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Phylogeny of the Botryosphaeriaceae reveals patterns of host association

Juanita De Wet [a,b], Bernard Slippers [a,c,*], Oliver Preisig [d], Brenda D. Wingfield [a,c], Michael J. Wingfield [a]

[a] *Forestry and Agricultural Biotechnology Institute (FABI), Tree Pathology Co-operative Programme (TPCP), University of Pretoria, Pretoria 0002, South Africa*
[b] *Department of Microbiology and Plant Pathology, University of Pretoria, South Africa*
[c] *Department of Genetics, University of Pretoria, South Africa*
[d] *Inqaba Biotec, Pretoria, South Africa*

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Abstract

Three anamorph genera of the Botryosphaeriaceae namely *Diplodia*, *Lasiodiplodia* and *Dothiorella* have typically dark, ovoid conidia with thick walls, and are consequently difficult to distinguish from each other. These genera are well-known pathogens of especially pine species. We generated a multiple gene genealogy to resolve the phylogenetic relationships of Botryosphaeriaceae with dark conidial anamorphs, and mapped host associations based on this phylogeny. The multiple gene genealogy separated *Diplodia*, *Lasiodiplodia* and *Dothiorella* and it revealed trends in the patterns of host association. The data set was expanded to include more lineages of the Botryosphaeriaceae, and included all isolates from different host species for which ITS sequence data are available. Results indicate that *Diplodia* species occur mainly on gymnosperms, with a few species on both gymnosperms and angiosperms. *Lasiodiplodia* species occur equally on both gymnosperms and angiosperms, *Dothiorella* species are restricted to angiosperms and *Neofusicoccum* species occur mainly on angiosperms with rare reports on Southern Hemisphere gymnosperms. *Botryosphaeria* species with *Fusicoccum* anamorphs occur mostly on angiosperms with rare reports on gymnosperms. Ancestral state reconstruction suggests that a putative ancestor of the Botryosphaeriaceae most likely evolved on the angiosperms. Another interesting observation was that both host generalist and specialist species were observed in all the lineages of the Botryosphaeriaceae, with little evidence of host associated co-evolution.

Keywords: *Diplodia*; *Lasiodiplodia*; *Dothiorella*; Host association

1. Introduction

Most of the species of the Botryosphaeriaceae cause disease symptoms such as die-back and cankers on numerous woody and non-woody hosts, especially in combination with stress-inducing environmental conditions (Eldridge, 1961; Buchanan, 1967; Punithalingam and Waterston, 1970). Species of the Botryosphaeriaceae include well-recognized pathogens of forestry trees including the important pine pathogen, *Diplodia pinea* (Desm.) J. Kickx f.) (Eldridge, 1961; Swart and Wingfield, 1991), and *Botryosphaeria dothidea* (Moug. Fr.) Ces. and De Not. and *Neofusicoccum eucalyptorum* Crous, H. Smith and M.J. Wingf. that cause serious canker diseases on *Eucalyptus* L'Hér (Smith et al., 1994, 2001). These fungi also include pathogens of fruit trees such as *Diplodia seriata* De Not. (=*Botryosphaeria obtusa*) and *D. mutila* (Fr.) Mont. (Phillips et al., 2007; Slippers et al., 2007), grape vines including *N. australe* Crous, Slippers and A.J.L. Phillips and *N. luteum* Crous, Slippers and A.J.L. Phillips (Van Niekerk et al., 2004) and the Proteaceae including *Saccharata proteae* (Wakef.) Denman and Crous (Denman et al., 2003).

* Corresponding author.
E-mail address: bernard.slippers@fabi.up.ac.za (B. Slippers).

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The taxonomy of species in the Botryosphaeriaceae is commonly based on the morphology of the anamorph states, which are most frequently encountered in nature. However, overlapping morphological characteristics has emphasized the utility of applying DNA sequence comparisons to resolve species. In a more recent and broadly based phylogenetic study, 10 lineages were identified for the Botryosphaeriaceae and these were shown to represent several newly described genera (Crous et al., 2006). The genera currently treated in the Botryosphaeriaceae are thus *Diplodia* Fr./*Lasiodiplodia* Ellis and Everh./*Tiarosporella* Höhn, *Botryosphaeria* Ces. and De Not. (*Fusicoccum* anamorphs), *Macrophomina* Petr., *Neoscytalidium* Crous and Slippers, *Dothidotthia* Höhn (*Dothiorella* anamorphs), *Neofusicoccum* Crous, Slippers and A.J.L. Phillips (*Botryosphaeria*-like teleomorphs, *Dichomera*-like synanamorphs), *Pseudofusicoccum* Mohali, Slippers and M.J. Wingf., *Saccharata* Denman and Crous (*Diplodia*- and *Fusicoccum*-like synanamorphs), "*Botryosphaeria*" *quercuum* (Schwein.) Sacc. (*Diplodia*-like anamorph) and *Guignardia* Viala and Ravaz (*Phyllosticta* anamorphs). The genus *Botryosphaeria* now applies only to *B. dothidea*, *B. mamane* D.E. Gardner and *B. corticis* (Demaree and Wilcox) Arx and E. Müll. Where the taxonomy remain uncertain the name "*Botryosphaeria*" is used in the broad sense and as is the case for "*Botryosphaeria*" *quercuum*. While the study of Crous et al. (2006) brought new clarity to the taxonomy of the Botryosphaeriaceae, it also highlighted many remaining taxonomic problems. Particularly the identity and phylogenetic relationships of genera with *Diplodia*-like anamorphs of the Botryosphaeriaceae that either belongs to *Diplodia*, *Dothiorella* and *Lasiodiplodia*, remains unclear.

The taxonomy of genera of the Botryosphaeriaceae with *Diplodia*-like anamorphs (*Diplodia*, *Lasiodiplodia* and *Dothiorella*) is commonly confused. Their conidia are similar in size and shape (mostly ovoid with a length:width ratio of 2–3:1), thick-walled, and often only becoming pigmented and dematiaceous as they age. These characters make the *Diplodia*-like anamorph genera distinctly different from other anamorph genera of the Botryosphaeriaceae having hyaline, *Fusicoccum*-like conidia, and they might thus be expected to be related. It is therefore, not surprising that they have also previously been treated as synonyms of each other (Punithalingam and Waterston, 1970; Denman et al., 2000). Phillips et al. (2005), however, provided strong evidence to re-erect *Dothiorella* to accommodate isolates with dark and single septate conidia early in development unlike conidia of *Diplodia*-like anamorphs turning dark and multi-septated over time. The finding that they are phylogenetically more closely related to *Neofusicoccum* than to *Diplodia* provided strong support for this view (Phillips et al., 2005; Crous et al., 2006). The taxonomic status of *Diplodia* and *Lasiodiplodia* remains uncertain (Crous et al., 2006).

One well studied example, which illustrates the complexities of identifying species of the Botryosphaeriaceae with *Diplodia*-like anamorphs, is found in the *D. pinea* species complex. All species with dematiaceous conidia associated with disease symptoms on *Pinus* L. spp. were initially treated as *D. pinea* (=*Sphaeropsis sapinea* (Fr.) Dyko and B. Sutton) (Waterman, 1943; Punithalingam and Waterston, 1970). *Diplodia pine*a has been differentiated based on different morphological types, that have been referred to as the A, B, C and I morphotypes (Wang et al., 1985; Palmer et al., 1987; Smith and Stanosz, 1995; Hausner et al., 1999; De Wet et al., 2000, 2002). Multiple gene genealogies for these fungi have, however, shown that the A, B and C morphotypes represent two distinct species. *Diplodia pinea* is the best known species and an important pine pathogen that occurs in two morphological forms referred to as the A and C morphotypes (De Wet et al., 2000, 2002). The B morphotype of *D. pinea* has been described as *D. scrobiculata* J. de Wet, Slippers and M.J. Wingf. (De Wet et al., 2003). Isolates designated as the I morphotype of *D. pinea* represent *D. seriata* (Burgess et al., 2001).

In the past, host association was often used to distinguish or describe species of the Botryosphaeriaceae. It has, however, become clear that host association is not always a good indication of species delineation in this family. Certain Botryosphaeriaceae are clearly generalist species, able to infect a wide range of unrelated hosts (e.g. *B. dothidea*, *L. theobromae* (Pat.) Griffon and Maubl. and *D. seriata*). Others are more specialized and appear to infect only a specific host genus or group of related host genera (e.g. *N. eucalyptorum* and *N. eucalypticola* Slippers, Crous and M.J. Wingf.). The difficulties associated with identifying many members of the Botryosphaeriaceae using morphological characteristics has, however, made it difficult to study host association patterns in the group. Such host association patterns are important when seeking to understand the driving forces of evolution in the group, patterns of co-evolution with specific hosts, as well as, for pathology and epidemiology studies. Large numbers of sequences are becoming available for species in the Botryosphaeriaceae, and a consideration of host association patterns has become possible.

The primary aim of this study was to generate a multiple gene genealogy for species of the Botryosphaeriaceae with *Diplodia*-like anamorphs. In order to further explore the host association patterns that became apparent amongst *Diplodia*-like anamorphs of the Botryosphaeriaceae, we expanded the initial sampling set by including all isolates of six of the 10 lineages of the Botryosphaeriaceae as described by Crous et al. (2006) with ITS sequence representation in GenBank, and for which host data are available.

2. Materials and methods

2.1. Fungal isolates

A collection of 23 *Diplodia*-like isolates from various regions and hosts was included in this study (Table 1). Sequence data for various Botryosphaeriaceae not

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Table 1
Diplodia and *Dothiorella* isolates included in this study

Isolates	Identification	Origin	Host	Other collections	Collector	GenBank Accession numbers		
						ITS	βT2	ACT
CMW1182	*D. cupressi*	Israel	*Cupressus sempervirens*		W. Swart (Swart et al., 1993)			
CMW1183	*D. cupressi*	Israel	*Cupressus sempervirens*		W. Swart (Swart et al., 1993)			
CMW8745	*D. pinea* (A)	Michigan	*Pseudotsuga menziesii*	150	M. Palmer			
CMW8750	*D. pinea* (A)	Great Britain	*Pseudotsuga menziesii*	94-165	J. Gibbs			
CMW13233	*D. seriata*	France	*Pseudotsuga menziesii*	BOT1109	P. Chandelier			
CMW8746	*D. scrobiculata*	France	*Cedrus deodora*	94-17	P. Chandelier			
CMW12514	*D. seriata*	France	*Cedrus* sp.	BOT1101	P. Chandelier			
CMW13234	*D. pinea* (A)	France	*C. deodora*	BOT1112	P. Chandelier			
CMW12513	*D. pinea* (A	France	*Larix* sp.	BOT1100	P. Chandelier			
CMW12516	*D. seriata*	France	*Abies grandis*	BOT1097	P. Chandelier			
CMW12284	*D. seriata*	Minnesota	*Picea abies*	MNS3/BOT2834	M.J. Wingfield			
CMW12283	*D. scrobiculata*	Ontario	*P. mariana*	U9596/BOT2833	J. Reid (Hausner et al., 1999)			
CMW4854	*Dothiorella* sp.	Canberra, Australia	*Casuarina* sp.		M.J. Wingfield			
CMW4855	*Dothiorella* sp.	Canberra, Australia	*Casuarina* sp.	CBS120688	M.J. Wingfield	DQ846773	DQ875340	DQ846781
CMW4856	*Dothiorella* sp.	Canberra, Australia	*Casuarina* sp.	CBS120689	M.J. Wingfield	DQ846772	DQ875339	DQ846780
CMW4857	*Dothiorella* sp.	Canberra, Australia	*Casuarina* sp.	CBS120690	M.J. Wingfield	DQ846774	DQ875341	DQ846782
CMW4858	*Dothiorella* sp.	Canberra, Australia	*Casuarina* sp.		M.J. Wingfield			
CMW14654	*D. pinea* (C)	Sulawesi, Indonesia	*P. merkusii*	[1]1	M.J. Wingfield			
CMW14655	*D. pinea* (C)	Sulawesi, Indonesia	*P. merkusii*	[1]6	M.J. Wingfield			
CMW14656	*D. pinea* (C)	Sulawesi, Indonesia	*P. merkusii*	[2]1	M.J. Wingfield			
CMW14657	*L. theobromae*	Cuba	*P. caribaea*	Cuba5	M.J. Wingfield			
CMW14658	*L. theobromae*	Cuba	*P. caribaea*	Cuba12	M.J. Wingfield			
CMW14659	*L. theobromae*	Cuba	*P. caribaea*	Cuba17	M.J. Wingfield			

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generated in this study were obtained from GenBank (Table 2). European isolates used in the study were provided by Dr. Pierre Chandelier (INRA-French National Institute for Agricultural Research, Nancy, France). All the other isolates were accessed from the Culture Collection (CMW) of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Isolates were transferred to 2% water agar (WA) (Biolab Diagnostics, Midrand, South Africa), with a few sterile pine needles placed on the agar surface, and incubated at 25 °C in constant light to induce sporulation. Single conidial isolates were generated, and these were grown on 2% malt extract agar (MEA) (Biolab Diagnostics, Midrand, South Africa) at 25 °C. All cultures were stored at 4 °C for further study.

2.2. DNA extractions, amplification and sequencing

DNA was extracted from the freeze-dried mycelium of the 23 single conidial isolates (Table 1). The isolates were grown in 500 µl of 2% ME broth in 1.5 ml Eppendorf tubes, incubated at 25 °C, one week prior to the DNA extraction. The broth was then removed through centrifugation, 20 min at 13,000 rpm, washed with distilled water and freeze-dried. DNA was extracted using the technique described by Raeder and Broda (1985).

The internally transcribed spacer (ITS) regions 1 and 2 and the 5.8S ribosomal subunit (White et al., 1990), Bt2 regions of the β-tubulin gene (Glass and Donaldson, 1995) and partial sequences of the protein-coding gene, actin (ACT) (Carbone and Kohn, 1999) were amplified (Table 1). The gene regions were amplified using primers and conditions as described previously (De Wet et al., 2000, 2003).

PCR products were visualized on a 1% agarose gel containing ethidium bromide using UV illumination. The PCR products were purified using the Roche High Pure PCR product purification kit (Roche Diagnostics, Germany). Both DNA strands were sequenced using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing kit and an ABI PRISM® 3100 DNA sequencer (Applied Biosystems, Foster City, CA 94404, USA). All the reactions were done using protocols recommended by the manufacturers. All the sequence data were processed using Sequence Navigator version 1.0.1 (Perkin-Elmer) and aligned using MAFFT version 5 (Katoh et al., 2005).

2.3. Phylogenetic analyses

BLAST searches in GenBank were performed using ITS sequence data. Two data sets were generated. One of these combined ITS, Bt2 of β-tubulin and ACT sequence data to distinguish between closely related *Diplodia*-like isolates from different coniferous hosts and geo-

Table 2
Isolates of *Diplodia pinea*, *D. scrobiculata* and various *Botryosphaeria* spp. included in this study for comparative purposes

Isolates	Identification	Origin	Host	Reference	GenBank Accession numbers		
					ITS	βT2	ACT
CMW190	*Diplodia pinea* (A)	United States	*P. resinosa*	Palmer et al., 1987; De Wet et al., 2000, 2003	AY253290	AY624256	AY624261
CMW4876	*D. pinea* (C)	Northern Sumatra, Indonesia	*P. patula*	De Wet et al., 2000, 2003	AY253294	AY624257	AY624262
CMW5870	*D. scrobiculata*	California	*P. radiata*	De Wet et al., 2003	AY623704	AY625259	AY624264
CMW4900	*D. scrobiculata*	Mexico	*P. greggii*	De Wet et al., 2003	AY623705	AY624260	AY624265
CMW189	*D. scrobiculata*	USA	*P. banksiana*	Palmer et al., 1987; De Wet et al., 2000, 2003	AY253292	AY624258	AY624263
CMW8230	*D. seriata*	Canada	*Picea glauca*	De Wet et al., 2003	AY972104	AY972119	AY972110
CMW8232	*D. seriata*	South Africa	*Malus domestica*	De Wet et al., 2003	AY972105	AY972120	AY972111
CMW9074	*Lasiodiplodia theobromae*	Mexico	*Pinus sp.*	Slippers et al., 2004a	AY236952	AY236930	AY972108
CMW10130	*Lasiodiplodia theobromae*	Uganda	*Vitex doniana*	Slippers et al., 2004a	AY236951	AY236929	AY972109
CMW7060	*D. mutila*	Netherlands	*Fraxinus excelsior*	Slippers et al., 2004a	AY236955	AY236933	AY972112
CMW7776	*D. mutila*	Pusiona, Italy	*Fraxinus excelsior*	Slippers et al., 2004a	AY972106	AY972121	AY972113
CMW7781	*D. mutila*	Porza, Switzerland	*Fraxinus excelsior*	Slippers et al., 2004a	AY972107	AY972122	AY972114
CMW7772	*Neofusicoccum ribis*	New York, USA	*Ribes sp.*	Slippers et al., 2004a	AY236935	AY236906	AY972115
CMW7773	*D. mutila*	New York, USA	*Ribes sp.*	Slippers et al., 2004a	AY236936	AY236907	AY972116
CMW7999	*B. dothidea*	Crocifisso, Switzerland	*Ostrya* sp.	Slippers et al., 2004a	AY236948	AY236926	AY972117
CMW8000	*B. dothidea*	Crocifisso, Switzerland	*Prunus* sp.	Slippers et al., 2004a	AY236949	AY236927	AY972118

graphical regions. The other data set was based only on ITS sequence data for selected species of the Botryosphaeriaceae, from all hosts available on GenBank. Six of the 10 lineages as described by Crous et al. (2006) were included. *Macrophomina*, *Guignardia*, "*Botryosphaeria*" *quercuum* and *Saccharata* were excluded as either their taxonomy is uncertain, or they group outside the phylogeny considered here. *Tiarosporella*, which grouped with *Diplodia* in Crous et al. (2006), was not included in this study as corresponding ITS sequence data was not available on GenBank.

At the time of analysis, 771 ITS sequences were available in GenBank for the Botryosphaeriaceae. A total of 134 of these sequences were used in this study, representing one ITS sequence for each species from a unique host. The aim of this analysis was to generate a global view of as many species of the Botryosphaeriaceae from unique hosts as possible and thus to consider their host associations. When more than one sequence was available representing the same species from the same host, one was chosen randomly. Because these data in GenBank was not expected to represent the full host ranges of all the species we compared the of host ranges represented by the ITS sequence data with published host ranges (e.g. SBML Fungus-Host Distribution Database http://nt.ars-grin.gov/fungaldatabases/fungushost/FungusHost.cfm and other published literature). The value of literature records of these species on various hosts is, however, weakened by the uncertainty surrounding reports of species of the Botryosphaeriaceae based solely on morphology. Following this process we were convinced that the overall patterns of host association for the genera were as accurate as possible.

Parsimony, distance (NJ), maximum likelihood (ML) and Bayesian analyses were applied to all data sets. Introns occurring in the partial gene sequences of Bt2 of β-tubulin and ACT were included in the phylogenetic analyses. All characters were treated as unordered and having equal weight. Partition homogeneity tests were performed on the combined data sets to determine whether there was congruency between the different phylogenies using PAUP* (Swofford, 2002). The phylogenetic signal ($G1$) of the data sets was determined using PAUP* and compared with critical values (Hillis and Huelsenbeck, 1992) at the 0.01 and 0.5 confidence levels.

Parsimony was based on strict heuristic searches with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum length is zero. Neighbor-joining distance analysis was done in PAUP* using the most appropriate model of DNA substitution as determined with MODELTEST 3.5 (Posada and Crandall, 1998). Maximum likelihood was also performed in PAUP* using the parameters as determined with MODELTEST 3.5 (Posada and Crandall, 1998). Bayesian analysis using MrBayes 3.0b4, implementing the Markov Chain Monte Carlo (MCMC) technique (Huelsenbeck and Ronquist, 2001) and the parameters predetermined with MODELTEST 3.5 was used. Trees were sampled every 100 generations. The first 500 of 500,000 trees were discarded (burnin = 200). The Bayesian analysis was repeated to test the independence of the results from topological priors. Bootstrap support for all four analyses was determined after 1000 replications and only groups with frequencies >50% were retained. The character state reconstruction was done in MacClade ver. 4 (Maddison and Maddison, 2000). All phylogenetic trees were viewed in TreeView and monophyletically rooted to *Mycosphaerella* spp. as outgroups (*M. konae* Crous, Joanne E. Taylor and M.E. Palm: ITS = AY260085, BT2 = AY725606, ACT = AY752213, EF-1α = AY752185 and *M. citri* Whiteside: ITS = AY752145; EF-1α = AY752179). *Mycosphaerella konae* was used in both data sets as an outgroup because it has sequences for all the relevant gene areas available on GenBank.

3. Results

3.1. Phylogenetic analysis of the Botryosphaeriaceae with Diplodia-like anamorphs (Fig. 1)

A collection of *Diplodia*-like isolates from coniferous hosts were included in this data set to determine their identity, as well as to derive information regarding specificity. The ITS region of the rDNA operon and parts of two protein-coding genes were successfully amplified for all the isolates included in this study (Table 1). Sequences generated from the amplification products ranged from 266 to 554 bp in length. A partition homogeneity test showed no significant conflict between the phylogenies of the rDNA, BT2 of β-tubulin or ACT ($P > 0.01$). The $G1$-value ($G1 = -0.73$) was lower than the predicted critical values at both the 95% ($P = -0.08$) and 99% ($P = -0.09$) confidence levels, implying strong phylogenetic signal for the data set. The combined data set contained 1306 characters of which 587 characters were constant, 296 were variable, parsimony uninformative characters and 423 were variable, parsimony informative characters. The data set had a consistency index (CI) of 0.65, a retension index (RI) of 0.81 and a homoplasy index (HI) of 0.35. MODELTEST 3.5 tested 56 models and predicted a transitional (TIM) model with a proportion of invariable sites (I) and gamma distribution shape parameter (G) as the most appropriate model of DNA substitution.

Two major clades emerged from the constructed phylogram (Fig. 1). One of these represented *Diplodia* and *Lasiodiplodia* and the other included *Botryosphaeria*, *Dothiorella* and *Neofusicoccum*. The *Diplodia*/*Lasiodiplodia* clade consisted of seven sub-clades including the A and C morphotypes of *D. pinea*, *D. scrobiculata*, *D. seriata*, *D. cupressi*, *D. mutila* and *L. theobromae*. The *Botryosphaeria*/*Neofusicoccum*/*Dothiorella* clade consisted of *B. dothidea*, *N. ribis* Slippers, Crous and M.J. Wingf., and an undescribed species of *Dothiorella* from *Casuarina*.

All isolates in the sub-clade containing the A morphotype of *D. pinea* were from various conifer hosts including *P. resinosa* Sol. ex Aiton, *Pseudotsuga menziesii* (Mirb.) Franco, *Cedrus deodora* (Roxb.) G. Don and a *Larix* Miller

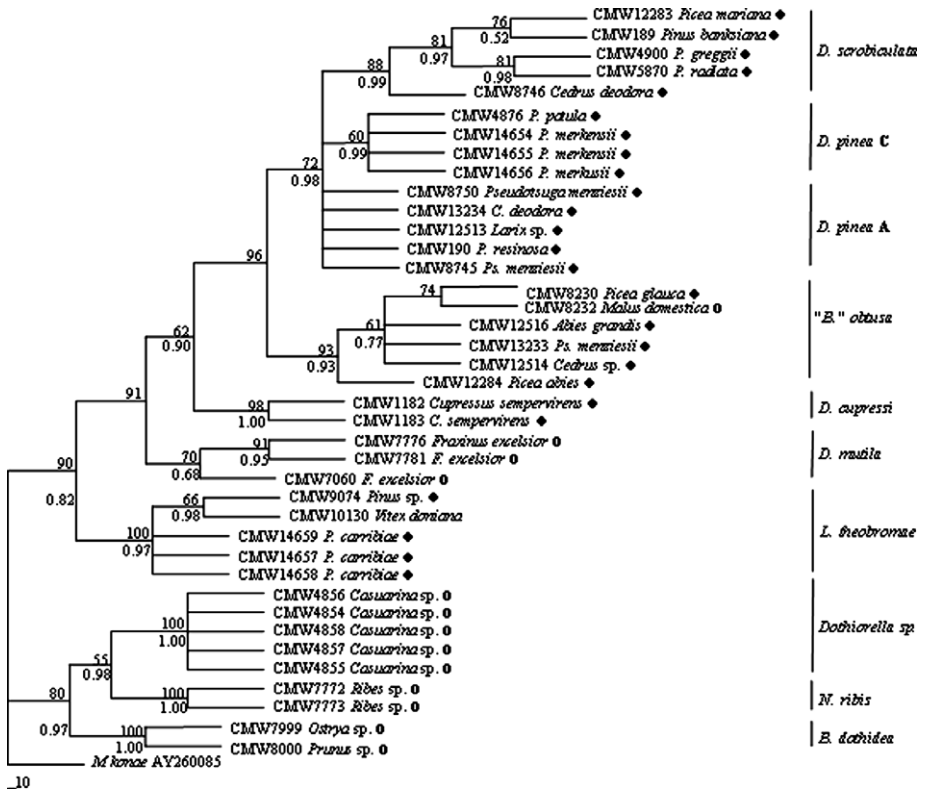

Fig. 1. Phylogram constructed for the combined sequence data of the ITS regions and 5.8S rDNA operon and two partial protein-coding genes (Bt2 of β-tubulin and ACT) based on neighbor-joining distance analysis with branch support values (maximum parsimony bootstrap proportions/Bayesian posterior probabilities). Bootstrap values were determined after 1000 replications using parsimony based on a strict heuristic search with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum length is zero. Only groups with frequencies Q2 >50% were retained. Isolates marked with ♦ are from Gymnosperms and isolates marked with o are from Angiosperms.

sp. These host species reside in the Pinales and Pinaceae, and they are represented by three sub-families, i.e. Pinoideae (*Pinus*), Laricoideae (*Larix* and *Pseudotsuga*) and Abietoideae (*Cedrus*).

The sub-clade representing *D. pinea* C morphotype, included three isolates (CMW14654, CMW14655 and CMW14656) recognized for the first time originating from *P. merkusii* in Sulawesi (Indonesia). They grouped with the previously described C morphotype isolate (CMW4876) from *P. patula* in Northern Sumatra (Indonesia).

The *D. scrobiculata* sub-clade contained isolates from *P. greggii* Engelm. ex Parl., *P. radiata* D. Don, *P. banksiana* Lamb., *Picea mariana* (Mill.) Britton, Sterns and Poggenburg and *C. deodora*. These hosts are all conifers residing in the Pinales and Pinaceae and they are represented by three sub-families, i.e. Pinoideae (*Pinus*), Piceoideae (*Picea*) and Abietoideae (*Cedrus*).

The *D. seriata* sub-clade contained isolates from a diverse range of hosts that includes angiosperms (*Malus domestica* Borkh.) as well as gymnosperms residing in the Pinales and Pinaceae and they are represented by three sub-families i.e. Piceoideae (*Picea*), Abietoideae (*Abies*, *Cedrus*) and Laricoideae (*Pseudotsuga*).

The *Lasiodiplodia* sub-clade is represented only by *L. theobromae* isolates from *Pinus* spp. and *Vitex doniana* Sweet.

3.2. Phylogenetic analysis for six lineages of the Botryosphaeriaceae (Fig. 2)

A total of 134 ITS sequences representing six of the 10 lineages of the Botryosphaeriaceae from every distinct host species available on GenBank were included. The *G*1-value ($G1 = -0.43$) was less than the predicted critical values at both the 95% ($P = -0.08$) and 99% ($P = -0.09$) confidence levels implying strong phylogenetic signal for the data set. The data set contained 564 characters of which 236 characters were constant, 51 were variable, parsimony uninformative characters and 277 were variable, parsimony informative characters. The data set had a consistency index (CI) of 0.52, a retension index (RI) of 0.90 and a homoplasy index (HI) of 0.48. MODELTEST 3.5 tested 56 models and predicted a transitional (TIM) model with a proportion of invariable sites (*I*) and a gamma distribution shape parameter (*G*) as the most appropriate model of DNA substitution.

In the resulting phylogram, seven lineages can be distinguished (Fig. 2). *Diplodia* and *Lasiodiplodia* isolates grouped in two separate lineages and were not unresolved as one lineage as was found based on large subunit sequence data (Crous et al., 2006). The *Diplodia* clade includes *D. seriata*, *D. pinea*, *D. scrobiculata* and *D. mutila*. *Diplodia seriata* occurs on a wide range of angiosperms and

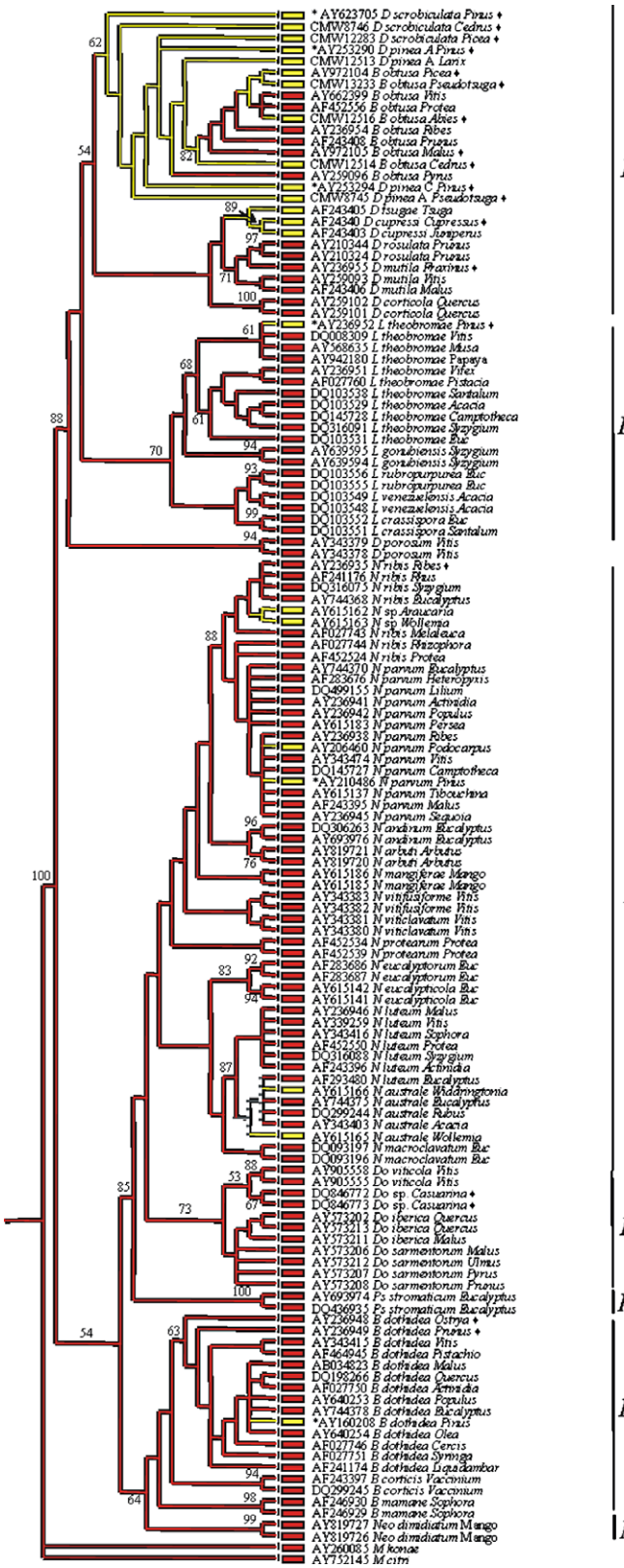

Fig. 2. Phylogram constructed for the ITS and 5.8S rDNA based on neighbor-joining distance analysis with branch support values (maximum parsimony bootstrap proportions). Bootstrap values were determined after 1000 replications using parsimony based on a strict heuristic search with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum length is zero. Only groups with frequencies >50% were retained. Gymnosperm/angiosperm character states were traced in MacClade. Isolates marked with ▬ are from Gymnosperms and isolates marked with ▬ are from Angiosperms. Isolates marked with an asterisk ∗ are from *Pinus* spp. *Pinus* is arguably the most extensively sampled host for the Botryosphaeriaceae. The dominating species are *D. pinea*, *D. scrobiculata* and *L. theobromae*. Reports of *B. dothidea* and *N. parvum* on this host are two rare exceptions, only observed once in each case. Isolates marked with ♦ were included in Fig. 1.

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gymnosperms. *Diplodia pinea* and *D. scrobiculata* occur only on gymnosperms, and *D. mutila* only on angiosperms. Some species such as *D. corticola* Phillips, Alves and Luque from *Quercus* L., *D. porosum* from *Vitis* L., *D. rosulata* Gure, Slippers and Stenlid from *Prunus* L. and *D. cupressi* from *Cupressus* appear to be restricted to a single host genus. In previous studies, isolates from cankers on *Juniperus* L. were identified as *D. mutila* and they were considered to be closely related to *D. cupressi* (Swart et al., 1993; Stanosz et al., 1998; Zhou and Stanosz, 2001). Results of this study, however, indicate that *D. mutila* from *Juniperus* represents *D. cupressi*.

In the *Lasiodiplodia* clade, isolates of *L. theobromae* all grouped together and they originated from a wide variety of hosts including both angiosperms and gymnosperms. *Lasiodiplodia venezuelensis* Burgess, Barber and Mohali from *Acacia* Miller, *L. rubropurpurea* Burgess, Barber and Pegg from *Eucalyptus*, *L. crassispora* Burgess and Barber from *Eucalyptus* and *Santalum* L., and *L. gonubiensis* Pavlic, Slippers and M.J. Wingf. from *Syzygium* Gaertn. also resided in this clade.

The *Neofusicoccum* clade included two species complexes. These were *N. ribis*/*N. parvum* and *N. luteum*/*N. australe* that occur on hosts including a wide variety of angiosperms and gymnosperms including *Araucaria* Juss., *Wollemia* Jones, Hill and Allen, *Widdringtonia* Endl., *Pinus* and *Podocarpus* Labill. Each of the other nine *Neofusicoccum* species in this clade was associated with only one host. These were *N. vitifusiforme* Crous, Slippers and A.J.L. Phillips from *Vitis*, *N. viticlavatum* Crous, Slippers and A.J.L. Phillips from *Vitis*, *N. eucalyptorum* from *Eucalyptus*, *N. eucalypticola* from *Eucalyptus*, *N. arbuti* Crous, Slippers and A.J.L. Phillips from *Arbutus* L., *N. andinum* Mohali, Slippers and M.J. Wingf. form *Eucalyptus*, *N. macroclavatum* T. Burgess, Barber and L.M. Hardy from *Eucalyptus*, *N. mangiferae* Crous, Slippers and A.J.L. Phillips from *Mangifera* L. and *N. protearum* Crous, Slippers and A.J.L. Phillips from *Protea* spp.

The *Dothiorella* clade included *Do. iberica* and *Do. sarmentorum*. These fungi are associated with various host genera but they are all angiosperms. The other two species in this clade were associated with only one host. They are *Do. viticola* from *Vitis* and a potentially undescribed species of *Dothiorella* from *Casuarina*.

The *Botryosphaeria* clade included two species. One of these is *B. dothidea* that occurs on a wide variety of angiosperms and occasionally on gymnosperms. The other species that resides in this clade is *Botryosphaeria corticis* (Demaree and Wilcox) Arx and E. Müll. from *Vaccinium* L.

The *Neoscytalidium* clade included two species, *N. dimidiatum* Crous and Slippers from *Mangifera* and "*Botryosphaeria*" *mamane* D.E. Gardner from *Sophora* L. They are known only from these hosts. The *Pseudofusicoccum* clade included *Ps. stromaticum* Mohali, Slippers and M.J. Wingf. only known from *Eucalyptus*.

4. Discussion

In this study, we provide strong supportive evidence for the distinction between *Diplodia*, *Lasiodiplodia* and *Dothiorella* as separate genera, based on sequence data from two protein-coding loci, as well as the ITS region of the rDNA operon. The study also confirms the phylogenetic relationship of these genera to genera with *Fusicoccum* anamorphs such as *Botryosphaeria* and *Neofusicoccum* (Jacobs and Rehner, 1998; Denman et al., 2000; Zhou and Stanosz, 2001). Furthermore, based on results of all available sequence data, *Diplodia* and *Lasiodiplodia* species are shown to commonly occur on both gymnosperms and angiosperms. All the other Botryosphaeriaceae lineages (excluding *Macrophomina*, *Guignardia*, *Saccharata* and "*Botryosphaeria*" *quercuum*) are predominantly found on angiosperms, with rare exceptions on gymnosperms. Interestingly, these are only from Southern Hemisphere conifers in the Araucariaceae and single reports from non-native pines in the Southern Hemisphere. These results suggest that the ancestors of the Botryosphaeriaceae evolved on angiosperms, and only later colonized and speciated on gymnosperms.

The multiple gene genealogy generated in this study, supports the separation of all three genera with *Diplodia*-like anamorphs. Despite the morphological similarities between *Diplodia*, *Lasiodiplodia* and *Dothiorella*, *Dothiorella* shares a more recent common ancestor with morphologically distinct genera such as *Neofusicoccum* and *Botryosphaeria*. This could be due to convergent evolution or simply because this character (*Diplodia*-like conidia) predates the separation of the main genera in Botryosphaeriaceae. The latter hypothesis might be most feasible because there are groups with both conidial forms for example *Saccharata* and *Dichomera* anamorphs of *Neofusicoccum* and *Botryosphaeria* that are superficially more similar to anamorphs with *Diplodia*-like conidia than those with *Fusicoccum*-like conidia.

Several species in the *Diplodia* clade could be distinguished in this study. These include both morphological forms (A and C morphotypes) of *D. pinea,* the well-known opportunistic, stress-associated die-back pathogen of pines (Swart and Wingfield, 1991; De Wet et al., 2000), *D. scrobiculata* that was previously known as the B morphotype of *D. pinea* (De Wet et al., 2003), *D. cupressi* previously treated as *D. pinea* f.sp. *cupressi* (Alves et al., 2006), *D. mutila* and *D. seriata* (Phillips et al., 2007). Many of these species have been confused in the past due to their morphological similarity (Wang et al., 1985; Swart et al., 1993; Smith and Stanosz, 1995; Stanosz et al., 1998; Burgess et al., 2001; Zhou and Stanosz, 2001). Cryptic species can, however, be distinguish when using multiple gene genealogies as has been shown previously (De Wet et al., 2000, 2003; Alves et al., 2006) and in the present study.

The multiple gene genealogy generated in this study confirms the wide host range of the A morphotype of *D. pinea* that includes various *Pinus* spp., *C. deodora*, *Pseudotsuga*

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menziesii and a *Larix* sp. This supports previous studies that have demonstrated a wide distribution and host range of the A morphotype of *D. pinea* (Stanosz et al., 1999; Zhou and Stanosz, 2001). The C morphotype of *D. pinea* is very closely related to the A morphotype based on DNA sequence data, but is morphologically distinct, more pathogenic and has a very restricted distribution (De Wet et al., 2000). This form of *D. pinea* was initially described from *P. patula* in Northern Sumatra, Indonesia (De Wet et al., 2000) and in this study it is also recognized from *P. merkusii* in Sulawesi, Indonesia. Unlike *P. patula*, this is a native pine in Asia and it is most likely the source of isolates found on the former species, which is grown as a non-native in plantations. Together these data strongly suggest that the C morphotype of *D. pinea* should be recognized and described as a distinct species.

Diplodia scrobiculata was initially found to be different from *D. pinea* (Palmer et al., 1987) and mainly associated with *P. resinosa* and *P. banksiana* in the North Central United States (Smith and Stanosz, 1995). It was later also reported from other *Pinus* spp., as well as *Cedrus* spp. in Europe and Israel (Stanosz et al., 1999; De Wet et al., 2000). Results of the present study have expanded the host range of *D. scrobiculata* to include *Picea mariana*. The host ranges of *D. pinea* and *D. scrobiculata* include only gymnosperms in the Pinaceae but both species appear not to be host-specific below this phylogenetic level.

Hosts of *D. seriata* include both gymnosperms and angiosperms. It is a generalist species reported from a wide variety of host genera (Punithalingam and Waller, 1973). *Diplodia mutila* is also a generalist species able to infect a wide range of angiosperms (Jacobs and Rehner, 1998; Zhou and Stanosz, 2001) and the single report of this fungus from a *Juniperus* sp. (Tisserat et al., 1988) was shown in this study to be *D. cupressi*. The host range of *D. cupressi* includes only gymnosperms in the Cupressaceae (Alves et al., 2006).

In most previous studies, the *Lasiodiplodia* clade of the Botryosphaeriaceae has been represented by sequence data from only one species, *L. theobromae*. In GenBank this species is represented by isolates from *Pinus*, *Vitis*, *Musa*, *Santalum*, *Carica papaya*, *Acacia*, *Camptotheca, Syzygium*, *Fraxinus*, *Vitex* and *Eucalyptus*. This fungus is thus a generalist species able to infect both angiosperms and gymnosperms. It is well known that *L. theobromae* is generally found in tropical and subtropical regions on an extremely wide host range (Punithalingam, 1976). Other *Lasiodiplodia* species are also predominant in tropical and subtropical regions, and most are also not host-specific. These include *L. gonubiensis* (Pavlic et al., 2004), *L. venezuelensis*, *L. rubropurpurea* and *L. crassispora* (Burgess et al., 2006). They do, however, seem to be associated only with angiosperms. *Lasiodiplodia* remains undersampled in most studies, including in this one, and needs dedicated collections and taxonomic attention if its true status is to be confirmed.

Dothiorella is represented by four species. These are *Do. sarmentorum* from *Malus*, *Ulmus*, *Pyrus* and *Prunus*, *Do. iberica* from species of *Quercus* and *Malus*, *Do. viticola* from *Vitis* spp and a potentially undescribed species from *Casuarina* spp. The latter species should be compared to other species described from this host and area to determine its species status, and be formally described if none exist. All the *Dothiorella* species, for which sequence data are available, are only known from angiosperms (Phillips et al., 2005).

Interesting trends were observed in host association for the lineages of the Botryosphaeriaceae investigated. Some *Diplodia* species (*D. pinea*, *D. scrobiculata* and *D. cupressi*) occur exclusively on gymnosperms, and other *Diplodia* species (*D. mutila* and *D. seriata*) on both gymnosperms and angiosperms. *Lasiodiplodia* species occur on both gymnosperms and angiosperms, and the phylogenetically more distant *Dothiorella* species only on angiosperms. *Neoscytalidium* and *Pseudofusicoccum* are known only from angiosperms. *Botryosphaeria* spp. are also known exclusively from angiosperms although there is a single report from *P. nigra* in Lexington, Kentucky (Flowers et al., 2003). This, however, represents only one isolate, and extensive world-wide studies on conifers in native and introduced environments have shown that this is not a general trend (De Wet et al., 2000; Burgess et al., 2004). Species of *Neofusicoccum* also occur mostly on angiosperms. There are, however, some interesting exceptions, all on Southern Hemisphere conifers. These include an undescribed *Neofusicoccum* sp. from *Wollemia* and *Araucaria*, *N. australe* from *Wollemia* and *Widdringtonia* in Australia and South Africa (Slippers et al., 2005b), and single reports of *N. parvum* on *P. patula* (Gezahgne et al., 2003) and *Podocarpus falcatus* (Gure et al., 2005) in Ethiopia.

Analyses of host association for the six lineages of the Botryosphaeriaceae have shown that most species have been reported only from angiosperms, or in a few cases both angiosperms and gymnosperms. Very few species are known exclusively from gymnosperms. Angiosperms thus appear to be the most common, and possibly ancestral, host group of the Botryosphaeriaceae (excluding *Macrophomina*, *Guignardia*, *Saccharata* and "*Botryosphaeria*" *quercuum*). Infection of gymnosperms most likely occurred more recently in specific groups via host shifts, as there appears to be little evidence for host associated co-evolution amongst species of the Botryosphaeriaceae. This is perhaps not surprising, given that many species are not host-specific. The close relationship between some species occurring predominantly on either gymnosperms or angiosperms (or different families within the gymnosperms) indicates that host shifts between distantly related groups of plants are not uncommon, and could have been an important driver of speciation in the group. Understanding these patterns of host shift is important, as they can often lead to disease or epidemic outbreaks (Slippers et al., 2005a).

Host association patterns in the Botryosphaeriaceae are largely unexplored. This is partly due to taxonomic problems that have been associated with the group and particularly a reliance on morphology to identify species. The

many recent reports of incorrectly identified or cryptic species aptly illustrates this view. The profusion of ITS sequence data that has become available for members of the Botryosphaeriaceae in recent years has made it possible here to explore general patterns of host association in the group. In some cases, the environment appears to be a dominating determinant (e.g. *L. theobromae*; Mohali et al., 2005; Punithalingam, 1976), while in others specificity might be restricted to a single host genus (e.g. *Eucalyptus* spp. for *N. eucalyptorum* and *N. eucalypticola*; Slippers et al., 2004b) or host families (e.g. Pinaceae for *D. pinea* and *D. scrobiculata*; De Wet et al., 2003; Stanosz et al., 1999). An improved understanding of these patterns and factors that drive them will be important determinants in understanding the evolution of this group of fungi, their epidemiology, the emergence of new diseases, and characterizing and managing their threat to forestry and agriculture.

Acknowledgments

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