CHAPTER 3

MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF *DOTHIORELLA CASUARINI* SP. NOV. AND OTHER BOTRYOSphaeriaceae WITH *Diploodia*-LIKE CONIDIA

Submitted to *Mycologia*: De Wet J, Slippers B, Preisig O, Wingfield BD, Tsopelas P & Wingfield MJ.
ABSTRACT

Following recent changes to the taxonomy of the Botryosphaeriaceae, species with Diplodia-like (dark, pigmented) conidia are considered to belong to at least three genera including Diplodia, Lasiodiplodia and Dothiorella. In a recent molecular phylogenetic study, it became apparent that two groups of isolates with Diplodia-like conidia required taxonomic revision. One group of isolates originated from Cupressus sempervirens in Greece and Cyprus and had previously been identified as D. pinea f.sp. cupressi based on morphological characteristics. The other isolates originated from a Casuarina sp. in Australia and were superficially similar to those in the first group based on their morphologically similar Diplodia-like conidia. The aim of this study was to resolve the taxonomy of these two groups of isolates by combining the information from the multiple gene genealogies with morphological characters. The results showed that the isolates from C. sempervirens in Greece and Cyprus represent D. cupressi. The isolates from Casuarina in Australia belong to the more distantly related genus Dothiorella and represent a distinct species that is described here as Do. casuarini sp. nov.
INTRODUCTION

Species of the Botryosphaeriaceae represent both pathogens and saprophytes of woody and non-woody plants (Denman et al. 2000; Crous et al. 2006). Some well-known species include the conifer pathogen, *Diplodia pinea* (Desm.) J. Kickx f. (Eldridge 1961; Swart & Wingfield 1991), the fruit tree pathogen, *D. seriata* De Not. (Phillips et al. 2007; Slippers et al. 2007), the blue stain-associated, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Mohali et al. 2005) and *Botryosphaeria dothidea* (Moug. Fr.) Ces. & De Not (Slippers et al. 2004a). In recent years, analyses of DNA sequence data have had a significant influence on the taxonomy of the Botryosphaeriaceae resulting in the description of ten generic lineages and various cryptic species (e.g. De Wet et al. 2003; Crous et al. 2006). Of particular relevance to this study is the fact that various investigations have shown that the genera *Diplodia*, *Lasiodiplodia* and *Dothiorella*, which all have anamorphs characterized by dark, pigmented conidia (Diplodia-like) and have been regarded as synonyms (Denman et al. 2000), are phylogenetically distinct (Phillips et al. 2005; Crous et al. 2006; De Wet et al. 2008).

*Diplodia* and *Lasiodiplodia* are well characterized genera of the Botryosphaeriaceae, but *Dothiorella* has only recently been re-erected as anamorph genus in this family (Phillips et al. 2005). Species of *Dothiorella* are morphologically most similar to those of *Diplodia*. However, the conidia of *Dothiorella* turn brown and 1-septate while still in the pycnidium and sometimes even when they are still attached to the conidiogenous cells. In contrast, those of *Diplodia* typically become dark and septate only after discharge from the pycnidium. Furthermore, in *Dothiorella* percurrent proliferation of the conidiogenous cells is extremely rare, while this form of conidium development is common in *Diplodia*. Interestingly, based on phylogenetic
inference, *Dothiorella* spp. are more closely related to *Neofusicoccum* spp. with hyaline conidia than they are to other genera with Diplodia-like conidia (Phillips et al. 2005).

*Dothiorella* is currently represented by four species namely *Do. pyrenophora* Sacc., *Do. sarmentorum* A.J.L. Phillips, Alves & Luque, *Do. iberica* A.J.L. Phillips, Luque & Alves and *Do. viticola* A.J.L. Phillips & Luque. *Dothiorella pyrenophora* is the type species of *Dothiorella* having conidia that are brown and one-septate while inside the pycnidial cavity and often still attached to the conidiogenous cells (Crous & Palm 1999; Phillips et al. 2005). *Dothiorella sarmentorum* has been reported from *Malus, Ulmus, Pyrus, Prunus* and *Menispermum*, and probably has a world-wide distribution (Phillips et al. 2005). *Dothiorella iberica* is known from *Quercus* and *Malus*, only in Italy and Spain (Phillips et al. 2005) and *Do. viticola* occurs on *Vitis vinifera* in South Africa and Spain (Luque et al. 2005).

In a recent molecular phylogenetic study (De Wet et al. 2008), it became apparent that two groups of isolates require taxonomic revision. Both had superficially similar Diplodia-like conidia. The one set of isolates from *Cupressus sempervirens* in Greece and Cyprus of which those from Greece have previously been identified as *D. pinea* f.sp *cupressi* based only on morphology (Xenopoulos & Tsopelas 2000). The other group of isolates originated from *Casuarina* in Canberra, Australia and appeared to represent an undescribed *Dothiorella* species. The aim of this study was to combine molecular phylogenetic data with morphological characters to characterize these isolates.

**MATERIALS AND METHODS**

**Fungal isolates and morphological characterization**

A collection of 11 isolates with Diplodia-like conidia were characterized (Table 1). Sequence data for various Botryosphaeriaceae not generated in this study were obtained from GenBank
(Table 1). All the isolates were accessed from the Culture Collection (CMW) of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates from this study have also been deposited in the Culture Collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Isolates were transferred to 2 % water agar (WA) (Biolab Diagnostics, Midrand, South Africa), to which a few sterile pine needles had been placed on the agar surface to induce sporulation, and incubated at 25 ºC in constant light to induce sporulation. Single conidial isolates were generated by breaking pycnidia that were formed on the pine needles, spreading the conidia out and allowing them to germinate. A single, germinating conidium was then transferred and grown on 2 % malt extract agar (MEA) (Biolab Diagnostics, Midrand, South Africa) at 25 ºC. All cultures were stored at 4 ºC for further study.

For morphological characterization, fruiting structures were sectioned by hand and mounted in clear lactic acid. Morphological observations were made and images were recorded using a Zeiss Axioskop light microscope and Axiocam digital camera (Carl Zeiss, Germany). Growth rate and colony morphology of the isolates were determined on 2 % MEA at 25 ºC. Color descriptions of cultures, mycelium and conidia were made according to Rayner (1970).

**DNA extractions**

DNA was extracted (Raeder & Broda 1985) from the freeze-dried mycelium of the 11 single conidial isolates (Table 1). The isolates were grown in 500 µl of 2 % malt extract (ME) (Biolab Diagnostics, Midrand, South Africa) broth in 1.5 ml Eppendorf tubes, incubated at 25 ºC, one week prior to the DNA extraction. The broth was then removed by centrifugation, 20 min at 13 000 rpm, washed with distilled water and freeze-dried.
DNA amplification and sequencing

Part of the elongation factor 1α (EF-1α) (Carbone & Kohn 1999) gene was amplified for 11 Diplodia-like isolates (Table 1) using primers and conditions as described previously (De Wet et al. 2000 & 2003). The ITS regions of the rDNA operon (White et al. 1990) for four of these isolates (Table 1) were also amplified, while those of the rest were obtained from a previous study (De Wet et al. 2008). 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, 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).

Phylogenetic analyses

ITS and EF-1α sequence data were combined after a partition homogeneity test was performed to determine whether there is congruency between the different phylogenies using PAUP* (Swofford 2002), and the combined dataset was submitted to TreeBase (SN3866). The homogeneity test was based on strict heuristic searches with a tree-bisection reconnection (TBR) branch swapping algorithm and 1000 replicates. Parsimony, distance (NJ) and Bayesian analyses were applied to the combined data set. Introns occurring in the partial EF-1α gene sequences were included in the phylogenetic analyses. All characters were treated as unordered and having equal weight. The phylogenetic signal (G1) of the data sets was determined using
PAUP* and compared with critical values (Hillis & Huelsenbeck 1992) at the 0.01 and 0.05 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.

Neighbour-joining distance analysis was done in PAUP* using the most appropriate model of DNA substitution as determined with MODELTEST 3.5 (Posada & Crandall 1998). Bayesian analysis using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) implementing the Markov Chain Monte Carlo (MCMC) technique and the parameters predetermined with MODELTEST 3.5 was performed. Four simultaneous Markov chains were run from random starting trees for 500 000 generations and trees were sampled every 100 generations. The first 700 of 5001 trees generated were discarded as burnin. The Bayesian analysis was repeated to test the independence of the results from topological priors. Bootstrap support was determined after 1000 replications and only groups with frequencies >50% were retained. All phylogenetic trees were viewed in TreeView and monophyletically rooted to Mycosphaerella spp. as outgroups (M. konae Crous, Joanne E. Taylor & M.E. Palm: ITS=AY260085, EF-1α=AY752185; and M. citri Whiteside: ITS=AY752145, EF-1α=AY752179).

RESULTS

Phylogenetic analyses

260 bp of the EF-1α gene were amplified and sequenced for 11 Diplodia-like isolates (Table 1). For four of these isolates, 540 bp of the rDNA operon including the ITS1, ITS2 and 5.8S sub-unit were also amplified and sequenced (Table 1). GenBank sequences of 26 isolates, representing Diplodia, Lasiodiplodia, Dothiorella, Botryosphaeria and Neofusicoccum were added for comparative purposes (Table 1). A partition homogeneity test showed that no significant conflict
exists between the phylogenies of the rDNA and EF-1α (P = 0.1). The G1-value (G1 = -0.33) was lower than the predicted critical values at both the 95% (P = -0.08) and 99% (P = -0.09) confidence levels, implying a strong phylogenetic signal. The combined data set contained 808 characters of which 327 characters were constant, 71 were variable, parsimony uninformative characters and 410 were variable, parsimony informative characters. The data set had a tree length of 1113, a consistency index (CI) of 0.73, a retention index (RI) of 0.92 and a homoplasy index (HI) of 0.27. These indices measure the level of homoplasy which is an indication of the reliability of the parsimonious cladograms. MODELTEST 3.5 tested 56 models and predicted the Tamura-Nei model with unequal frequencies (TrN) and a gamma distribution shape parameter (G) as the most appropriate model of DNA substitution.

Two major clades were observed after analyses of the combined dataset (Fig. 1) and these results were confirmed when analyses were done on the two datasets independently. One major clade represented Diplodia and Lasiodiplodia and the other Botryosphaeria, Dothiorella and Neofusicoccum. The Diplodia/Lasiodiplodia clade was comprised of seven sub-clades namely D. cupressi, D. mutila, D. scrobiculata, D. pinea, D. seriata, L. theobromae and D. porosum Van Niekerk & Crous. The Dothiorella/Neofusicoccum/Botryosphaeria clade also consisted of seven sub-clades including Do. sarmentorum, Do. iberica, an undescribed Dothiorella species, N. eucalyptorum, N. luteum, N. ribis and B. dothidea.

The isolates from C. sempervirens from Greece and Cyprus grouped with D. cupressi from Israel. While isolates from Casuarina in Australia grouped in a distinct clade representing an undescribed Dothiorella species, with strong bootstrap and Bayesian posterior probability support (100 % and 1.0, respectively). Support for the undescribed Dothiorella species as a
distinct member of the genus *Dothiorella* was also provided when the two datasets were analyzed separately.

**Taxonomy**

Results of the phylogenetic and morphological analyses provide robust evidence to support treatment of the isolates from a *Casuarina* sp. as a discrete taxon for which the following description is provided:

*Dothiorella casuarini* J. De Wet, Slippers & M.J. Wingfield anam. sp. nov. (Figs. 2—7)

(Mycobank 510856)

Etym.: named for *Casuarina* the host from which the fungus was isolated.

Margines coloniarum irregulariter rosulatae. Mycelium cum seriebis tumorum hyphorum chlamydosporas semblantium. Conidiomata pycnidialia, nigra, globosa. Cellulae conidiogenae cellulis pycnidiorum proxime portatae, holoblasticae, hyalinae, subcylindricae, in plano eodem in concretionibus periclinalibus proliferantes, raro percurrente proliferantes bis vel ter indistincte annulatae. Conidia 22—38 x 8—13.5 µm (mediocr 27.1 x 10.8 µm), primo non septata hyalina subcylindrica, dum etiam in pycnidio brunnescentia vel atrobrunnescentia, unisepata raro 2—3 septata, ellipoidea vel ovoidea, raro anguste ellipsoidea, apice late rotundata, basi truncata.

*Cultures* smooth to fluffy, pale greenish grey to greenish grey from above, becoming lighter or white around the edges, light bluish of sky grey from below, colony margins irregular, rosette-like. *Mycelium* thick walled, branched, septate, melanized light to dark brown, with strings of dark brown chlamydospore-like hyphal swellings. *Conidiomata* pycnidia, black, globose, ostiole central, solitary, scattered and immersed in water agar, few on pine needles supplied as substrate. *Conidiophores* absent. *Conidiogenous cells* emerging directly from cells
lining the pycnidial cavity, holoblastic, hyaline, smooth-walled, sub-cylindrical, determinate or indeterminate and proliferating at the same level resulting in periclinal thickening, very rarely proliferating percurrently to produce two or three indistinct annellations. *Conidia* (22—)23—31(—38) × (8—)9—12 (—13.5) μm (ave. of 60 conidia = 27.1 × 10.8 μm), initially aseptate and hyaline, becoming brown to dark brown or sepia and 1-septate within the pycnidium, rarely 2-3 septate, ellipsoid to ovoid, rarely narrow ellipsoid, as obtuse apex and truncate base.

**Known host.** *Casuarina* sp.

**Known geographical range.** Canberra, Australia.

**Holotype: Australia:** Canberra: Cotter River. On *Casuarina* sp., 2000, M.J. Wingfield (CMW4855/CBS120688); in Herb. PREM59650.

**Paratypes: Australia:** Canberra: Cotter River. On *Casuarina* sp., 2000, M.J. Wingfield (CMW4856/CBS120689, CMW4857/CBS120690, CMW4854, CMW4858); all in Herb. PREM59651, PREM59652, PREM59649, PREM59653.

**DISCUSSION**

The gene genealogy generated from ITS rDNA and partial EF-1α sequence data, combined with morphological observations provide robust evidence to justify the description of a set of Diplodia-like isolates from *Casuarina* in Australia as the new species, *Dothiorella casuarini*. This is the fifth species to be described in *Dothiorella*. All except the type species, *Do. pyrenophora* for which no cultures are available, are phylogenetically distinct. In contrast, it would be very difficult to distinguish them based only on morphological characteristics as these often overlap and the more easily distinguishable teleomorphs are rare. This is a problem that is encountered increasingly commonly for fungi (Crous 2005), with the Botryosphaeriaceae providing an excellent example (Crous et al. 2006).
Dothiorella are distinguished from other anamorph genera of the Botryosphaeriaceae based on conidial morphology and DNA sequence comparisons (Luque et al. 2005; Phillips et al. 2005). In this regard, Do. casuarini has conidia that are ellipsoid to ovoid, initially aseptate and hyaline turning brown to dark brown and 1-septate while still in the pycnidium. Conidia of this species are longer than those of Do. sarmentorum, Do. iberica and Do. viticola. It is also characterized by chlamydospore-like hyphal swellings, which are frequently observed and that have not been reported in other Dothiorella spp. Furthermore, Do. casuarini has very obvious smooth to fluffy grey-green cultures with typical irregular, rosette-like borders.

No teleomorph structures have been observed for Do. casuarini. This is not unusual as sexual states are typically less common in the Botryosphaeriaceae than anamorph states. The known teleomorphs of other Dothiorella sp. were previously described as “Botryosphaeria” sarmentorum A.J.L. Phillips, Alves & Luque, “Botryosphaeria” iberica A.J.L. Phillips, Luque & Alves and “Botryosphaeria” viticola A.J.L. Phillips & Luque (Phillips et al. 2005). The teleomorph of Dothiorella has since been placed in the genus Dothidotthia, but the above mentioned teleomorphs have not been formally renamed (Crous et al. 2006). If a teleomorph were to be found for Do. casuarini this would be expected to have the characteristics of Dothidotthia.

Phylogenetic analyses of the ITS rDNA and partial EF-1α sequence data, grouped a set of isolates from Greece and Cyprus with the ex-type cultures of D. cupressi from Israel. This fungus was recently described by Alves et al. (2006) and was previously known as D. pinea f. sp. cupressi, the causal agent of a canker disease on Cupressus sempervirens in Israel (Solel et al. 1987), South Africa (Linde et al. 1997), Greece (Xenopoulos & Tsopelas 2000) and Tunisia (Intini & Panconesi 2005). This is the first report of the pathogen from C. sempervirens in
Cyprus. *Diplodia cupressi* is phylogenetically most closely related to *B. tsugae* and *D. mutila* (Alves *et al.* 2006) and clearly has no logical association with *D. pinea*. *Diplodia cupressi* is also the name given to the pathogen found on *Juniperus* spp. previously identified as *D. mutila* (Alves *et al.* 2006, De Wet *et al.* 2008).

Phylogenetic analyses in this study showed that *D. cupressi* is more closely related to species from hardwoods, such as *D. mutila* from *Fraxinus*, than to *D. pinea*. Interestingly, *D. pinea* is also more closely related to the hardwood-infecting species, *D. seriata*, than to other softwood-infecting species. Clearly, distantly related hosts have been colonized by ancestors of these fungi. These host jumps (Slippers *et al.* 2005), rather than co-evolution with the hosts, most likely contributed to the speciation of the taxa. These results also support results of a recent study (De Wet *et al.* 2008) in which we showed that species of *Diplodia* and *Lasiodiplodia* were common on both gymnosperms and angiosperms (*D. seriata*, *D. porosum*, *L. theobromae*). This was in contrast to species of *Dothiorella*, *Neofusicoccum* and *Botryosphaeria* that were virtually all from angiosperms, which is the likely ancestral host group of the Botryosphaeriaceae (De Wet *et al.* 2008).

*Diplodia*, *Lasiodiplodia* and *Dothiorella* are all morphologically similar members of the Botryosphaeriaceae. These genera all have conidia that are similar in size and shape (ellipsoidal to ovoid), initially hyaline, but becoming pigmented with age, and sometimes septate. Isolates belonging to these three genera included in this study could, however, easily be assigned to these genera using a multiple gene sequence comparisons. This underscores the importance of combining morphological and DNA sequence data when identifying and describing new species with Diplodia-like characteristics (Denman *et al.* 2000; De Wet *et al.* 2003; Alves *et al.* 2004; Pavlic *et al.* 2004; Alves *et al.* 2006).
Diplodia and Lasiodiplodia are clearly sister genera and it is not surprising that they share similar conidial morphology. Dothiorella is, however, more closely related to morphologically distinct genera such as Neofusicoccum and Botryosphaeria. The latter taxa have conidia that are mostly hyaline and fusoid in shape and only rarely become pigmented, thus very different from those of Dothiorella. Pigmented older conidia that are ovoid to ellipsoid thus represent a polyphyletic character, which has been lost or gained independently amongst the lineages of the Botryosphaeriaceae.

Results of this study, confirm the value of generating multiple gene genealogies to resolve the status of species of the Botryosphaeriaceae with Diplodia-like anamorphs. It has further shown that neither morphology, nor host association, necessarily reflect the evolutionary history of the genera of the Botryosphaeriaceae. Much remains to be understood regarding the role of host association in shaping the diversity and distribution of species in this group of fungi. Studies considering conidial morphology, and factors that influence this character based on a more complete taxon set are likely to reflect important aspects of the evolutionary histories for members of the Botryosphaeriaceae.
REFERENCES


Taxonomy, phylogeny and identification of Botryosphaeriaceae associated with pome and stone fruit trees in South Africa and other regions of the world. *Plant Pathology* 56, 128-139.


Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. *Mycologia* 96, 83-101.


*Forest Pathology* 30, 121-126.
Table 1. *Diplodia* and *Dothiorella* isolates included in this study as well as other members of the Botryosphaeriaceae used for comparative purposes.

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<th>Isolates</th>
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<th>Reference/Collector</th>
<th>GenBank Accession numbers</th>
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\(^a\) CMW refers to the Culture Collection (CMW) of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.  
\(^b\) Reference refers to previous publications where the same isolates were used and collector refers to the collector and isolation numbers of isolates not previously published.  
\(^c\) Sequences for isolates in bold were generated in the present study while the remainder were obtained from GenBank.
Figure 1. Phylogram constructed from the sequences of the rDNA operon (ITS regions and 5.8S ribosomal sub-unit) and partial elongation factor 1 alpha (EF-1α) 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 with branch support values (maximum parsimony bootstrap proportions/Bayesian posterior probabilities). Bootstrap values were determined after 1000 replications in PAUP*. Only groups with frequencies >50% were retained. The Bayesian posterior support values were determined using MrBayes 3.1.2 with the Tamura-Nei model and a gamma distribution shape parameter (TrN+ G). The bar represents 10 changes.
Figures 2-7. *Dothiorella casuarini* sp. nov. **Fig. 2.** Pycnidium formed on a sterile pine needle in culture on water agar. **Fig. 3.** Pigmented chlamydospore-like hyphal cells in chains. **Fig. 4-5.** Conidiogenous cells and immature developing conidia. **Fig. 6-7.** Mature, septate, dark conidia.

Bars = 10 µm
CHAPTER 4

PHYLOGENY OF THE BOTRYOSPHAERIACEAE REVEALS PATTERNS OF HOST ASSOCIATION

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 dataset 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.
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 & 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 & Wingfield 1991), and *Botryosphaeria dothidea* (Moug. Fr.) Ces. & De Not. and *Neofusicoccum eucalyptorum* Crous, H. Smith & M.J. Wingf. that cause serious canker diseases on *Eucalyptus* L’Hér (Smith *et al.* 1994; Smith *et al.* 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 & A.J.L. Phillips and *N. luteum* Crous, Slippers & A.J.L. Phillips (Van Niekerk *et al.* 2004) and the Proteaceae including *Saccharata proteae* (Wakef.) Denman & Crous (Denman *et al.* 2003).

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, ten 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 & Everh./*Tiarosporella* Höhn, *Botryosphaeria* Ces. & De Not. (*Fusicoccum* anamorphs), *Macrophomina* Petr., *Neoscytalidium* Crous & Slippers, *Dothidotthia* Höhn (*Dothiorella* anamorphs), *Neofusicoccum* Crous, Slippers & A.J.L. Phillips...
(Botryosphaeria-like teleomorphs, Dichomera-like synanamorphs), Pseudofusicoccum Mohali, Slippers & M.J. Wingf., Saccharata Denman & Crous (Diplodia- and Fusicoccum-like synanamorphs), “Botryosphaeria” quercuum (Schwein.) Sacc. (Diplodia-like anamorph) and Guignardia Viala & Ravaz (Phyllosticta anamorphs). The genus Botryosphaeria now applies only to B. dothidea, B. mamane D.E. Gardner and B. corticis (Demaree & Wilcox) Arx & 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 or 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 & 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 & B. Sutton) (Waterman 1943; Punithalingam & Waterston 1970). *Diplodia pinea* 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 & 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 & 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 & 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 & 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 ten lineages of the Botryosphaeriaceae as described by Crous *et al.* (2006) with ITS sequence representation in GenBank, and for which host data are available.

**MATERIAL AND METHODS**

**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 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.

**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 & 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 & Donaldson 1995) and part of the protein-coding gene, actin (ACT) (Carbone & 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 visualised 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).

**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 geographical 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 ten 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 & 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. Neighbour-joining distance analysis was done in PAUP* using the most appropriate model of DNA substitution as determined with MODELTEST 3.5 (Posada & Crandall 1998). Maximum likelihood was also performed in PAUP* using the parameters as determined with MODELTEST 3.5 (Posada & Crandall, 1998). Bayesian analysis using MrBayes 3.0b4, implementing the Markov Chain Monte Carlo (MCMC) technique (Huelsenbeck & 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 & Maddison 2000). All phylogenetic trees were viewed in TreeView and monophyletically rooted to *Mycosphaerella* spp. as outgroups (*M. konae* Crous, Joanne E. Taylor & 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.
RESULTS

Phylogenetic analyses 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 – 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 retention 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 & 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 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 & 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.

**Phylogenetic analyses for six lineages of the Botryosphaeriaceae (Fig. 2)**

A total of 134 ITS sequences representing six of the ten lineages of the Botryosphaeriaceae from every distinct host species available on GenBank were included. The G1-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 dataset. 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 retention 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 gymnosperms. Diplodia pinea and D. scrobiculata occur only on gymnosperms, and D. mutila only on angiosperms. Some species such as D. corticola Phillips, Alves & Luque from Quercus L., D. porosum from Vitis L., D. rosulata Gure, Slippers & 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 & 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 & Mohali from Acacia Miller, L. rubropurpurea Burgess, Barber & Pegg from Eucalyptus, L. crassispora Burgess & Barber from Eucalyptus and Santalum L., and L. gonubiensis Pavlic, Slippers & 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 & 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 & A.J.L. Phillips from *Vitis*, *N. viticlavatum* Crous, Slippers & A.J.L. Phillips from *Vitis*, *N. eucalyptorum* from *Eucalyptus*, *N. eucalypticola* from *Eucalyptus*, *N. arbuti* Crous, Slippers & A.J.L. Phillips from *Arbutus* L., *N. andinum* Mohali, Slippers & M.J. Wingf. form *Eucalyptus*, *N. macroclavatum* T. Burgess, Barber & L.M. Hardy from *Eucalyptus*, *N. mangiferae* Crous, Slippers & A.J.L. Phillips from *Mangifera* L. and *N. protearum* Crous, Slippers & 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 & Wilcox) Arx & E. Müll. from *Vaccinium* L.

The Neoscytalidium clade included two species, *N. dimidiatum* Crous & 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 & M.J. Wingf. only known from *Eucalyptus*. 
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 & Rehner 1998; Denman et al. 2000; Zhou & 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 & 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 & Stanosz 1995; Stanosz *et al.* 1998; Burgess *et al.* 2001; Zhou & 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 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 & 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 & 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 & Waller 1973). *Diplodia mutila* is also a generalist species able to infect a wide range of angiosperms (Jacobs & Rehner 1998; Zhou & 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. austral* 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*; Punithalingam 1976; Mohali *et al*. 2005), 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*; Stanosz *et al*. 1999; De Wet *et al*. 2003). 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.
REFERENCES


Combined multiple gene genealogies and phenotypic characters differentiate several
species previously identified as Botryosphaeria dothidea. Mycologia 96, 83-101.

Taxonomy, phylogeny and identification of Botryosphaeriaceae associated with pome
and stone fruit trees in South Africa and other regions of the world. Plant Pathology 56,
128-139.

Preliminary studies on Botryosphaeria species from Southern Hemisphere conifers in
Australasia and South Africa. Australasian Plant Pathology 34, 213-220.

2004b. Speciation and distribution of Botryosphaeria spp. on native and introduced

Smith DR, Stanosz GR, 1995. Confirmation of two distinct populations of Sphaeropsis sapinea
in the North Central United States using RAPDs. Phytopathology 85, 699-704.


eucalyptorum sp. nov., a new species in the B. dothidea-complex on Eucalyptus in
South Africa. Mycologia 93, 277-285.

Stanosz GR, Swart WJ, Smith DR, 1998. Similarity between fungi identified as Diplodia pinea
f.sp. cupressi in Israel and Botryosphaeria stevensii or Diplodia mutila on Juniperus in


*Mycologia* 93, 516-527.
Table 1. *Diplodia* and *Dothiorella* isolates included in this study.

<table>
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<th>Isolates</th>
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<th>Host</th>
<th>Other collections</th>
<th>Collector</th>
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Table 2. Isolates of *Diplodia pinea*, *D. scrobiculata* and various *Botryosphaeria* spp. included in this study for comparative purposes.

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<th>Identification</th>
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**Figure 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 neighbour-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 >50% were retained. Isolates marked with ♦ are from Gymnosperms and isolates marked with ◆ are from Angiosperms.
**Figure 2.** Phylogram constructed for the ITS and 5.8S rDNA based on neighbour-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 Figure 1.