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

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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 regarding the occurrence and species diversity of Botryosphaeriaceae in native Myrtaceae forests or what their relationship is to those species infecting *Eucalyptus* in plantations. The objectives of this study 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. Single spore cultures were identified based on conidial morphology and comparisons of DNA sequences of the ITS and EF1-α regions. Six Botryosphaeriaceae species were identified. *Botryosphaeria dothidea*, *N. eucalyptorum* and specimens residing in the *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 pseudoseriata* sp. nov. and *Spencermartinsia uruguayensis* sp. nov. are novel species found only on native myrtaceous hosts. Pathogenicity tests showed that isolates obtained from native trees and identified as *L. pseudotheobromae*, *N. eucalyptorum* and the N. parvum-N.ribis complex are pathogenic to *E. grandis*. Interestingly, *Lasiodiplodia pseudotheobromae* has not previously been found on *Eucalyptus* in Uruguay and represents a potential threat to this host.

Introduction

The Botryosphaeriaceae is a very diverse group of fungi that includes endophytes and plant pathogens of trees and plants (Alves *et al*. 2008; Huang *et al*. 2008; Lazzizera *et al*. 2008). It is well known that certain endophytic fungi may become pathogenic when trees and plants become stressed (Old *et al*. 1990; Pusey 1989; Wene and Schoeneweiss 1980). In this regard, 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 (Zhou *et al*. 2008). Stem cankers and die-back of *Eucalyptus* spp. have commonly 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 loss, tree mortality, and coppice failure (Balmelli and Resquin 2005). Additionally, due to the explosive increase in the area planted to introduced species, the biotic interactions 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 occur between introduced *Eucalyptus* and native trees, which could result in negative economic impact as well as an ecological disturbance. 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 on which a species of Botryosphaeriaceae, *B. dothidea*, has been found (Bettucci *et al*. 2004).

Eucalyptus spp. are non-native in Uruguay and pathogens affecting these trees could have been introduced. 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 (Rodas *et al*. 2008; 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; Pavlic *et al*. 2007; Seixas *et al*. 2004; Wingfield *et al*. 2001; Wingfield 2003). Very little is known about the Botryosphaeriaceae species occurring on introduced and native Myrtaceae hosts in Uruguay. The aim of this study was, therefore, to gain 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

Between 2005 and 2008 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 trees (less than 500 m away). Endophytic microorganisms were isolated from asymptomatic 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 (~20°C) for 1 week. Colonies resembling Botryosphaeriaceae were selected for this study, and maintained on 2% MEA at 8°C. To verify the efficacy of the surface disinfestation and to assure the growth of only endophytic microorganisms, imprints of sample surfaces were made on MEA plates and observed for 1 week to confirm that fungi did not grow (Hyde and Soytong 2008).

Isolations from cankers were done from wood tissue at the advancing zone of the lesion, which was surface-disinfested in 70% ethyl alcohol for 30 s, 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 (~20°C) for 1 week. Colonies resembling Botryosphaeriaceae were subcultured to a fresh 2% MEA plate for further investigation.

Morphological characterization

Isolates were stimulated to produce fruiting structures (pycnidia) and conidia, by growing them on 1.5% water agar (WA) (Sigma Chemicals, St. Louis, MO) with sterilized pine needles placed onto the medium surface. Plates were incubated at 22°C under black light until pycnidia were observed on the pine needles (approx. 3 weeks after plating). Monoconidial cultures were obtained by plating a conidial suspension from two pycnidia, suspended in 300 µl of sterile water, and plated onto 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.

DNA extraction, PCR, sequencing and phylogenetic analysis

DNA extraction from the 49 isolates listed in Table 1 was done as described in Pérez *et al*. (2009).The phylogenetic analyses were performed in two 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 confirm the identification of the *Diplodia* sp. clade and the *Spencermartinsia* sp. isolate with the analysis of the rDNA ITS region along with part of the EF1-α region. Analyzes of both DNA regions were performed separately and combined, to test congruence.

Table 1. List of isolates used in this study.

Culture ID[a]	Species	Host	GenBank accession no.	
			ITS	EF
UY9	*Botryosphaeria dothidea*	*Blepharocalyx salicifolius*	EU080907	–
UY16	*Neofusicoccum parvum-N. ribis* complex	*B. salicifolius*	EU080908	–
UY37	*N. parvum-N. ribis* complex	*Eucalyptus grandis*	EU080910	–
UY48	*Botryosphaeria dothidea*	*Eucalyptus grandis*	EU080911	–
UY52	*N. parvum-N. ribis* complex	*Eucalyptus grandis*	EU080912	–
UY99	*N. parvum-N. ribis* complex	*Eucalyptus grandis*	EU860378	–
UY107	*Diplodia pseudoseriata*	*Myrcianthes cisplatensis*	EU080914	EU863178
UY118	*N. parvum-N. ribis* complex	*Eugenia uruguayensis*	EU080915	–
UY119	*B. dothidea*	*E. uruguayensis*	EU080916	–
UY129	*N. parvum-N. ribis* complex	*Myrrhinium atropurpureum* var. *octandrum*	EU860379	–
UY180	*Diplodia pseudoseriata*	*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	–
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 pseudoseriata*	*Hexachlamis edulis*	EU080922	EU863179
UY672	*Spencermartinia uruguayensis*	*Hexachlamis edulis*	EU080923	EU863180
UY693	*Diplodia pseudoseriata*	*Eugenia unifora*	EU080924	–
UY719	*B. dothidea*	*Myrrhinium atropurpureum* var. *octandrum*	EU080925	–
UY754	*N. parvum-N. ribis* complex	*Eucalyptus ficifolia*	EU080926	–
UY788	*Diplodia pseudoseriata*	*Blepharocalyx salicifolius*	EU080927	EU863181
UY956	*Diplodia pseudoseriata*	*Blepharocalyx salicifolius*	EU860383	–
UY1050	*N. parvum-N. ribis* complex	*Eucalyptus globulus*	EU080928	–
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	*Dip. pseudoseriata*	*Acca sellowiana*	EU080931	–
UY1233	*N. eucalyptorum*	*Eucalyptus viminalis*	EU080932	–
UY1263	*Dip. pseudoseriata*	*Myrciaria tenella*	EU080933	EU863182
UY1267	*N. parvum-N. ribis* complex	*Blepharocalyx salicifolius*	EU860385	
UY1285	*Dip. pseudoseriata*	*Myrcianthes cisplatensis*	EU860386	–
UY1298	*N. eucalyptorum*	*Myrrhinium atropurpureum* var. *octandrum*	EU080934	–
UY1313	*N. parvum-N. ribis* complex	*Myrciaria tenella*	EU860387	–
UY1324	*Dip. pseudoseriata*	*Myrcianthes cisplatensis*	EU860388	–
UY1325	*N. parvum-N. ribis* complex	*Myrcianthes cisplatensis*	EU860389	–
UY1335	*Dip. pseudoseriata*	*Blepharocalyx salicifolius*	EU860390	–
UY1356	*Lasiodiplodia pseudotheobromae*	*Myrcianthes pungens*	EU860391	–
UY1366	*N. parvum-N. ribis* complex	*Blepharocalyx salicifolius*	EU080935	–
UY1581	*B. dothidea*	*Myrceugenia exsuma*	EU860392	–
UY1602	*N. parvum-N. ribis* complex	*Eugenia involucrata*	EU860393	–
UY1605	*Dip. pseudoseriata*	*Eugenia involucrata*	EU860394	–
UY1609	*N. parvum-N. ribis* complex	*Eucalyptus cinerea*	EU860395	–
UY1611	*B. dothidea*	*Eucalyptus cinerea*	EU860396	–
UY1636	*Dip. pseudoseriata*	*Myrceugenia exsuma*	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	–
CMW15198	*Dichomera eucalypti*	*Eucalyptus diversicolor*	AY344371	–
CMW15952	*Dic. eucalypti*	*Eucalyptus diversicolor*	DQ093194	–
VPRI31988	*Dic. versiformis*	*Eucalyptus pauciflora*	AY344377	–
WAC12403	*Dic. versiformis*	*Eucalyptus camaldulensis*	AY344376	–
CBS112547	*Dip. 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
CBS112583	*Dip. matila*	*Vitis vinifera*	AY259093	AY573219
CBS230.30	*Dip. matila*	*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	–
CBS110574	*Dip. porosum*	*Vitis vinifera*	AY343378	–
CBS116470	*Dip. rosulata*	*Prunus africana*	EU430265	–
CBS116472	*Dip. rosulata*	*Prunus africana*	EU430266	–
CBS109944	*Dip. scrobiculata*	*Pinus greggii*	DQ458899	DQ458884
CBS113424	*Dip. scrobiculata*	*Pinus greggii*	DQ458900	DQ458885
CBS112585	*Dip. seriata*	*Vitis vinifera*	AY259094	AY573220
CMW7774	*Dip. seriata*	*Ribes* sp.	AY236953	–
CBS119049	*Dip. seriata*	*Vitis* sp.	DQ458889	DQ458874
CBS910.73	*Dothiorella acerina*	*Acer pseudoplatanus*	EU673315	EU673282
CBS242.51	*Do. coryli*	unknown	EU673317	EU673284
CBS115805	*Do. iberica*	*Quercus ilex*	AY573213	AY573228
CBS115039	*Do. iberica*	*Quercus* sp.	AY573210	AY573234
CBS188.87	*Do. juglandis*	*Juglans regia*	EU673316	EU673283
CBS165.33	*Do. sarmentorum*	*Prunus armeniaca*	AY573208	AY573225
IMI163581b	*Do. sarmentorum*	*Ulmus* sp.	AY573212	AY573235
CMW15947	*Fusicoccum macroclavatum*	*Eucalyptus saligna*	DQ093199	–
CMW15955	*F. macroclavatum*	*Eucalyptus globulus*	DQ093196	–
CMW13488	*Lasiodiplodia crassispora*	*Eucalyptus urophylla*	DQ103552	DQ103559
WAC12533	*L. crassispora*	*Santalum album*	DQ103550	DQ103557
CMW14077	*L. gonubiensis*	*Syzygium cordatum*	AY639595	–
CMW14078	*L. gonubiensis*	*Syzygium cordatum*	AY639594	–
CBS356.59	*L. parva*	*Theobroma cacao*	EF622082	–
CBS456.78	*L. parva*	Cassava-field soil	EF622083	–
CBS116459	*L. pseudotheobromae*	*Gmelina arborea*	EF622077	–
CBS116460	*L. pseudotheobromae*	*Acacia mangium*	EF622078	–
WAC12535	*L. subtropurpurea*	*Eucalyptus grandis*	DQ103553	–
WAC12536	*L. subtropurpurea*	*Eucalyptus grandis*	DQ103554	–
CMW10130	*L. theobromae*	*Vitis donniana*	AY236951	AY236900
CMW9074	*L. theobromae*	*Pinus* sp.	AY236952	AY236901
CBS169.34	*Neodeightonia phoenicum*	*Phoenix dactylifera*	EU673338	EU673307
CBS123168	*Neod. phoenicum*	*Phoenix canariensis*	EU673339	EU673308
CBS448.91	*Neod. subglobosa*	Keratomycosis in eye	EU673337	EU673306
CMW15954	*Neofusicoccum australe*	*Eucalyptus diversicolor*	DQ093200	DQ093222
CMW6837	*N. australe*	*Acacia* sp.	AY339262	–
CBS115679	*N. eucalypticola*	*Eucalyptus grandis*	AY615141	–
CBS115767	*N. eucalypticola*	*Eucalyptus rostii*	AY615143	–
CBS115768	*N. eucalyptorum*	*Eucalyptus nitens*	AY615138	–
CBS115791	*N. eucalyptorum*	*Eucalyptus grandis*	AF283686	–
CMW10126	*N. eucalyptorum*	*Eucalyptus grandis*	AF283687	–
CMW6804	*N. eucalyptorum*	*Eucalyptus dunnii*	AY615139	–
CBS110299	*N. luteum*	*Vitis vinifera*	AY259091	–
CBS118842	*N. luteum*	*Syzygium cordatum*	DQ116088	–
CBS118531	*N. mangiferae*	*Mangifera indica*	AY615185	–
CMW13998	*N. mangiferae*	*Syzygium cordatum*	DQ116081	–
CMW9078	*N. parvum*	*Actinidia deliciosa*	AY236940	–
CMW9079	*N. parvum*	*Actinidia deliciosa*	AY236941	–
CMW9080	*N. parvum*	*Populus nigra*	AY236942	–
CMW9081	*N. parvum*	*Populus nigra*	AY236943	–
CBS115475	*N. ribis*	*Ribes* sp.	AY236935	–
CBS121.26	*N. ribis*	*Ribes rubrum*	AF241177	–
CMW7773	*N. ribis*	*Ribes* sp.	AY236936	–
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
CBS302.75	*Spencermartinia* sp.	*Poniciana gelliesii*	EU673319	EU673286
CBS500.72	*Spencermartinia* sp.	*Medicago sativa*	EU673318	EU673285
ICMP16819	*Spencermartinia* sp.	*Citrus sinensis*	EU673320	EU673287
ICMP16824	*Spencermartinia* sp.	*Citrus sinensis*	EU673321	EU673288
ICMP16827	*Spencermartinia* sp.	*Citrus sinensis*	EU673322	EU673289
ICMP16828	*Spencermartinia* sp.	*Citrus sinensis*	EU673323	EU673290
CBS117089	*Spencermartinia viticola*	*Vitis vinifera*	AY905554	AY905559
CBS117110	*S. viticola*	*Vitis vinifera*	AY905558	AY905561
CBS447.68	*Guignardia philoprina*	*Taxus baccata*	AY236956	–
CBS117449	*Pseudofusicoccum stromaticum*	*Eucalyptus* hybrid	DQ436935	DQ436936

[a]Isolates sequenced in this study are indicated with the prefix "UY" and ex-type cultures are shown in bold

The ITS region was amplified for all isolates using primers ITS1 and ITS4 (White *et al*., 1990). Polymerase Chain Reaction (PCR) amplifications were performed as described in Pérez *et al*. (2009). PCR products were then 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), and sequences of the closest matching species were downloaded. 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/) (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.

The EF1-α region was amplified to confirm the identity of the *Diplodia* sp. clade and the *Spencermartinsia* sp. isolate. The EF1-α was amplified using primers EF-AF (5′ CATCGAGAAGTTCGAGAAGG 3′) and EF-BR (5′ CRATGGTGATACCRCGCTC 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 MgCl2, 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 10°C. 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 (Farris *et al*. 1995; Huelsenbeck *et al*. 1996). Thus isolates UY107, UY671, UY788 and UY1263 from the *Diplodia* sp. clade plus isolate UY672 were subjected to ITS, EF1-α and combined analyses using neighbor-joining and maximum parsimony. The original alignment was populated with corresponding sequence data of all the species residing in *Diplodia*,

Neodeightonia, *Dothiorella*, *Spencermartinsia*, and representative species of *Neofusicoccum* and *Lasiodiplodia* available in GenBank. Phylogenetic analysis was performed using PAUP Version 4.0b10. Best models for neighbor-joining analysis was determined from the AIC Modeltest version 3.7 (Posada and Crandall 1998) as K81uf+I+G, TrN+I+G and TrN+I+G for the ITS, EF1-α and combined dataset, respectively. Gaps generated in the alignment process during the comparison were treated as missing data and all characters were treated as unordered and of equal weight. Ties were broken randomly when found. All the sequences obtained in this study were deposited in GenBank (Table 1). In addition, corresponding alignments were deposited in TreeBASE (SN3975, for alignments presented in Figs. 1 and 2).

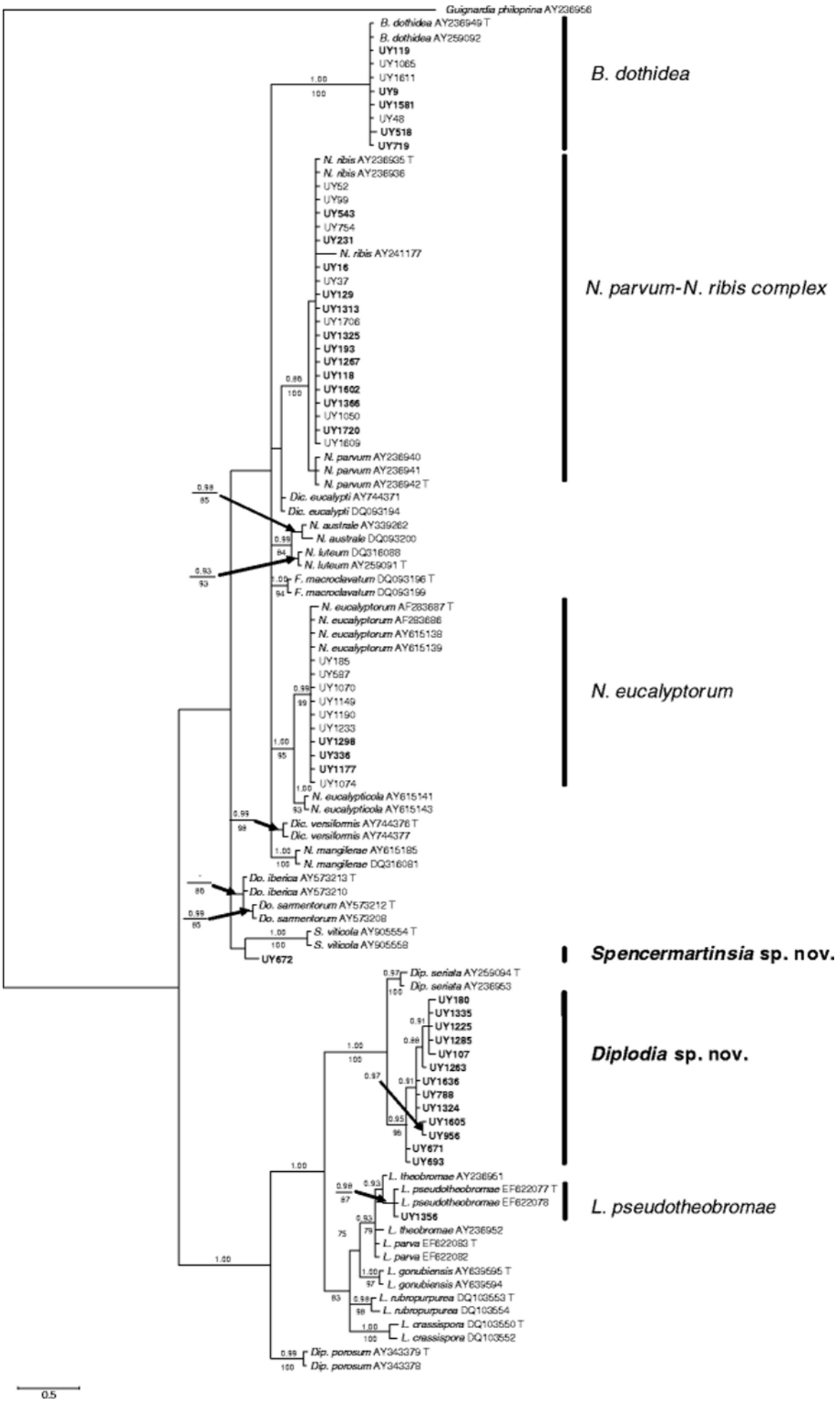
Guignardia philoprina AY236956
B. dothidea AY236949 T
B. dothidea AY259092
UY119
UY1065
UY1611
UY9
UY1581
UY48
UY518
UY719
B. dothidea
N. ribis AY236935 T
N. ribis AY236936
UY52
UY99
UY543
UY754
UY231
N. ribis AY241177
UY16
UY37
UY129
UY1313
UY1706
UY1325
UY193
UY1267
UY118
UY1602
UY1366
UY1050
UY1720
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. eucalypticola AY615141
N. eucalypticola AY615143
N. eucalyptorum
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
S. viticola AY905554 T
S. viticola AY905558
UY672
Spencermartinsia sp. nov.
Dip. seriata AY259094 T
Dip. seriata AY236953
UY180
UY1335
UY1225
UY1285
UY107
UY1263
UY1636
UY788
UY1324
UY1605
UY956
UY671
UY693
Diplodia sp. nov.
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. 1. A Bayesian tree based on ITS sequences showing the phylogenetic relationship among the isolates obtained in the present study and Botryosphaeriaceae species obtained from GenBank (Table 1). 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. The scale bar indicates 0.5 substitutions per site.

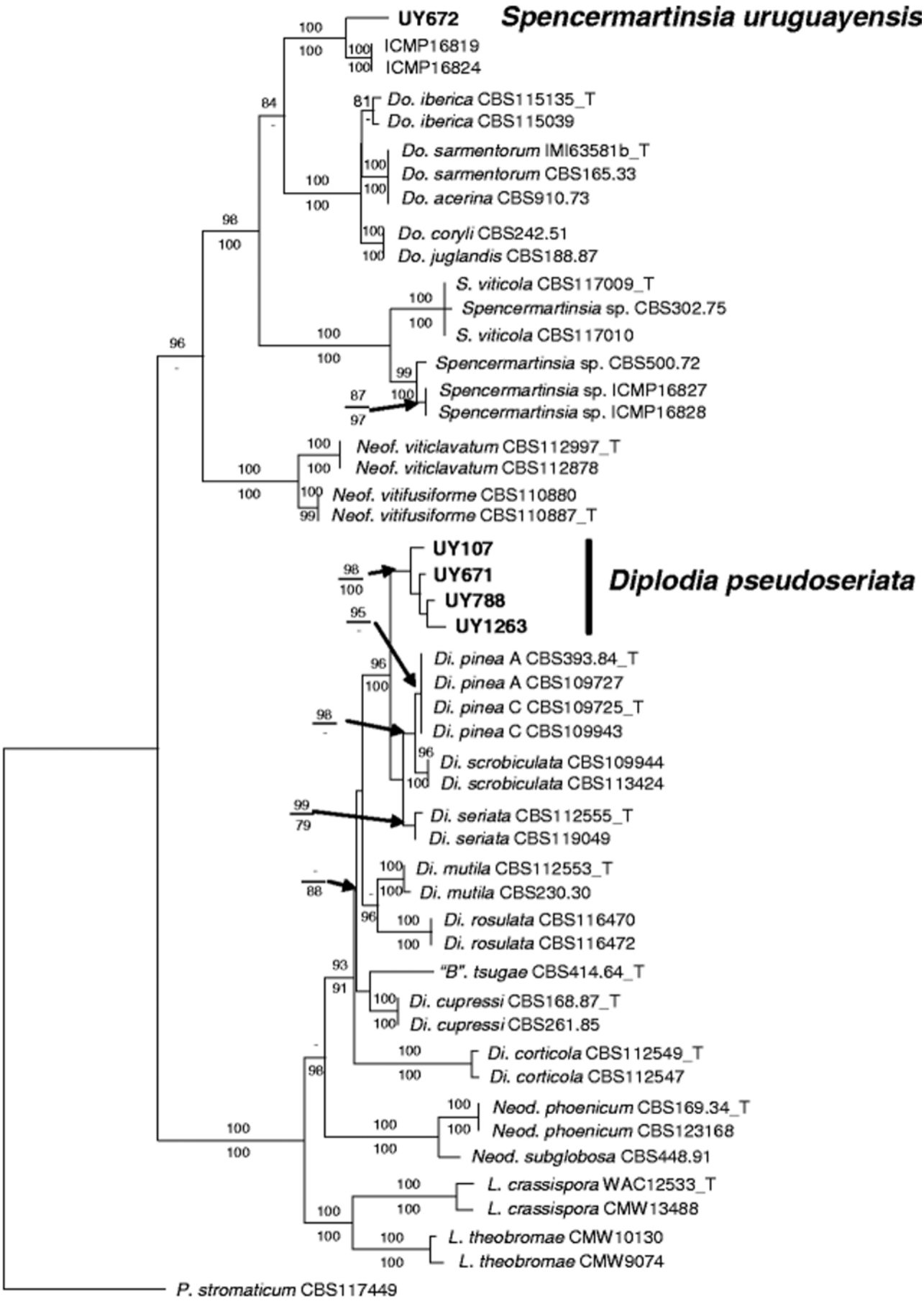
Spencermartinsia uruguayensis
UY672
ICMP16819
ICMP16824
Do. iberica CBS115135_T
Do. iberica CBS115039
Do. sarmentorum IMI63581b_T
Do. sarmentorum CBS165.33
Do. acerina CBS910.73
Do. coryli CBS242.51
Do. juglandis CBS188.87
S. viticola CBS117009_T
Spencermartinsia sp. CBS302.75
S. viticola CBS117010
Spencermartinsia sp. CBS500.72
Spencermartinsia sp. ICMP16827
Spencermartinsia sp. ICMP16828
Neof. viticlavatum CBS112997_T
Neof. viticlavatum CBS112878
Neof. vitifusiforme CBS110880
Neof. vitifusiforme CBS110887_T
Diplodia pseudoseriata
UY107
UY671
UY788
UY1263
Di. pinea A CBS393.84_T
Di. pinea A CBS109727
Di. pinea C CBS109725_T
Di. pinea C CBS109943
Di. scrobiculata CBS109944
Di. scrobiculata CBS113424
Di. seriata CBS112555_T
Di. seriata CBS119049
Di. mutila CBS112553_T
Di. mutila CBS230.30
Di. rosulata CBS116470
Di. rosulata CBS116472
"B". tsugae CBS414.64_T
Di. cupressi CBS168.87_T
Di. cupressi CBS261.85
Di. corticola CBS112549_T
Di. corticola CBS112547
Neod. phoenicum CBS169.34_T
Neod. phoenicum CBS123168
Neod. subglobosa CBS448.91
L. crassispora WAC12533_T
L. crassispora CMW13488
L. theobromae CMW10130
L. theobromae CMW9074
P. stromaticum CBS117449
0.01

Fig. 2. Distance tree obtained from neighbor-joining analysis of the combined ITS and EF1-α dataset using model TrN+I+G indicating the location of the "UY" unknowns. 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 neighbor-joining and maximum parsimony analyses are shown above and below branches, respectively. Only bootstrap values higher than 75% are shown. *Pseudofusicoccum stromaticum* was used as the outgroup taxon. Branch lengths are scaled and scale bar is 0.01 nucleotide substitutions per site.

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 have been presented previously (Pérez *et al*. 2009). Inoculations were performed on 4 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 surface disinfested 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. Five millimeter mycelial plugs from 1 week old pure cultures, on 2% MEA, were 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 N° 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 temperatures ranging from 15 to 25°C. 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 (~20°C) for 1 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 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 associated with 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 3 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 conidial morphology. A total of 52 isolates representing the six groups were further investigated using DNA sequence comparisons, including 17 obtained from *Eucalyptus* and 35 from native myrtaceous hosts (Table 1). Phylogenetic analysis of DNA sequence data confirmed 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 maximum parsimony and Bayesian analyses resulted in trees of similar topology (Fig. 1).

Based on the ITS sequences, six different Botryosphaeriaceae species were represented among the 52 isolates analyzed, in agreement with the grouping obtained based on morphological characteristics. Eight of the isolates 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, and the remaining isolate formed a distinct branch amongst clades representing *Dothiorella* and *Spencermartinsia* species respectively.

Botryosphaeria dothidea occurred as an endophyte in four different native Myrtaceae species and two *Eucalyptus* species (Table 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 and two species of native Myrtaceae (Table 1). It was also associated with a stem canker in *Myrceugenia glaucescens*. *Lasiodiplodia pseudotheobromae* was found associated with a stem canker on *Myrcianthes pungens*. In addition, isolates of the unknown *Diplodia* sp. 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 from a stem canker observed on *Myrciaria tenella*. The isolate of the unidentified *Spencermartinsia* sp. was found as an endophyte in the native Myrtaceous tree, *Hexachlamis edulis* (Table 1).
The DNA sequence alignments of the ITS, EF1-α and the combined dataset of both regions (tree parameters in Table 2) showed consistency among trees and confirmed that the group of isolates in the *Diplodia* sp. cluster obtained from native trees grouped consistently in a strongly supported distinct clade (Fig. 2). Additionally, isolate UY672 grouped separately from other *Dothiorella* and *Spencermartinsia* species with significant sequence divergence between it and the closest related clade (*Spencermartinsia* sp. ICMP16819 and ICMP16824).

Table 2. Tree parameters obtained from the maximum parsimony analysis of the ITS region, partial EF1-α gene and combined dataset of both regions.

Dataset	Total characters	Parsimony-uninformative	Constant characters	Parsimony informative	Tree Length	Consistency Index	Retention Index	Homoplasy Index
ITS	556	33	367	156	426	0.716	0.937	0.284
EF1-α	352	26	93	233	659	0.700	0.924	0.300
Combined	908	59	460	389	1,111	0.689	0.924	0.311

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* seedlings (Fig. 3). 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 ($P < 0.05$; Fig. 4). Isolates of the unknown *Diplodia* sp. and *Spencermartinsia* sp. produced lesions not significantly different from the controls. Similar results were observed for inoculations with *B. dothidea*.

Fig. 3. Stem lesions observed 1 week after inoculation of selected isolates on 4-month old *E. grandis* seedlings, a. Control, b. *Diplodia pseudoseriata* (isolate UY788), c. *Spencermartinsia uruguayensis* (isolate UY672), d. *N. parvum*/*N. ribis* (isolate UY543), e. *B. dothidea* (isolate UY719) and f. *L. pseudotheobromae* (isolate UY1356). Scale bar = 5 mm.

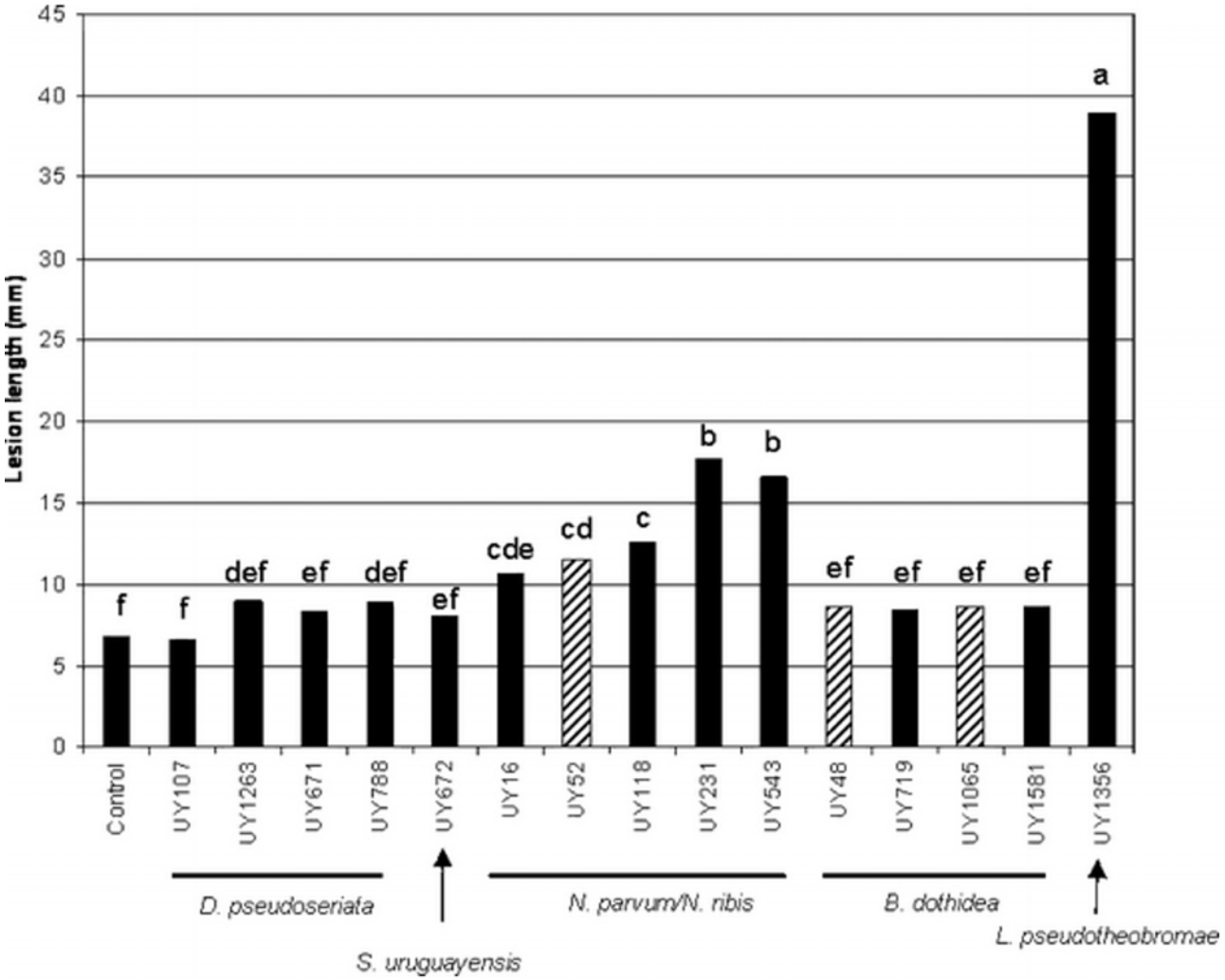

Fig. 4. Lesion length (average of 10 replicates) observed 1 week after inoculation on stems of *E. grandis* for selected isolates of Botryosphaeriaceae species found on Myrtaceae hosts in Uruguay. Letters indicate mean separation based on LSD (P=0.05). Isolates UY52, UY48 and UY1065 shown with downward diagonal bars were obtained from *Eucalyptus* and randomly selected and included in this study for reference.

Isolates were grouped by species and mean lesion length for species inoculated were compared using orthogonal contrasts, although only a limited number of isolates were analyzed for some species. Results indicated that *L. pseudotheobromae* was the most pathogenic species followed by isolates of the *N. parvum-N. ribis* complex, whereas the unidentified *Diplodia* sp. and *Spencermartinsia* sp. together with *B. dothidea* showed no differences (P>0.10) compared to the control treatment (Fig. 5). Stem diameter determined 1 week after inoculation ranged between 3 and 4 mm and showed no significant differences among treatments (data not shown), indicating there was no effect associated with seedling size.

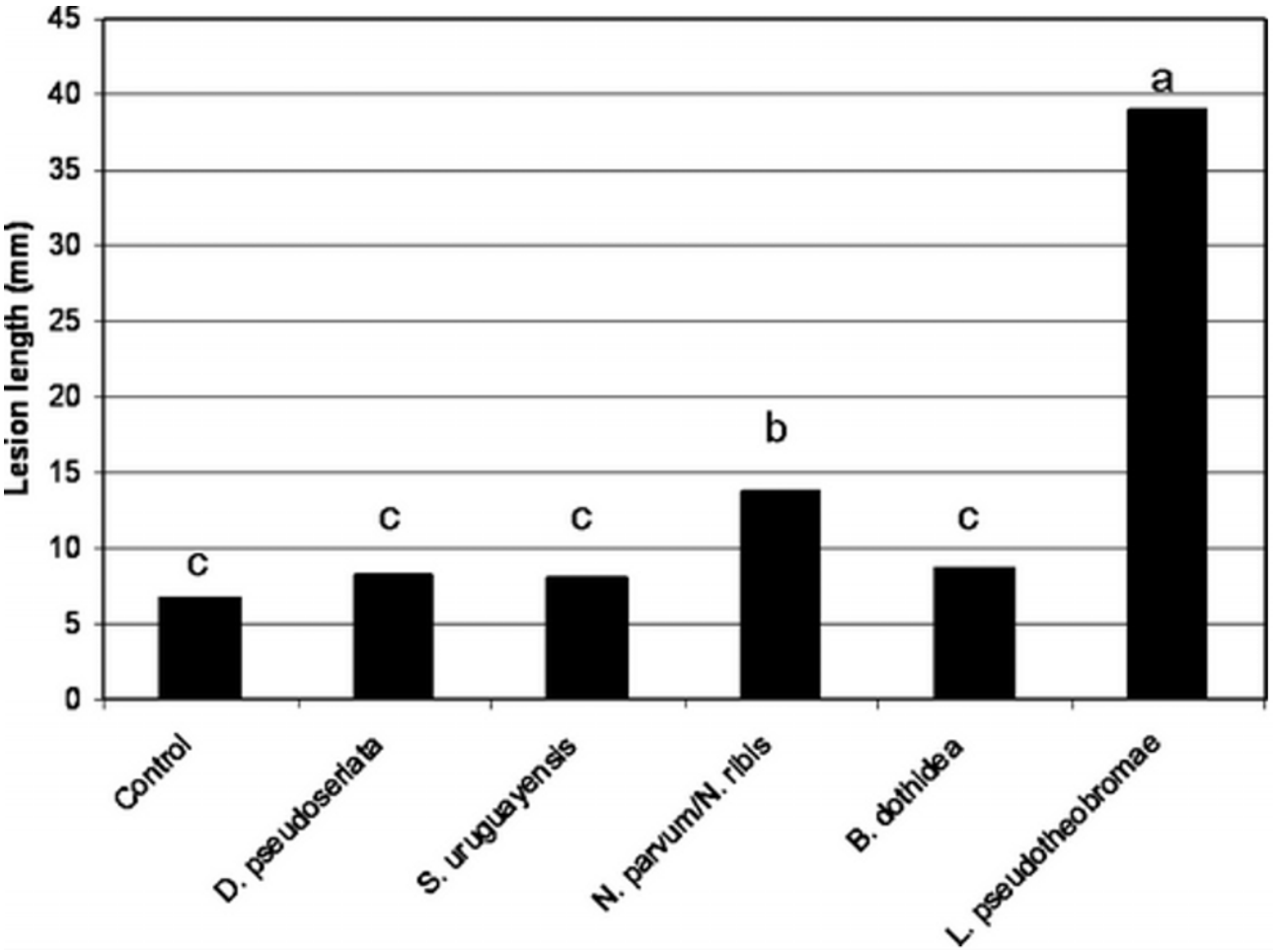

Fig. 5. Mean lesion length (mm) observed for those Botryosphaeriaceae species obtained from native Myrtaceae hosts 1 week after inoculated on *E. grandis* stems. Isolates were grouped by species and mean comparison between groups was performed using orthogonal contrasts. Different letters indicate significant differences ($P<0.001$).

Taxonomy

Based on morphology and combined multiple gene genealogies, we conclude that the isolates representing the unidentified *Diplodia* sp. and *Spencermartinsia* sp. represent previously undescribed species in the Botryosphaeriaceae. We provide the following description for these two species.

Diplodia pseudoseriata C.A. Pérez, R.A. Blanchette, B. Slippers & M.J. Wingfield, sp. nov. (Fig. 6).

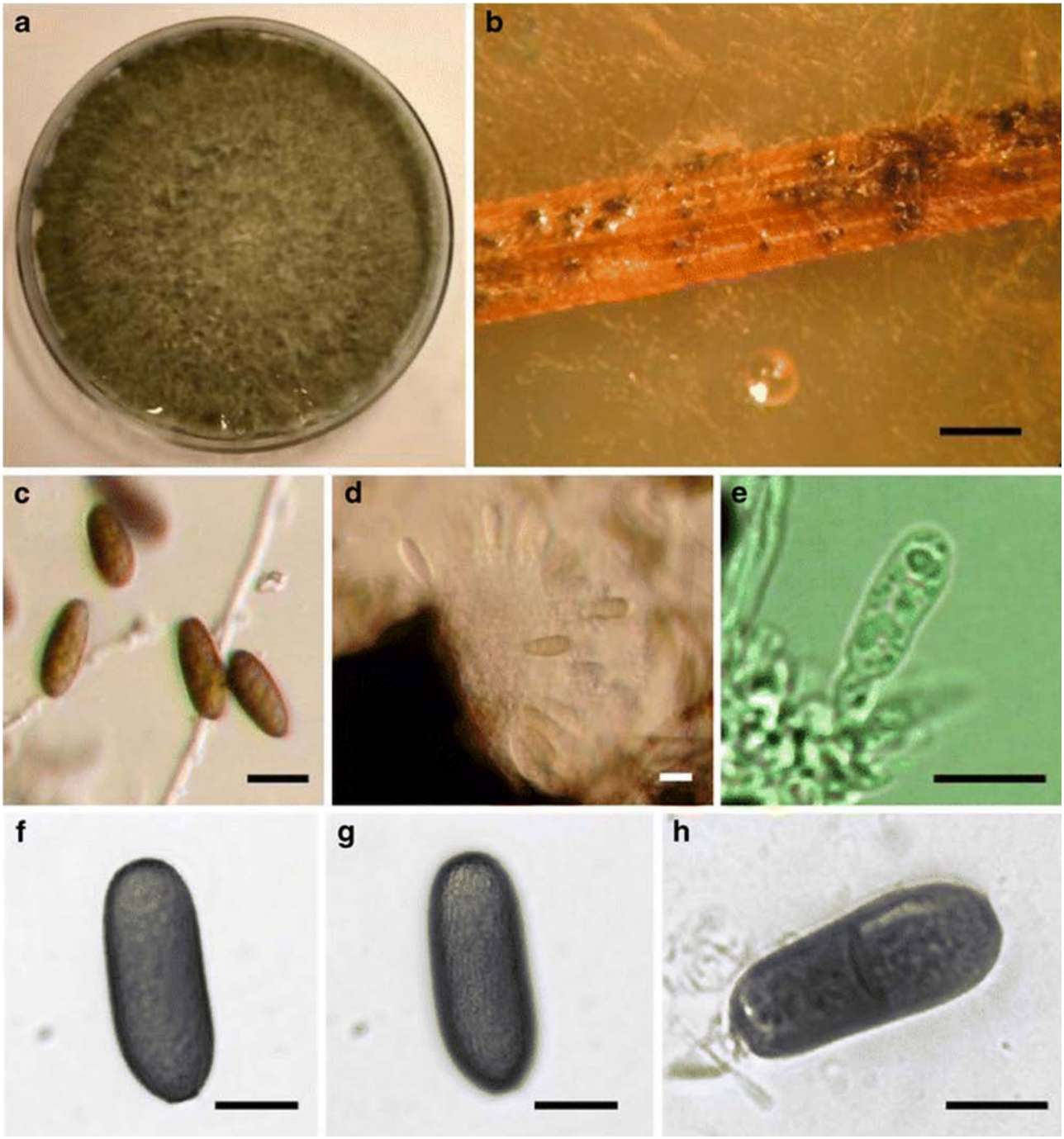

Fig. 6. Micrographs of fruiting structures of *Diplodia pseudoseriata* a. 1 week old colony grown on PDA; b. semi-immersed and superficial pycnidia formed on pine needles; c. brown mature conidia; d. conidiophores with immature conidium; e. conidiogenous cell with immature conidium; f. and g. conidium 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

MycoBank: MB 513545

Etymology: Named for its resemblance to *D. seriata*.

Pycnidia (formed in culture on sterilized pine needles) semi-immersed or superficial, solitary, globose, black, covered by mycelium, up to 430 μm diam. Conidiogenous cells cylindrical, discrete, producing a single conidium at the tip, with no evident annelations. Conidia (23–) 25.5–26.5 (–30.5) × (10–) 11.5–12 (–14) μm, initially hyaline becoming dark brown, wall externally smooth,

roughened on the inner surface, sometimes 1-septate, ovoid, apex obtuse, base truncate.

Pycnidia (in foliis sterilibus pini culta) subimmersa vel superficialia, solitaria globosa nigra mycelio tecta usque ad 430 µm diametro. Cellulae conidiogenae cylindricae discretae apice conidium unicum sine annelationibus manifestis facientes. Conidia (23–) 25.5–26.5 (–30.5) × (10–) 11.5–12 (–14) µm primo hyalina atrobrunnescentia parietibus extus laevibus, intus exasperatis, interdum semel septata ovoidea apice obtusa basi truncata.

Teleomorph: unknown

Hosts: *Acca sellowiana*, *Blepharocalyx salicifolius*, *Eugenia uniflora*, *Eugenia involucrata*, *Hexachlamis edulis*, *Myrceugenia euosma*, *Myrciaria tenella*, and *Myrcianthes cisplatensis*.

Known distribution: Uruguay

Specimen examined: URUGUAY. Paysandu, Guaviyu. Isolated from asymptomatic twig of *Blepharocalyx salicifolius*, August 2006, C. Perez, holotype PREM 60264, living cultures UY788, CMW26771, CBS 124906.

Additional specimens: URUGUAY. Paysandu, Guaviyu. Isolated from asymptomatic twig of *Myrcianthes cisplatensis*, asymptomatic twig of *Hexachlamis edulis*, and expanding lesion of stem canker on *Myrciaria tenella*, August 2006, C. Perez, paratype, living cultures UY107/CMW26742, herbarium PREM 60265, UY671/CMW26762/CBS 124907, herbarium PREM 60266, UY1263/CMW26788, herbarium PREM 60267, respectively.

Spencermartinsia uruguayensis C.A. Pérez, R.A. Blanchette, B. Slippers & M.J. Wingfield, sp. nov. (Fig. 7)

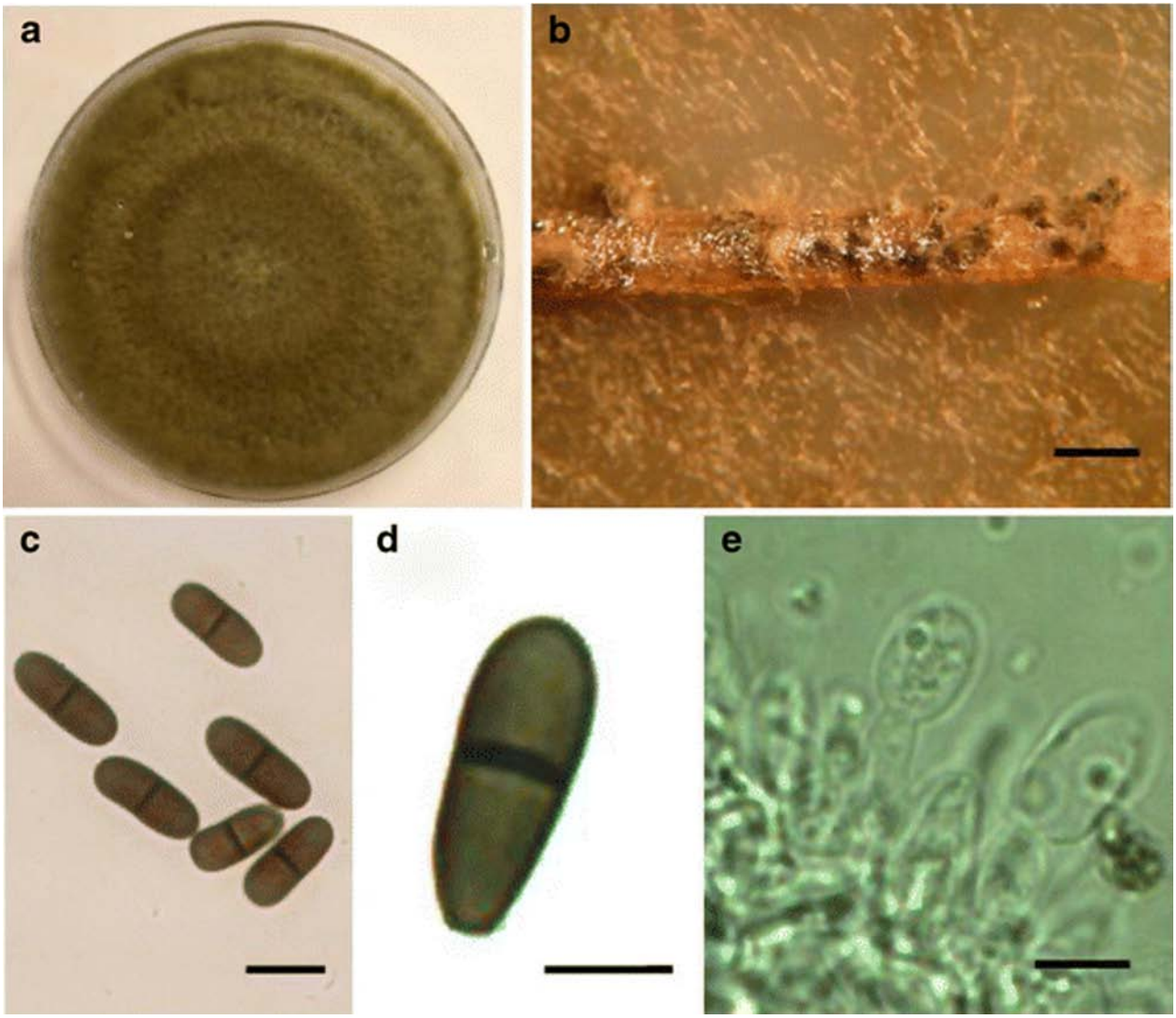

Fig. 7. Micrographs of fruiting structures of *Spencermartinsia uruguayensis* a. 1 week old colony grown on PDA; b. superficial pycnidia formed on pine needles; c. dark brown walled conidia, 1-septate; d. conidium slightly constricted at the septum, with broadly rounded apex and truncate base; e. conidiophores with immature conidia. Scale bars: b = 1 mm; c = 20 µm; d–e = 10 µm

MycoBank: MB 514538

Etymology: Name refers to the country Uruguay where this fungus was first found.

Pycnidia (formed in culture on sterilized pine needles) superficial, solitary, globose, black, non-papillate, covered by mycelium, up to 350 µm diam. Conidiogenous cells hyaline, subcylindrical. Conidia (17–) 22–22.5 (–26.5) × (7–) 9–9.5 (–12) µm, dark brown walled, 1-septate, slightly constricted at the septum, ovoid with broadly rounded apex and truncate base.

Pycnidia (in foliis sterilibus pini culta) superficialia, solitaria globosa nigra non papillata, mycelio tecta usque ad 350 µm diametro. Cellulae conidiogenae hyalinae subcylindricae. Conidia (17–) 22–22.5 (–26.5) × (7–) 9–9.5 (–12) µm, parietibus atrobrunneis, semel septata in septo parum constricta, ovoidea apice late rotundata basi truncata.

Teleomorph: unknown

Host: *Hexachlamis edulis*.

Known distribution: Uruguay

Specimen examined: URUGUAY. Paysandu, Tres Bocas. Endophytic infections on twigs of *Hexachlamis edulis*, August 2006, C. Perez, holotype PREM 60268, living cultures UY672, CMW26763, CBS 124908.

Discussion

Results of this study provide evidence that a diverse group of Botryosphaeriaceae occurs on both introduced *Eucalyptus* and native Myrtaceae trees in Uruguay. *Botryosphaeria dothidea*, *N. eucalyptorum* and members of the *N. parvum-N. ribis* complex were isolated from both *Eucalyptus* and native Myrtaceae, demonstrating biotic exchange between native and introduced Myrtaceae. In contrast, *L. pseudotheobromae* was restricted to *Myrcianthes pungens*. In addition, two novel species of the Botryosphaeriaceae were isolated from Myrtaceae native to Uruguay. Of these, *D. pseudoseriata* occurred in healthy tissue of six different Myrtaceae species and it was also associated with a stem canker observed on *Myrciaria tenella*. *Spencermartinsia uruguayensis* was obtained from endophytic infections on *Hexachlamis edulis*.

Pathogenicity tests showed that isolates obtained from native Myrtaceae are able to infect *Eucalyptus*. Of these, isolates in the *N. parvum-N. ribis* group and *L. pseudotheobromae* were highly pathogenic, killing a significant area of stem tissue and resulting in large cankers. In contrast, *D. pseudoseriata* and *S. uruguayensis* showed no differences from the control ($P>0.10$) and this may indicate that these isolates are not Eucalyptus pathogens. To the best of our knowledge *L. pseudotheobromae* has not been found occurring on *Eucalyptus* in Uruguay and these results highlight the importance of considering native forests when assessing the potential for host shifts between pathogens.

Botryosphaeria dothidea was confirmed as an endophyte in *Eucalyptus* and native Myrtaceae hosts, but it was also found associated with stem cankers in *Eucalyptus*. This fungus was previously reported as an endophyte infecting *Eucalyptus* spp. (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, identifications of Botryosphaeriaceae prior to the application of DNA sequence comparisons must be considered with some circumspection as the name used most probably refers to a suite of different species and not one fungus. Thus, some of the isolates previously considered to be *B. dothidea* have subsequently been shown to represent *N. parvum* and N. ribis (Slippers *et al*. 2004a). Using a modern taxonomic concept for Botryosphaeriaceae, *B. dothidea* has rarely been 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). Consistent with this view, *B. dothidea* was not the most common Botryosphaeriaceae species isolated from Eucalyptus samples in the present study. Inoculation tests on *E. grandis* also showed that this fungus had a very low level of pathogenicity to these trees.

Neofusicoccum eucalyptorum has recently been shown to infect native Myrtaceae in Uruguay and cross pathogenicity was also shown to occur (Pérez *et al*. 2009). Prior to that study, this fungus was reported in Uruguay as an endophyte in *E. globulus* and it was also collected from bark lesions (Alonso 2004). This fungus was found in six different *Eucalyptus* species and it also represented 63% of the Botryosphaeriaceae isolated in this study. Smith *et al*. (2001) considered the pathogenicity of several isolates of *N. eucalyptorum* and showed that this fungus was consistently more pathogenic than *B. dothidea*.

It was not surprising to find isolates in the *N. parvum-N. ribis* complex in this study, as members of this group are commonly known to 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* represents different species. They also recommend caution when distinguishing between these two species based on morphological or single locus DNA sequence data. Preliminary evidence using ITS and EF-1α data (data not shown) could not conclusively resolve their identity and they possibly represent a distinct cryptic species in this complex. Pavlic *et al*. (2009) provided evidence confirming that *N. parvum* and *N. ribis* represent distinct species and also reported the presence of cryptic species into this complex. 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, most likely using RPB2 sequences in combination with ITS sequences as proposed by Pavlic *et al*. (2009).

The *N. parvum-N. ribis* group warrants further investigation to resolve the correct identification of isolates obtained in this study. This is especially so, because it was one of the most pathogenic species in this and previous studies (Pavlic *et al*. 2007; Mohali *et al*. 2009). Our results indicate that representatives of the *N. parvum-N. ribis* complex are widely present in both *Eucalyptus* and native Myrtaceae. The clear association of this complex with stem cankers on both hosts, together with the pathogenicity observed in inoculation tests, suggests that this group represents the most significant threat to trees in Uruguay.

Interestingly, *L. pseudotheobromae* was found in a single sample association with a stem canker on the native Uruguayan tree, *Myrcianthes pungens*. Inoculation tests also showed that it is pathogenic to *Eucalyptus*. This species, along with *L. parva*, has recently been shown to be a cryptic species previously misidentified as *L. theobromae* (Alves *et al*. 2008). Thus, previous references to *L. theobromae* must be considered with caution because they may actually refer to *L. pseudotheobromae* or other species.

Lasiodiplodia theobromae sensu lato 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). 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. Mohali *et al*. (2005) further demonstrated that there was no evidence of host specificity for this fungus and a high level of gene flow was found between populations occurring on different hosts.

Diplodia pseudoseriata was widely distributed in native Myrtaceae forests in Uruguay. Despite its common occurrence in close proximity to *Eucalyptus* plantations, it was not detected on this host in this study. The weak reactions observed after inoculation on *E. grandis* also suggest that this species is not pathogenic to this host. This fungus, thus far only known from native Myrtaceae in Uruguay, is possibly native to this region and unable to infect Eucalyptus. It is the only *Diplodia* species known from native Myrtaceae, and there are few records of this fungus on Angiosperms in the Southern Hemisphere (De Wet *et al*. 2008; Slippers and Wingfield 2007).

The presence of *S. uruguayensis* on the native *H. edulis* is intriguing. Despite the examination of a very large number of Myrtaceae samples, this species was found on a single sample suggesting that

it is a rare fungus on this host and in the area. This may indicate that this species was only very recently introduced, or if it is native to Uruguay, it may be more common on non-Myrtaceae hosts. Inoculation results suggest that it has only minor levels of pathogenicity.

The study here used traditional techniques to isolate botryosphaeriaceous endophytes. It is therefore likely that only quickly growing taxa were isolated and those that are slow growing or cannot grow in culture were not detected (Hyde and Soytong 2008). It would be interesting to apply techniques where DNA is isolated directly from samples (e.g. Guo *et al*. 2000, 2001; Nikolcheva and Bärlocher 2005; Duong *et al*. 2006; Seena *et al*. 2008; Tao *et al*. 2008; Curlevski *et al*. 2009; Nilsson *et al*. 2009), to reveal if other botryosphaericeous endophytes are present.

Although 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 provide a foundation to monitor the development of such diseases on native and non-native Myrtaceae in Uruguay in the future. In particular it will be important to study the 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, discovery off the relatively aggressive species, *L. pseudotheobromae*, on a native host demonstrates the relevance of surveying native forest trees for early detection of potential threats to *Eucalyptus* plantations.

Acknowledgments

This work was partially funded by INIA-Uruguay, project FPTA221. We also thank Forestal Oriental, Rivermol, Stora Enso and Weyerhaeuser financial and logistic support. We appreciate the assistance of Andrés Berrutti, Ana Terzaghi, Oscar Bentancur, Sofia Simeto, Gustavo Balmelli, Hugh Glen and Marieka Gryzenhout.

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