyzed using molecular techniques.

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DNA extraction, PCR, sequencing and phylogenetic analysis

For DNA extraction, the 49 isolates listed in Table 6.1 were grown in 2% malt extract agar (MEA) at room temperature for 10 days. Mycelium was scrapped directly from the colonies on the plates and transferred to microfuge (1.5 ml) with 1-mm glass beads and extraction buffer of the Qiagen Plant DNeasy Mini Kit (Qiagen Inc., Valencia, CA). These were vigorously shaken using a vortex mixer and placed in a water bath at 60ºC for 1 hr. DNA extraction was performed using the Qiagen Plant DNeasy Mini Kit following manufacturer's instructions.

The phylogenetic analyses were performed in three steps. First, the internal transcribed spacer region of the ribosomal DNA operon (ITS) was amplified for all isolates and compared with Botryosphaeriaceae species found on *Eucalyptus* spp. worldwide. The second step was to fully resolve identification of those isolates grouped in the *Neofusicoccum parvum-N. ribis* complex via multigene analysis of the regions ITS, translation elongation factor 1-alpha (EF1-α) and part of the RNA polymerase II subunit (RPB2). Lastly, to achieve a better resolution of the *Diplodia* sp. 1 clade and the *Dothiorella* sp. 1 isolate a combined analysis of the rDNA ITS region along with part of the EF1-α region was performed.

The entire ITS region was amplified for all isolates using primers ITS1 (5' TTC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al.*, 1990). Polymerase Chain Reactions (PCR) amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) with the following parameters: 5 min at 94ºC; 1 min at 94ºC; 1 min at 50ºC; 1 min at 72ºC; cycle to step 2, 35 times; 5 min at 72ºC; hold at 10ºC. A 25-µl reaction mixture of 1.0 µl of 0.05% casein, 12.5 µl of Amplitaq Gold PCR Master-Mix (Applied Biosystems, Foster City, CA), 1.0 µl of 10 mM ITS1, 1.0 µl of 10 mM ITS4, 8.5 µl of ddH_2O and 1.0 µl of DNA template was used.

PCR products were stained with SYBR Green nucleic acid dye (MBL International, Woburn, MA) and visualized on 1.5% agarose gel under UV light. Amplicons were then purified and prepared for sequencing using ExoSAP-IT PCR clean-up kit (USB Corp., Cleveland, OH) following manufacturer's instructions. The same primers were used for sequencing reactions performed with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) 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). ITS sequences were subjected to BLAST searches in NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, verified 30 June 2008), and sequences of the closest matching species were download. Sequences of ex-type cultures were preferred when available, along with sequences of all the Botryosphaeriaceae species previously reported on Myrtaceous hosts. 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 30 June 2008) (Katoh et al., 2005).

Phylogenetic analysis was performed using PAUP Version 4.0b10 (Swofford, 2002) for maximum parsimony analysis, and Mr. Bayes v3.1.2 (Ronquist and Huelsenbeck, 2003) for Bayesian analysis. Maximum parsimony analysis was performed using the heuristic search option with simple taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. Gaps were treated as missing data and all characters were treated as unordered and of equal weight. Support for the nodes of the shortest trees was determined by analysis of 1,000 bootstrap replicates (Hillis and Bull, 1993). Tree length (TL), consistency index (CI), retention index (RI), and homoplasy index (HI) were calculated.

The best nucleotide substitution model for the Bayesian analysis was selected using MrModeltest v2.2 (Nylander, 2004) from which the SYM+I+G model was selected using Akaike Information Criterion (AIC). Four MCMC chains starting from a random tree topology were run over 10 million generations. Trees were sampled every 100th generation and 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 99,801 trees was computed from a total of 199,602 sampled trees.

To have a better resolution of the *Neofusicoccum parvum-N.ribis* complex, a combined analysis was also conducted based on ITS region, EF1-α and part of the RNA polymerase II subunit (RPB2). The EF1-α was amplified using primers EF-AF (5′ CATCGAGAAGTTCGAGAAGG 3’) and EF-BR (5′ CRATGGTGATACCRCGCTC 3’) (Sakalidis, 2004) whereas the RPB2 region was amplified using primers RPB2bot6F (5′ GGTAGCGACGTCACTCCC 3’) and RPB2bot7R (5′ GGATGGATCTCGCAATGCG 3’) (Sakalidis, 2004). PCRs were performed in a 25-µl reaction mixture of 0.5 µl of *Taq* DNA polymerase (Roche Molecular Biochemicals, Almeda, CA), 1X buffer and $MgCl_2$ mixture (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM of each dNTP, 0.15 mM of each primer and made up to a final volume of 25-µl with water. PCR amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) with the following parameters: 94 °C for 2 min initial denaturation; 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and 72 °C for 7 min final extension; hold at 10ºC. Forward and reverse sequences from each locus were obtained with the same primers and assembled using ChromasPro software version 1.33. Sequences obtained in this study including sequences of the ex-type cultures of *N. parvum* and *N. ribis* and *Dothiorella sarmentorum* as outgroup taxon were aligned online using the E-INS-i strategy in MAFFT version 6. Alignments of each of the three loci were aligned separately and then combined in a single data set. Combined dataset was examined by using Partition Homogeneity Test (Farris *et al*., 1995; Huelsenbeck *et al.*, 1996) in PAUP to determine statistical congruence between data sets in order to proceed with the combined analysis.

Neighbor-joining analysis was performed using the model TrN+G selected from the AIC by Modeltest version 3.7 (Posada and Crandall, 1998). 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. Maximum parsimony analysis of the combined data set was performed using the heuristic search option with simple taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. Support for the nodes of the shortest trees was determined by analysis of 1,000 bootstrap replicates. Tree length (TL), consistency index (CI), retention index (RI), and homoplasy index (HI) were calculated.

As mentioned above, the EF1-α region was amplified to resolve the identity of the *Diplodia* sp.1 clade and *Dothiorella* sp.1 isolate. Combined analysis of ITS and EF1-α nucleotide sequences have proved to be useful for distinction of close related Botryosphaeriaceae (e.g. Luque *et al*., 2005; Phillips *et al.*, 2005, Slippers *et al.,* 2004a). PCRs were performed as described above. Sequences were obtained in both directions and assembled using ChromasPro and aligned with sequences of the closest related species obtained from GenBank; sequences of ex-type cultures were utilized when available. Multiple sequence alignments were also made online using the E-INS-i strategy in MAFFT version 6. ITS and EF1-α sequence datasets were examined for congruence by using the Partition Homogeneity Test in PAUP.

Multigene analyses was based on the ITS and EF1-α of selected isolates. Thus isolates UY107, UY671, UY788 and UY1263 from the *Diplodia* sp.1 clade plus isolate UY672 were subjected to combined analyses using neighbor-joining and maximum parsimony. The original alignment was populated with corresponding sequence data of all the *Diplodia, Lasiodiplodia* and *Dothiorella* species available in GenBank. Phylogenetic analysis was performed using PAUP Version 4.0b10. Best model for neighbor-joining analysis was determined in Modeltest version 3.7 where the TrN+G model was selected from the AIC. 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 6.1). In addition, corresponding alignments were deposited in TreeBASE (SN3975, for alignments presented in Figures 6.1, 6.2 and 6.3).

Pathogenicity tests

Selected isolates representing the six species of Botryosphaeriaceae obtained from native trees were tested for pathogenicity on *Eucalyptus*. Results obtained with *N. eucalyptorum* inoculations were presented in Chapter 5 of this thesis and not included in this study. Inoculations were performed on four month-old *E. grandis* seedlings using an adaptation of the method described by Simeto *et al.* (2007). Briefly, the region of the stem to be wounded was previously surface disinfected with 70% ethyl alcohol. A wound was made on the stem of each seedling at approximately 10 cm above the soil level and between two nodes using a cork borer of 5 mm diameter to remove the bark and expose the cambium. Mycelial plugs from pure cultures grown for a week on 2% MEA at room temperature were taken using the same cork borer size and placed into the wound with the mycelial surface facing the cambium. A piece of sterile cotton soaked in sterile water was attached to the inoculated wound with Ready Por Nº 545 tape (Sagrin S.A., Montevideo, Uruguay) to prevent desiccation of the plug. Each isolate was inoculated into the stems of ten seedlings. Plugs of sterile MEA were inoculated into stems of 10 trees as controls. Inoculated trees were maintained outside under a structure with a plastic roof and open sides with temperature ranging from 15 to 25 ºC. Stem diameter at the site of the inoculation and lesion length were determined and photographed for records a week after inoculation.

To complete Koch's postulates, three inoculated stems per isolate were randomly selected for re-isolation of the inoculated fungus. Thus, pieces of wood from the edges of the lesions were surface-disinfested in 70% ethyl alcohol for 1 min, immersed in 0.4% sodium hypochlorite for 2 min, then rinsed twice in sterile distilled water and blotted dry on sterile filter paper. Disinfested plant tissue was placed on 2% MEA and incubated at room temperature (~20ºC) for one week. Fungal identification was based on colony and conidial morphology.

Data were subjected to analysis of variance (ANOVA) using the Generalized Linear Model procedure (PROC GLM) of SAS (release 9.1; SAS Institute, Inc., Cary, NC). The assumptions used in the ANOVA were tested using PROC UNIVARIATE. When the F test was significant ($P<0.05$) the treatment means were compared using Fisher's least significant differences (LSD) at *P*=0.05. Isolates were grouped by species and comparisons between groups were performed using orthogonal contrasts described by Gomez and Gomez (1984).

RESULTS

Sampling and fungal isolates

A total of nine *Eucalyptus* species and 14 native Myrtaceae species were surveyed countrywide (Table 6.1). One hundred and thirty four isolates resembling Botryosphaeriaceae were obtained from both groups of hosts. Isolates UY37 and UY185 were isolated from dead tissue from *E. grandis* and *E. maidenii* pruning residue, respectively. Specimens UY336, UY1050, UY1065, UY1263, UY1356 and UY1366 were isolated from expanding lesions of stem cankers on *Myrceugenia glaucescens, E. globulus, E. maidenii, Myrciaria tenella, Myrcianthes pungens,* and *Blepharocalyx salicifolius,* respectively. The remaining isolates were obtained from asymptomatic plant material. All isolates produced conidiomata after three weeks of incubation on water agar with pine needles under continuous black light.

Morphology and DNA sequence comparisons

The 134 isolates were placed in six groups based on colony and conidia morphology. A total of 52 representative isolates of each group were further investigated with molecular techniques, including 17 obtained from *Eucalyptus* and 35 from native Myrtaceous hosts (Table 6.1). Phylogenetic analysis showed that the 52 analyzed isolates reside in the Botryosphaeriaceae. ITS sequences from all isolates were then aligned with Botryosphaeriaceae species previously reported for Myrtaceae, including *Eucalyptus*. The alignment contained 100 ingroup taxa and *Guignardia philoprina* as the outgroup taxon. Out of 556 total characters, 292 were constant, 115 variable characters were parsimony-uninformative and 149 were parsimony informative. Heuristic search analysis of the data resulted in one tree (TL = 543 steps; CI = 0.715; RI = 0.949; HI = 0.285). The two analyses, namely maximum parsimony and Bayesian, resulted in trees of similar topology. The phylogenetic tree obtained from the Bayesian analysis is shown in Figure 6.1.

Based on the ITS sequences, six different Botryosphaeriaceae species are represented in the 52 isolates analyzed, in agreement with the grouping obtained by morphological characteristics. Eight of them clustered with *B. dothidea*, ten isolates clustered with *N. eucalyptorum*, 19 isolates clustered within the *N. parvum-N. ribis* complex, one isolate (UY1356) grouped with *Lasiodiplodia pseudotheobromae*, 13 isolates were closely related to

Diplodia seriata (='*B'. obtusa*), but grouped clearly distinct from it (*Diplodia* sp.1), and the remaining isolate formed a distinct branch (*Dothiorella* sp.1) amongst clades representing *Dothiorella* species.

Botryosphaeria dothidea was found endophytically in four different native Myrtaceae species and two *Eucalyptus* species (Table 6.1) and was also associated with a stem canker on *E. maidenii*. Isolates belonging to the *N. parvum-N. ribis* complex were found in five distinct *Eucalyptus* species and eight native Myrtaceae species. These were obtained from asymptomatic plant tissue except isolates UY1050 and UY1366 which were obtained from stem cankers on *E. globulus* and *Blepharocalyx salicifolius*, respectively. *Neofusicoccum eucalyptorum* was found as an endophyte in six different *Eucalyptus* species, namely *E. dunnii, E. globulus, E. grandis, E. maidenii, E. tereticornis* and *E. viminalis.* It was additionally isolated from healthy tissues of two species of Myrtaceae including *Blepharocalyx salicifolius* and *Myrrhinium atropurpureum* var. *octandrum*, and associated with a stem canker in *Myrceugenia glaucescens. Lasiodiplodia pseudotheobromae* was found associated with a stem canker on *Myrcianthes pungens*. In addition, *Diplodia* sp.1 were obtained from Myrtaceous trees, but not found on *Eucalyptus* samples. Most of these isolates were obtained from healthy tissue with the exception of isolate UY1263 which was isolated from a stem canker observed on *Myrciaria tenella*. Finally, a *Dothiorella* sp.1 was found as an endophyte in *Hexachlamis edulis*, a native Myrtaceous tree.

Multigene genealogy resulted in a better resolution of the group *N. parvum-N. ribis*. The alignment combining the ITS, EF1-α and RPB2 regions contained 12 ingroup taxa and *Dothiorella sarmentorum* was the outgroup taxon. The 518 characters of the ITS region, 317 characters of the EF1-α and the 542 characters of the RPB2 region resulted in a total of 1377 characters, of which 1157 were constant, 178 variable characters were parsimony-uniformative, and 42 characters were parsimony informative. Heuristic search analysis resulted in a most parsimony tree (TL = 230, CI = 0.991, RI = 0.979, HI = 0.009) of identical topology with the one obtained in the neighbor-joining analysis (Figure 6.2). Results indicate that the isolates obtained in this study form two distinct groups, also distinct from *N. parvum* and *N. ribis*. Isolates UY52 and UY231 are more closely related to *N. ribis*, while the rest of the isolates included in the multigene analysis group intermediate to the two species.

The alignment of the combined datasets of the ITS and EF1-α DNA sequences for the resolution of the putative undescribed *Diplodia* and *Dothiorella* species yielded a total of 874 characters, of which 445 were constant, 31 variable characters were parsimony-uninformative and 398 were parsimony informative. The alignment contained 46 ingroup taxa plus *Botryosphaeria dothidea* as the outgroup taxon and the heuristic search analysis of the data resulted in one tree (TL = 1065 steps; CI = 0.697; RI = 0.911; HI = 0.303) (Figure 6.3). Results confirmed that the group of isolates (*Diplodia* sp.1) obtained from native trees grouped consistently in a strongly supported distinct clade in *Diplodia.* In addition, the combined analyses showed that isolate UY672 group separated from other *Dothiorella* spp. with significant sequence divergence between it and the closest related clade (*Do. iberica*). Micrographs of fruiting structures for these two undescribed species are presented in Figures 6.7 and 6.8.

Pathogenicity tests

Selected isolates representing all the Botryosphaeriaceae species found on Myrtaceae hosts were able to produce lesions within a week after inoculation on stems of *E. grandis* seedling (Figure 6.4). Significant differences in lesion length were observed among isolates of different species, and isolate UY1356 identified as *L. pseudotheobromae* collected from *Myrcianthes pungens* showed the largest lesions (P<0.05; Figure 6.5). Isolates of *Diplodia* sp.1 and *Dothiorella* sp.1 showed lesions not significantly different from the control treatment. Similar results were observed for inoculations with *B. dothidea*.

Isolates were grouped by species and mean lesion length of species were compared using orthogonal contrasts, although only a limited number of isolates were analyzed for some species. Results indicate that *L. pseudotheobromae* was the most aggressive species followed by isolates of the complex *N. parvum-N. ribis* complex whereas *Diplodia* sp.1, *Dothiorella* sp.1 and *B. dothidea* showed no differences (P>0.10) with the control (Figure 6.6). Stem diameter determined 1 week after inoculation ranged between 3-4 mm and showed no significant differences among treatments (data not shown), indicating there was no effect due to seedling size.

DISCUSSION

Our results provide evidence that a diverse group of Botryosphaeriaceae species are occurring on both introduced *Eucalyptus* and native Myrtaceae trees in Uruguay. *Botryosphaeria dothidea, N. eucalyptorum* and the complex *N. parvum-N. ribis* were isolated from both hosts demonstrating biotic exchange between native and introduced Myrtaceae. In contrast, *L. pseudotheobromae* was restricted to one host, *Myrcianthes pungens.* In addition, two novel species of the Botryosphaeriaceae were isolated from Myrtaceae native to Uruguay. *Diplodia* sp.1 was isolated endophytically from six distinct Myrtaceae species and also associated with a stem canker observed on *Myrciaria tenella*. *Dothiorella* sp.1 was obtained from endophytic infection in *Hexachlamis edulis*. Pathogenicity tests revealed the cross pathogenicity (on *Eucalyptus*) of isolates obtained from native trees, among which *N. parvum-ribis* and *L. pseudotheobromae* were highly aggressive, killing a significant area of stem tissue and resulting in large cankers. On the other hand, *Diplodia* sp.1 and *Dothiorella* sp.1 showed no differences from the control ($P>0.10$) and this may indicate that these isolates are not a serious threat to *Eucalyptus.* To our knowledge *L. pseudotheobromae* has not been found occurring on *Eucalyptus* in Uruguay and these results highlight the importance of scouting native forest and assessing cross pathogenicity to find other potential threats to *Eucalyptus*.

Botryosphaeria dothidea was previously reported as an endophyte infecting eucalypts (Bettucci and Alonso, 1997; Smith *et al.*, 1996) and also causing stem cankers on *Eucalyptus* in Uruguay (Balmelli *et al*., 2004) and other countries (Smith *et al.*, 1994). In addition, Bettucci *et al.* (2004) reported the presence of endophytic *B. dothidea* in *Myrceugenia glaucescens*, a Myrtaceous tree native to Uruguay. However, identification of Botryosphaeriaceae species prior to the application of DNA sequence comparisons indicates that reference to *B. dothidea* probably implies a suite of different species and not one fungus. Thus, some of the isolates previously considered to be *B. dothidea* have subsequently been identified as *N. parvum* and *N. ribis* (Slippers *et al*., 2004a). Using a modern taxonomic concept for Botryosphaeriaceae (Crous *et al*., 2006), *B. dothidea* has been rarely isolated from *Eucalyptus* spp. and it has been suggested that this fungus may not be an important pathogen of these trees (Slippers *et al.*, 2004b; Pavlic *et al.*, 2007). Our results confirm the occurrence of *B. dothidea* as an endophyte in *Eucalyptus* and native Myrtaceae hosts, but it was also found associated with stem cankers in *Eucalyptus*. *Botryosphaeria dothidea* was not the most common Botryosphaeriaceae species isolated from *Eucalyptus* samples in the present study. Although a wide host range of this fungus was previously reported, cross pathogenicity between unrelated hosts, involving *Eucalyptus*, has been only recently tested by Pavlic *et al.* (2007) using one isolate. The pathogenicity tests on *Eucalyptus* carried out in the present study with selected isolates from native trees and *Eucalyptus* suggest that the aggressiveness of the isolates tested was limited.

Neofusicoccum eucalyptorum has previously been reported in Uruguay as an endophyte in *E. globulus* and also from bark lesions (Alonso, 2004). This fungus was found in six different *Eucalyptus* species and it is evident that this was the most common species of Botryosphaeriaceae isolated from *Eucalyptus* in this study. Smith *et al.* (2001) analyzed the pathogenicity of several isolates of *N. eucalyptorum,* and concluded that even when isolates of *N. eucalyptorum* were less virulent than those of *B. dothidea*, it was clear that *N.*

eucalyptorum is pathogenic to eucalypts. Interestingly, *N. eucalyptorum* was found for the first time infecting hosts outside of the genus *Eucalyptus*. A deeper examination of this fungus along with cross pathogenicity tests on *Eucalyptus* is presented in Chapter 5 of this thesis.

The finding of isolates in the *N. parvum-N. ribis* complex was not surprising as this group is known to commonly occur on *Eucalyptus* and other hosts including certain Myrtaceae trees worldwide (Barber *et al.*, 2005; Burgess *et al.*, 2005; Gure *et al.,* 2005; Mohali *et al.*, 2007; Pavlic *et al.,* 2007; Slippers *et al*., 2004b). Slippers *et al.* (2004a) used a multiple gene genealogy approach to confirm that *N. parvum* and *N. ribis* represent different species. They also recommend caution when distinguishing between these two species based on morphological or single locus DNA sequence data. Our results based on multigene genealogy analyses indicate that the Uruguayan isolates grouped in-between both species, but were consistently separated from them, although by small margins. This group warrants further investigation, because our results suggest that a recent speciation event may have occurred. Until more information is obtained, however, this group cannot be described as distinct taxon.

Slippers *et al.* (2004b) showed that *N. parvum*, rather than other species of the Botryosphaeriaceae, was associated with disease of *Eucalyptus* in South Africa. In addition, *N. parvum* was reported as an important die-back and stem canker pathogen of *Eucalyptus* in Ethiopia, Republic of Congo, and Uganda (Gezahgne *et al*., 2004; Smith *et al.*, 1994, Roux *et al.*, 2001). *Neofusicoccum ribis* also has a wide host range and it has been found on certain *Eucalyptus* spp. (Barber *et al.*, 2005; Mohali *et al.*, 2007), Myrtaceae species (Pavlic *et al.*, 2007) and other non-Myrtaceous hosts (Denman *et al.*, 2003; Zhou *et al.*, 2001). This fungus was associated with the death of *E. radiata* in Australia (Shearer *et al.*, 1987), and Pavlic *et al.* (2007) concluded that *N. ribis* is the most pathogenic species of Botryosphaeriaceae on *Eucalyptus* clones used in their study. These reports reinforce the need for correct identification, as well as to assess the pathogenicity of Botryosphaeriaceae isolates occurring on eucalypts.

Alonso (2004) reported the presence of *N. ribis* on *E. globulus* based on the morphology and comparisons of sequence data for the ITS region of the rDNA operon, but further analyses are required to confirm this report. Our results indicate that *N. parvum-ribis* is widely present in both *Eucalyptus* and native Myrtaceae. The clear association of this complex with stem cankers in both hosts, together with the pathogenicity observed in inoculation tests, suggests that this group is a major threat to trees in Uruguay.

Interestingly, *L. pseudotheobromae* was found infecting a native Myrtaceous tree. It has been recently shown that *L. pseudotheobromae,* along with *L. parva,* is a cryptic species previously identified as *L. theobromae* (Alves *et al.,* 2008). Because of the recent identification, very little is known regarding the biology and etiology of this fungus, and most likely previous references to *L. theobromae* must be considered with caution because they may actually be referring to *L. pseudotheobromae*. *Lasiodiplodia theobromae* has been referred to as a widely distributed fungus in tropical and subtropical regions and is reported to infect more than 500 plant species (Punithalingam, 1976). This fungus has been associated with shoot blight, die-back, wood discoloration, and stem cankers on a diverse group of hosts (Mohali *et al*., 2005). Although it is considered an opportunistic pathogen, it has been demonstrated to have a devastating effect on stressed plants (Müllen *et al*., 1991). In accordance with the results obtained in our study, Pavlic *et al.* (2007) concluded that *L. theobromae* isolated from *Syzygium cordatum*, a Myrtaceae species native to South Africa, was the most pathogenic Botryosphaeriaceae species to the *Eucalyptus* clone tested in that study. These results raise concern about this species because Mohali *et al.* (2005) demonstrated that there was no evidence of host specificity for this fungus and a high gene flow was found between populations occurring on different hosts. *Lasiodiplodia pseudotheobromae* was found in a single sample occurring in association with a stem canker on *Myrcianthes pungens*. Inoculation tests conducted in this study suggest that it could represent a serious threat to *Eucalyptus* plantations. Future surveys are needed to monitor the movement of this fungus and determine how prevalent it is in both native forest and plantations.

Diplodia sp.1 seems to be widely distributed in native Myrtaceae forests in Uruguay. However, its occurrence in *Eucalyptus* plantations was not detected in this study. The weak reactions observed after inoculation on *E. grandis* suggest that this species does not represent a serious threat to *E. grandis*. Future studies should focus on using other *E. grandis* seed sources and other *Eucalyptus* spp. to determine its potential threat to plantation forestry in the country.

The finding of *Dothiorella* sp.1 on the native *Hexachlamis edulis* is intriguing. After the examination of a very large number of Myrtaceae samples, this species was found on a single sample suggesting that its distribution on these various hosts and regions is restricted. This may indicate that this species was only very recently introduced, or if native to Uruguay, it may have jumped from another non-Myrtaceae host to *Hexachlamis edulis*. Pathogenicity results suggest that it does not represent a major threat to *Eucalyptus*, although further investigation is needed to fully assess the importance of this fungus to *Eucalyptus* plantations.

Although to date no extensive diseases outbreaks caused by Botryosphaeriaceae have been observed in Uruguay, the situation could change. The explosive expansion of *Eucalyptus* plantations and the association of Botryosphaeriaceae with extreme weather conditions, primarily drought, along with the additional pressure and stresses from other pathogens, raise concerns about the threat of Botryosphaeriaceae-related diseases worldwide (Desprez-Loustau *et al*., 2006; Slippers and Wingfield, 2007). Results presented here lay the foundation of monitoring the development of such diseases on native and non-native Myrtaceae in Uruguay in the future. In particular it is important to study the current gene flow between both hosts of *B. dothidea, N. eucalyptorum* and the *N. parvum-N. ribis* complex to better assist breeding programs aimed at elevating resistance to diseases. In addition, the finding of the more aggressive species, *L. pseudotheobromae*, on a native host show the relevance of scouting native forest trees for early detection of potential threats to *Eucalyptus* plantations.

ACKNOWLEDGMENTS

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Table 6.1: List of isolates used in this study.

Culture ID*	Species	Host	GenBank accession no.		
			ITS	EF	RPB2
UY9	*Botryosphaeria dothidea*	*Blepharocalyx salicifolius*	EU080907	-	-
UY16	*Neofusicoccum parvum-N. ribis* complex	*B. salicifolius*	EU080908	EU863160	EU863177
UY37	*N. parvum-N. ribis* complex	*Eucalyptus grandis*	EU080910	EU863161	EU863176
UY48	*Botryosphaeria dothidea*	*Eucalyptus grandis*	EU080911	-	-
UY52	*N. parvum-N. ribis* complex	*Eucalyptus grandis*	EU080912	EU863162	EU863175
UY99	*N. parvum-N. ribis* complex	*Eucalyptus grandis*	EU860378	-	-
UY107	*Diplodia* sp.1	*Myrcianthes cisplatensis*	EU080914	EU863178	-
UY118	*N. parvum-N. ribis* complex	*Eugenia uruguayensis*	EU080915	EU863163	EU863174
UY119	*B. dothidea*	*E. uruguayensis*	EU080916	-	-
UY129	*N. parvum-N. ribis* complex	*Myrrhinium atropurpureum* var. *octandrum*	EU860379	-	-
UY180	*Diplodia* sp.1	*Acca sellowiana*	EU860380	-	-
UY185	*N. eucalyptorum*	*Eucalyptus maidenii*	EU860371	-	-
UY193	*N. parvum-N. ribis* complex	*Psidium pubifolium*	EU860381	-	-
UY231	*N. parvum-N. ribis* complex	*Blepharocalyx salicifolius*	EU080917	EU863164	EU863173
UY336	*N. eucalyptorum*	*Myrceugenia glaucescens*	EU860372	-	-
UY518	*B. dothidea*	*Myrceugenia glaucescens*	EU860382	-	-
UY543	*N. parvum-N. ribis* complex	*Eugenia repanda*	EU080920	-	-
UY587	*N. eucalyptorum*	*Eucalyptus tereticornis*	EU080921	-	-
UY671	*Diplodia* sp.1	*Hexachlamis edulis*	EU080922	EU863179	-
UY672	*Dothiorella* sp.1	*Hexachlamis edulis*	EU080923	EU863180	-
UY693	*Diplodia* sp.1	*Eugenia uniflora*	EU080924	-	-
UY719	*B. dothidea*	*Myrrhinium atropurpureum* var. *octandrum*	EU080925	-	-
UY754	*N. parvum-N. ribis* complex	*Eucalyptus ficifolia*	EU080926	-	-
UY788	*Diplodia* sp.1	*Blepharocalyx salicifolius*	EU080927	EU863181	-

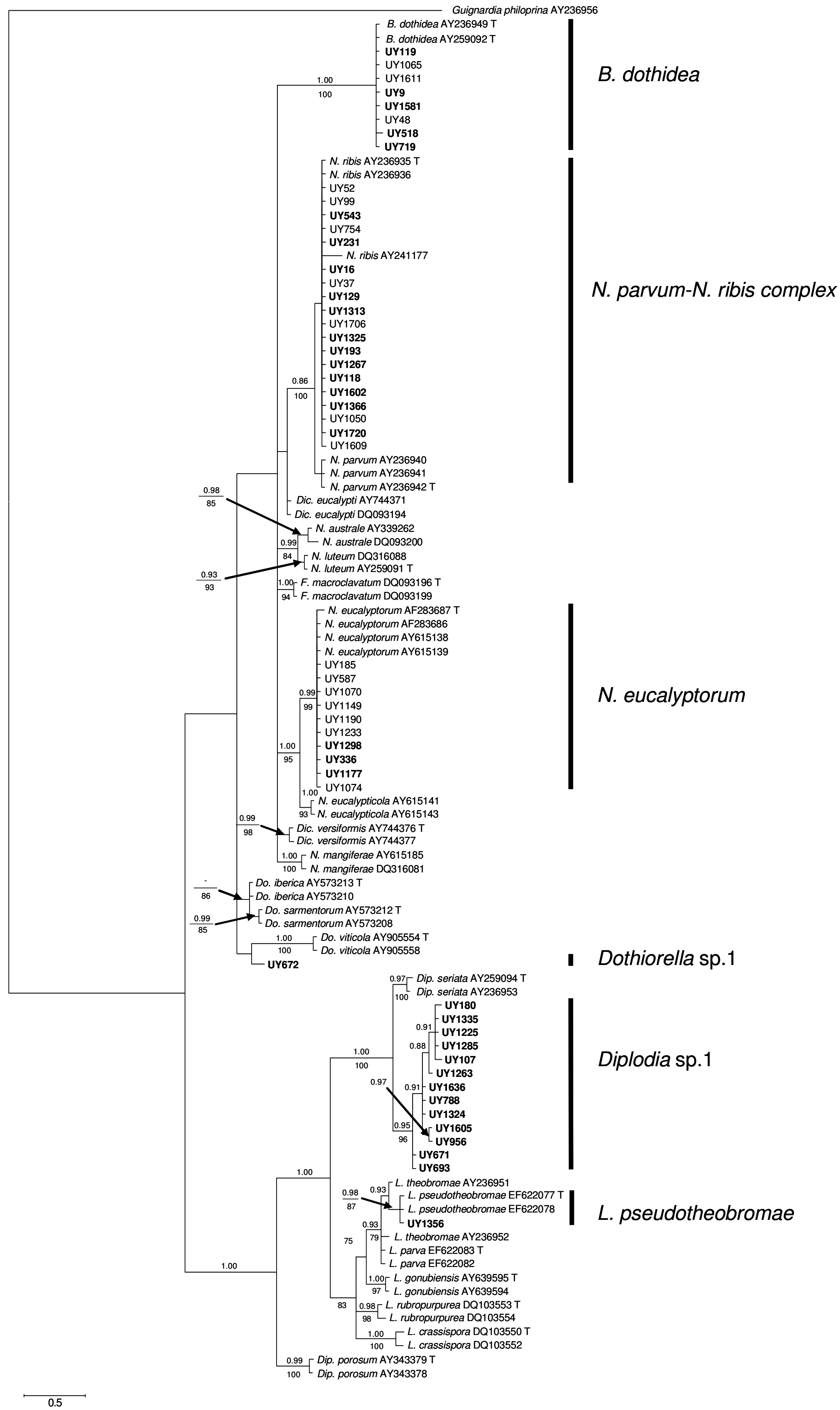
Culture ID*	Species	Host	GenBank accession no. ITS	EF	RPB2
UY956	*Diplodia* sp.1	*Blepharocalyx salicifolius*	EU860383	-	-
UY1050	*N. parvum-N. ribis* complex	*Eucalyptus globulus*	EU080928	EU863165	EU863172
UY1065	*B. dothidea*	*Eucalyptus maidenii*	EU860384	-	-
UY1070	*N. eucalyptorum*	*Eucalyptus maidenii*	EU080929	-	-
UY1074	*N. eucalyptorum*	*Eucalyptus grandis*	EU860374	-	-
UY1149	*N. eucalyptorum*	*Eucalyptus dunnii*	EU860375	-	-
UY1177	*N. eucalyptorum*	*Blepharocalyx salicifolius*	EU860376	-	-
UY1190	*N. eucalyptorum*	*Eucalyptus globulus*	EU080930	-	-
UY1225	*Diplodia* sp.1	*Acca sellowiana*	EU080931	-	-
UY1233	*N. eucalyptorum*	*Eucalyptus viminalis*	EU080932	-	-
UY1263	*Diplodia* sp.1	*Myrciaria tenella*	EU080933	-	-
UY1267	*N. parvum-N. ribis* complex	*Blepharocalyx salicifolius*	EU860385	EU863182	-
UY1285	*Diplodia* sp.1	*Myrcianthes cisplatensis*	EU860386	-	-
UY1298	*N. eucalyptorum*	*Myrrhinium atropurpureum* var. *octandrum*	EU080934	-	-
UY1313	*N. parvum-N. ribis* complex	*Myrciaria tenella*	EU860387	-	-
UY1324	*Diplodia* sp.1	*Myrcianthes cisplatensis*	EU860388	-	-
UY1325	*N. parvum-N. ribis* complex	*Myrcianthes cisplatensis*	EU860389	-	-
UY1335	*Diplodia* sp.1	*Blepharocalyx salicifolius*	EU860390	-	-
UY1356	*Lasiodiplodia pseudotheobromae*	*Myrcianthes pungens*	EU860391	-	-
UY1366	*N. parvum-N. ribis* complex	*Blepharocalyx salicifolius*	EU080935	-	-
UY1581	*B. dothidea*	*Myrceugenia euosma*	EU860392	-	-
UY1602	*N. parvum-N. ribis* complex	*Eugenia involucrata*	EU860393	-	-
UY1605	*Diplodia* sp.1	*Eugenia involucrata*	EU860394	-	-
UY1609	*N. parvum-N. ribis* complex	*Eucalyptus cinerea*	EU860395	-	-
UY1611	*B. dothidea*	*Eucalyptus cinerea*	EU860396	-	-

Culture ID*	Species	Host	GenBank accession no. ITS	EF	RPB2
UY1636	*Diplodia* sp.1	*Myrceugenia euosma*	EU860397	-	-
UY1706	*N. parvum-N. ribis* complex	*Eucalyptus robusta*	EU860398	-	-
UY1720	*N. parvum-N. ribis* complex	*Eugenia involucrata*	EU860399	-	-
CBS414.64	*"Botryosphaeria" tsugae*	*Tsuga heterophylla*	DQ458888	DQ458873	-
CBS110302	*B. dothidea*	*Vitis vinifera*	AY259092	-	-
CBS115476	*B. dothidea*	*Prunus* sp.	AY236949	AY236898	-
CMW15198	*Dichomera eucalypti*	*Eucalyptus diversicolor*	AY744371	DQ093214	-
CMW15952	*Dic. eucalypti*	*Eucalyptus diversicolor*	DQ093194	DQ093215	-
VPRI31988	*Dic. versiformis*	*Eucalyptus pauciflora*	AY744377	-	-
WAC12403	*Dic. versiformis*	*Eucalyptus camaldulensis*	AY744376	-	-
CBS112547	*Diplodia corticola*	*Quercus ilex*	AY259110	DQ458872	-
CBS112549	*Dip. corticola*	*Quercus suber*	AY259100	AY573227	-
CBS168.87	*Dip. cupressi*	*Cupressus sempervirens*	DQ458893	DQ458878	-
CBS261.85	*Dip. cupressi*	*Cupressus sempervirens*	DQ458894	DQ458879	-
CBS112553	*Dip. mutila*	*Vitis vinifera*	AY259093	AY573219	-
CBS230.30	*Dip. mutila*	*Phoenix dactylifera*	DQ458886	DQ458869	-
CBS109727	*Dip. pinea A*	*Pinus radiata*	DQ458897	DQ458882	-
CBS393.84	*Dip. pinea A*	*Pinus nigra*	DQ458895	DQ458880	-
CBS109725	*Dip. pinea C*	*Pinus patula*	DQ458896	DQ458881	-
CBS109943	*Dip. pinea C*	*Pinus patula*	DQ458898	DQ458883	-
CBS110496	*Dip. porosum*	*Vitis vinifera*	AY343379	AY343340	-
CBS110574	*Dip. porosum*	*Vitis vinifera*	AY343378	AY343339	-
CBS109944	*Dip. scrobiculata*	*Pinus greggii*	DQ458899	DQ458884	-
CBS113424	*Dip. scrobiculata*	*Pinus greggii*	DQ458900	DQ458885	-
CBS112555	*Dip. seriata*	*Vitis vinifera*	AY259094	AY573220	-

Culture ID*	Species	Host	GenBank accession no. ITS	EF	RPB2
CMW7774	*Dip. seriata*	*Ribes* sp.	AY236953	AY236902	-
CBS115035	*Dothiorella iberica*	*Quercus ilex*	AY573213	AY573228	-
CBS115039	*Do. iberica*	*Quercus* sp.	AY573210	AY573234	-
CBS120.41	*Do. sarmentorum*	*Pyrus communis*	AY573207	AY573224	EF204482
CBS165.33	*Do. sarmentorum*	*Prunus armeniaca*	AY573208	AY573225	-
IMI63581b	*Do. sarmentorum*	*Ulmus* sp.	AY573212	AY573235	-
CBS117009	*Do. viticola*	*Vitis vinifera*	AY905554	AY905559	-
CBS117110	*Do. viticola*	*Vitis vinifera*	AY905558	AY905561	-
CMW15947	*Fusicoccum macroclavatum*	*Eucalyptus saligna*	DQ093199	-	-
CMW15955	*F. macroclavatum*	*Eucalyptus globulus*	DQ093196	DQ093217	-
CMW13488	*Lasiodiplodia crassispora*	*Eucalyptus urophylla*	DQ103552	DQ103559	-
WAC12533	*L. crassispora*	*Santalum album*	DQ103550	DQ103557	-
CMW14077	*L. gonubiensis*	*Syzygium cordatum*	AY639595	DQ103566	-
CMW14078	*L. gonubiensis*	*Syzygium cordatum*	AY639594	DQ103567	-
CBS356.59	*L. parva*	*Theobroma cacao*	EF622082	EF622062	-
CBS456.78	*L. parva*	Cassava-field soil	EF622083	EF622063	-
CBS116459	*L. pseudotheobromae*	*Gmelina arborea*	EF622077	EF622057	-
CBS116460	*L. pseudotheobromae*	*Acacia mangium*	EF622078	EF622058	-
WAC12535	*L. rubropurpurea*	*Eucalyptus grandis*	DQ103553	DQ103571	-
WAC12536	*L. rubropurpurea*	*Eucalyptus grandis*	DQ103554	DQ103572	-
CMW10130	*L. theobromae*	*Vitex donniana*	AY236951	AY236900	-
CMW9074	*L. theobromae*	*Pinus* sp.	AY236952	AY236901	-
WAC12539	*L. venezuelensis*	*Acacia mangium*	DQ103547	DQ103568	-
WAC12540	*L. venezuelensis*	*Acacia mangium*	DQ103548	DQ103569	-
CMW15954	*Neofusicoccum australe*	*Eucalyptus diversicolor*	DQ093200	DQ093222	-

			GenBank accession no.		
Culture ID*	Species	Host	ITS	EF	RPB2
CMW6837	*N. australe*	*Acacia* sp.	AY339262	AY339270	-
CBS115679	*N. eucalypticola*	*Eucalyptus grandis*	AY615141	AY615133	-
CBS115767	*N. eucalypticola*	*Eucalyptus rossii*	AY615143	AY615135	-
CBS115768	*N. eucalyptorum*	*Eucalyptus nitens*	AY615138	AY615130	-
CBS115791	*N. eucalyptorum*	*Eucalyptus grandis*	AF283686	AY236891	-
CMW10126	*N. eucalyptorum*	*Eucalyptus grandis*	AF283687	AY236892	-
CMW6804	*N. eucalyptorum*	*Eucalyptus dunnii*	AY615139	AY615131	-
CBS110299	*N. luteum*	*Vitis vinifera*	AY259091	AY573222	-
CBS118842	*N. luteum*	*Syzygium cordatum*	DQ316088	-	-
CBS118531	*N. mangiferae*	*Mangifera indica*	AY615185	-	-
CMW13998	*N. mangiferae*	*Syzygium cordatum*	DQ316081	-	-
CMW9078	*N. parvum*	*Actinidia deliciosa*	AY236940	AY236885	-
CMW9079	*N. parvum*	*Actinidia deliciosa*	AY236941	AY236886	EU863166
CMW9080	*N. parvum*	*Populus nigra*	AY236942	AY236887	EU863167
CMW9081	*N. parvum*	*Populus nigra*	AY236943	AY236888	EU863168
CBS115475	*N. ribis*	*Ribes* sp.	AY236935	AY236877	EU863170
CBS121.26	*N. ribis*	*Ribes rubum*	AF241177	AY236879	EU863171
CMW7773	*N. ribis*	*Ribes* sp.	AY236936	AY236878	EU863169
CBS112878	*N. viticlavatum*	*Vitis vinifera*	AY343380	AY343342	-
CBS112997	*N. viticlavatum*	*Vitis vinifera*	AY343381	AY343341	-
CBS110880	*N. vitifusiforme*	*Vitis vinifera*	AY343382	AY343344	-
CBS110887	*N. vitifusiforme*	*Vitis vinifera*	AY343383	AY343343	-
CBS447.68	*Guignardia philoprina*	*Taxus baccata*	AY236956	AY236905	-

(*) Isolates sequenced in this study are indicated with the prefix "UY" and ex-type cultures are shown in bold.


Guignardia philoprina AY236956
B. dothidea AY236949 T
B. dothidea AY259092 T
UY119
UY1065
UY1611
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UY48
UY518
UY719
B. dothidea
N. ribis AY236935 T
N. ribis AY236936
UY52
UY99
UY543
UY754
UY231
N. ribis AY241177
UY16
UY37
UY129
UY1313
UY1706
UY1325
UY193
UY1267
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UY1602
UY1366
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UY1720
UY1609
N. parvum AY236940
N. parvum AY236941
N. parvum AY236942 T
N. parvum-N. ribis complex
Dic. eucalypti AY744371
Dic. eucalypti DQ093194
N. australe AY339262
N. australe DQ093200
N. luteum DQ316088
N. luteum AY259091 T
F. macroclavatum DQ093196 T
F. macroclavatum DQ093199
N. eucalyptorum AF283687 T
N. eucalyptorum AF283686
N. eucalyptorum AY615138
N. eucalyptorum AY615139
UY185
UY587
UY1070
UY1149
UY1190
UY1233
UY1298
UY336
UY1177
UY1074
N. eucalyptorum
N. eucalypticola AY615141
N. eucalypticola AY615143
Dic. versiformis AY744376 T
Dic. versiformis AY744377
N. mangiferae AY615185
N. mangiferae DQ316081
Do. iberica AY573213 T
Do. iberica AY573210
Do. sarmentorum AY573212 T
Do. sarmentorum AY573208
Do. viticola AY905554 T
Do. viticola AY905558
UY672
Dothiorella sp.1
Dip. seriata AY259094 T
Dip. seriata AY236953
UY180
UY1335
UY1225
UY1285
UY107
UY1263
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UY1324
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UY956
UY671
UY693
Diplodia sp.1
L. theobromae AY236951
L. pseudotheobromae EF622077 T
L. pseudotheobromae EF622078
UY1356
L. pseudotheobromae
L. theobromae AY236952
L. parva EF622083 T
L. parva EF622082
L. gonubiensis AY639595 T
L. gonubiensis AY639594
L. rubropurpurea DQ103553 T
L. rubropurpurea DQ103554
L. crassispora DQ103550 T
L. crassispora DQ103552
Dip. porosum AY343379 T
Dip. porosum AY343378
0.5

Fig. 6.1: Phylogenetic relationship among the isolates obtained in the present study and Botryosphaeriaceae species obtained from GenBank (Table 1). Bayesian tree based on ITS sequences was constructed using a SYM+I+G model. Posterior probabilities (10 million generations) of the Bayesian analysis and bootstrap values (1,000 replicates) of the maximum parsimony analysis are shown above and below branches, respectively. *Guignardia philoprina* was the outgroup taxon. Sequences obtained in this study are indicated with a prefix "UY", and those obtained from native Myrtaceae hosts are in bold. Ex-type cultures are labeled with a "T" at the end. Scale bar indicates 0.2 substitutions per site.

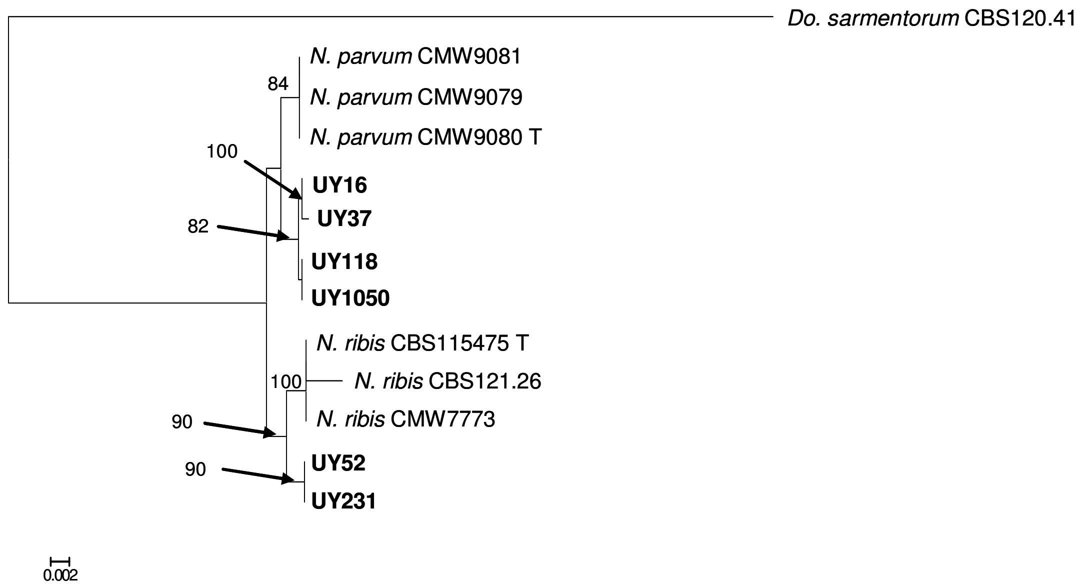

Fig. 6.2: Neighbor-joining tree obtained from the combined dataset of the ITS, EF1-α and RPB2 DNA sequence alignment of the *N. parvum-N. ribis* complex showing the location of the isolates obtained in the present study indicated in bold. Species name and culture ID is shown for each sequence. Sequences labeled with a "T" at the end correspond to the ex-type culture. Bootstrap values of 1,000 replicates of the maximum parsimony analysis are shown at the branches. Only bootstrap values higher than 75% are shown. *Dothiorella sarmentorum* was used as the outgroup taxon.

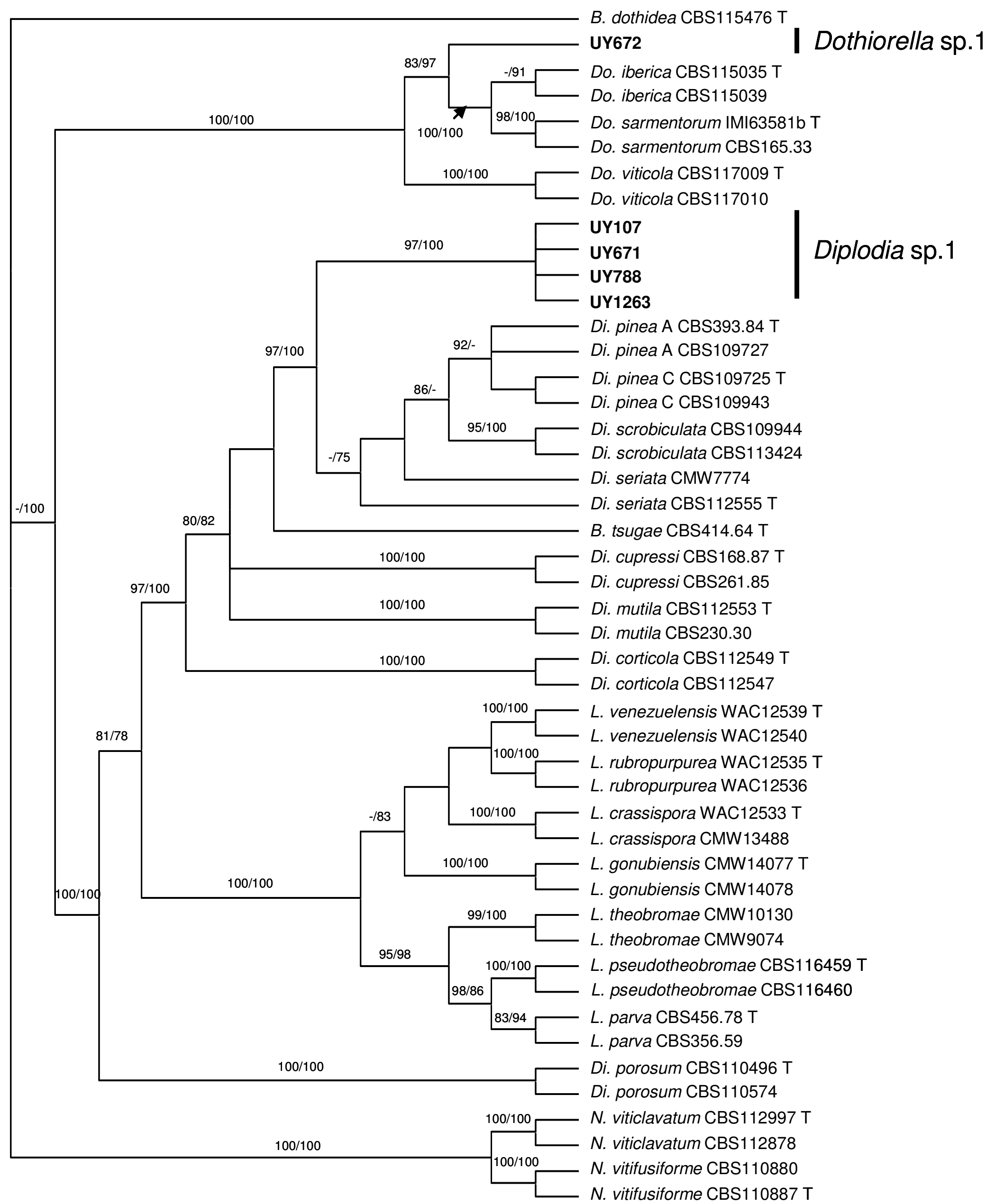

Fig. 6.3: Strict consensus tree of the six most parsimony trees obtained with the combined dataset of ITS and EF1-α genes showing the phylogenetic location of the isolates obtained in the present study, indicated in bold. Species name and culture ID are shown for each sequence. Sequences labeled with a "T" at the end correspond to the ex-type culture. Bootstrap values of 1000 replicates of neighbor-joining and maximum parsimony analyses are shown at the branches, respectively. Only bootstrap values higher than 75% are shown. *Botryosphaeria dothidea* was used as an outgroup taxon.

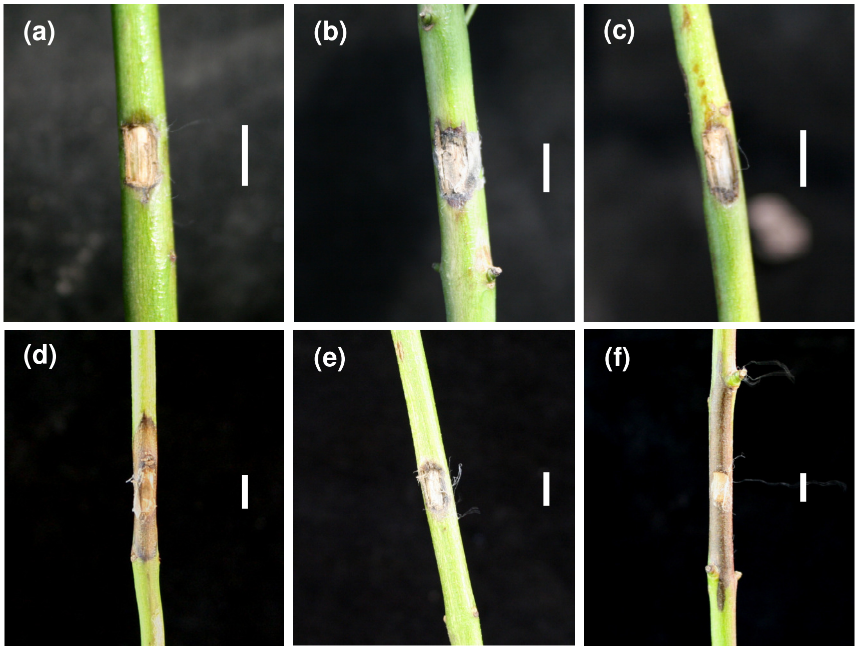

Fig. 6.4: Stem lesions observed one week after inoculation of selected isolates on four-month old *E. grandis* seedlings, a) Control, b) *Diplodia* sp.1 (isolate UY788), c) *Dothiorella* sp.1 (isolate UY672), d) *N. parvum/N. ribis* (isolate UY543), e) *B. dothidea* (isolate UY719) and f) *L. theobromae* (isolate UY1356). Scale bar = 5 mm.

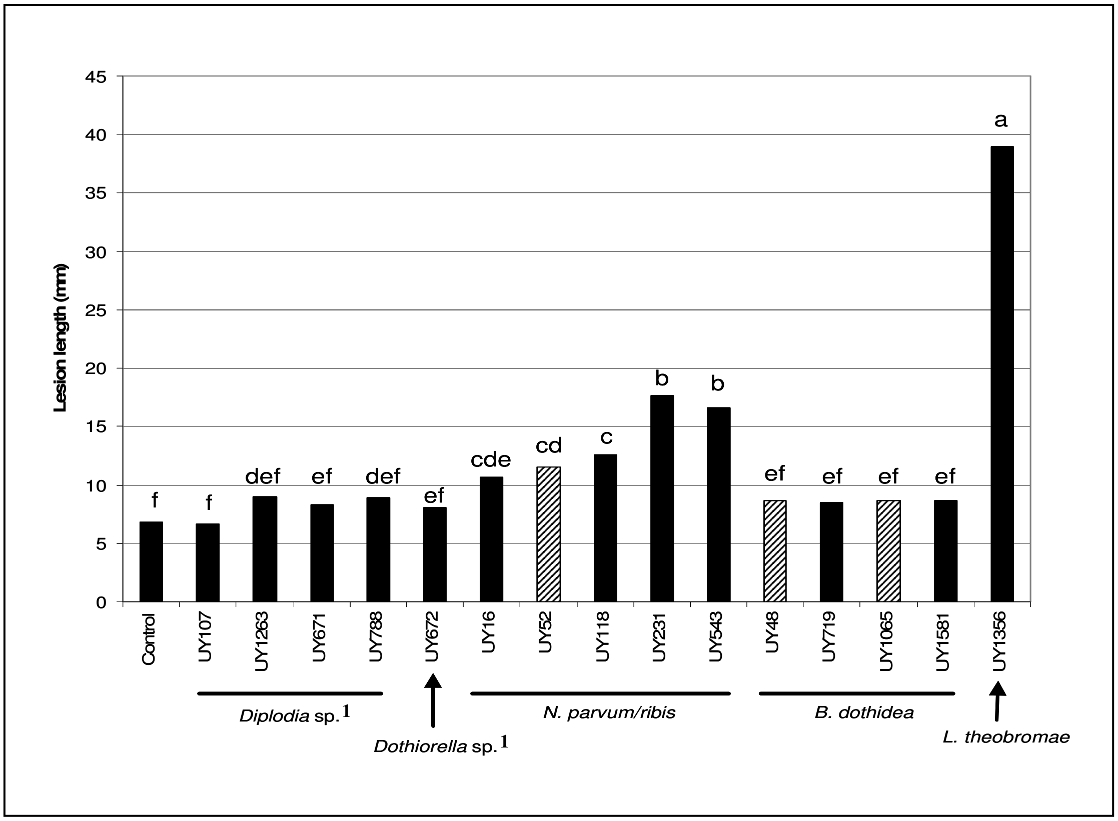

Fig. 6.5: Lesion length (average of 10 replicates) observed one week after inoculation on stems of *E. grandis* for selected isolates of Botryosphaeriaceae species found on Myrtaceae hosts in Uruguay. Letters indicate mean separation based on LSD ($P = 0.05$). Isolates UY52, UY48 and UY1065 shown with downward diagonal bars were obtained from *Eucalyptus* and randomly selected and included in this study for reference.

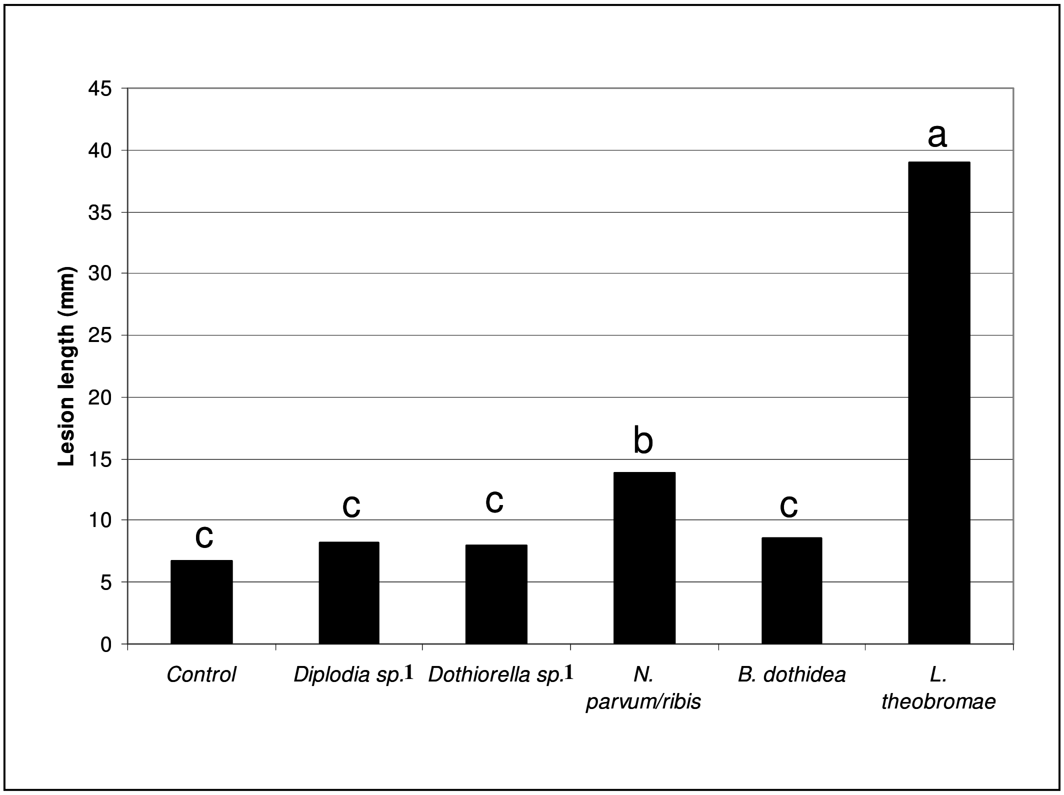

Fig. 6.6: Mean lesion length (mm) observed for those Botryosphaeriaceae species obtained from native Myrtaceae hosts one week after inoculated on *E. grandis* stems. Isolates were grouped by species and mean comparison between groups was performed using orthogonal contrasts. Different letters indicate significant differences ($P < 0.001$).

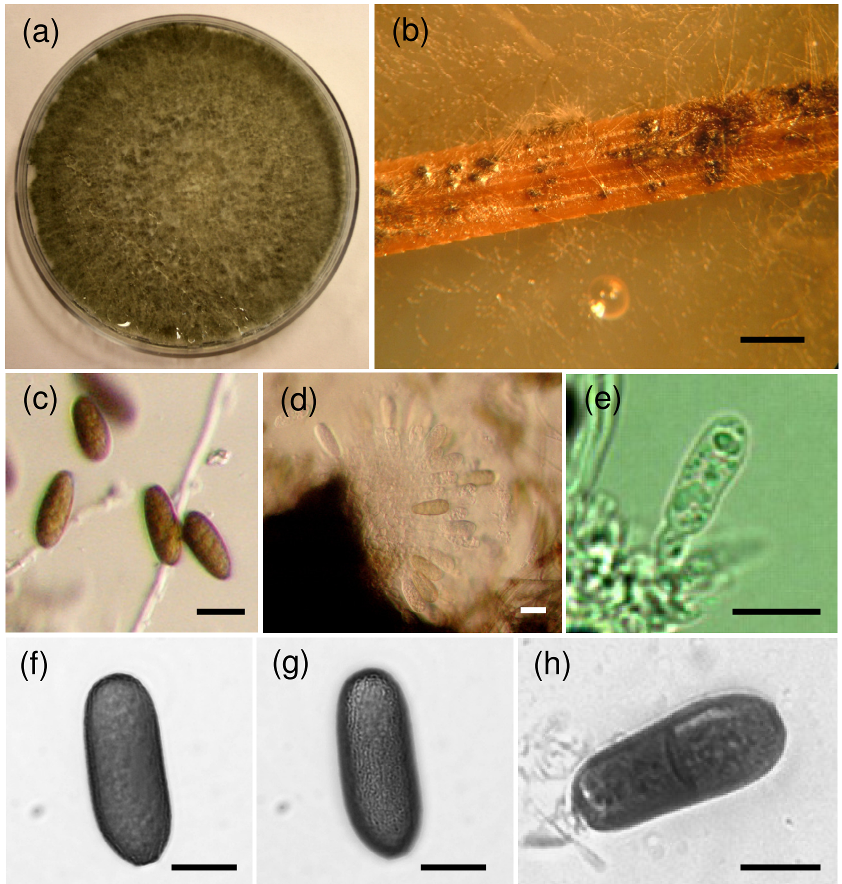

Fig. 6.7: Micrographs of fruiting structures of *Diplodia* sp.1 **a)** top view of an one week old colony grown on PDA; **b)** semi-immersed and superficial pycnidia formed on pine needles; **c)** brown mature conidia; **d)** conidiophore cells with immature conidium; **e)** a close-up of conidiophore cell with immature conidium; **f)** and **g)** conidium with obtuse apex and truncate base photographed at two different levels of focus to show the conidium wall with a smooth outer surface (f) and the roughened inner surface (g); **h)** 1-septate conidium. Scale bars: b = 1 mm; c-e = 20 µm; f-h = 10 µm.

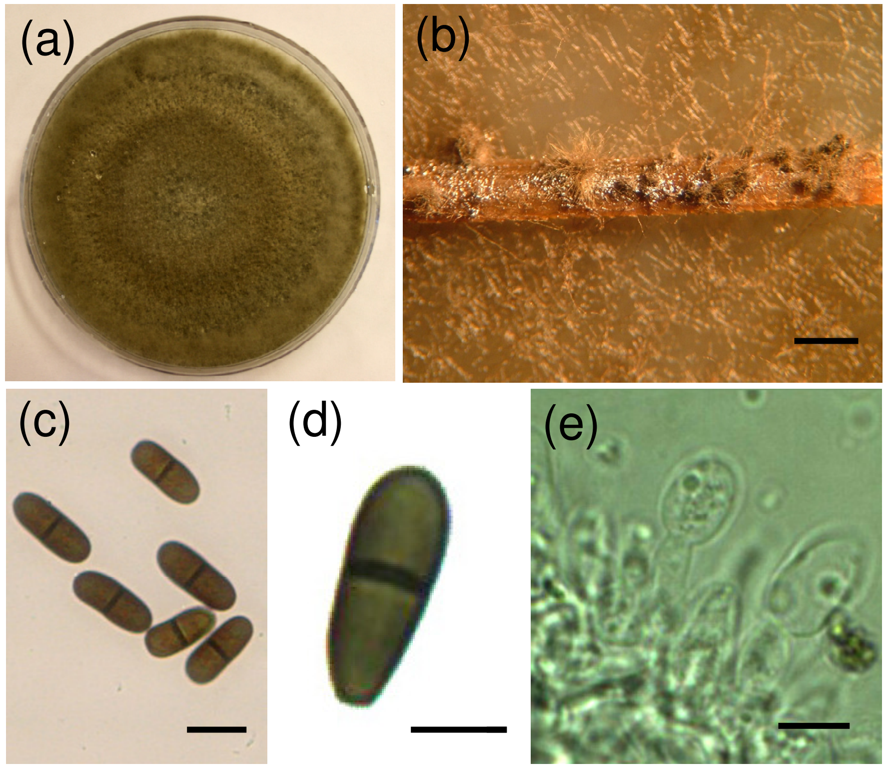

Fig. 6.8: Micrographs of fruiting structures of *Dothiorella* sp.1 **a)** top view of an one week old colony grown on PDA; **b)** superficial pycnidia formed on pine needles; **c)** dark brown walled conidia, 1-septate; **d)** conidium slightly constricted at the septum, with broadly rounded apex and truncate base; **e)** conidiophore cells with immature conidia. Scale bars: b = 1 mm; c = 20 µm; d-e = 10 µm

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