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Molecular and morphological characterization of *Dothiorella casuarini* sp. nov. and other Botryosphaeriaceae with diplodia-like conidia

Juanita de Wet[1]
Forestry and Agricultural Biotechnology Institute (FABI), Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, South Africa

Bernard Slippers
Forestry and Agricultural Biotechnology Institute (FABI) and Department of Genetics, University of Pretoria, South Africa

Oliver Preisig
Forestry and Agricultural Biotechnology Institute (FABI)

Brenda D. Wingfield
Forestry and Agricultural Biotechnology Institute (FABI) and Department of Genetics, University of Pretoria, South Africa

Panaghiotis Tsopelas
NAGREF-Institute of Mediterranean Forest Ecosystems, Terma Alkmanos, Athens, Greece

Michael J. Wingfield
Forestry and Agricultural Biotechnology Institute (FABI)

Abstract: After recent changes to the taxonomy of the Botryosphaeriaceae species with diplodia-like (= dark, ovoid, often 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 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.

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[1] Corresponding author. E-mail: juanita.dewet@fabi.up.ac.za

Key words: Botryosphaeriaceae, *Diplodia*, *Dothiorella*

INTRODUCTION

Species of the Botryosphaeriaceae represent both pathogens and saprophytes of woody and nonwoody plants (Slippers and Wingfield 2007). Some well known species include the conifer pathogen *Diplodia pinea* (Desm.) J. Kickx f. (Eldridge 1961, Swart and 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 10 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 genera *Diplodia*, *Lasiodiplodia* and *Dothiorella*, all of which have anamorphs characterized by dark, ovoid, often 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 been resurrected only recently as an anamorph genus in this family (Phillips et al 2005). Species of *Dothiorella* are morphologically most similar to those of *Diplodia*. However *Dothiorella* conidia 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*. Of note, 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 1-septate while inside the pycnidial cavity and often still attached to the conidiogenous cells (Crous and Palm 1999, Phillips et al 2005). *Dothiorella sarmentorum* has been reported from *Malus*, *Ulmus*, *Pyrus*, *Prunus* and *Menispermum* and probably has worldwide 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 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 are from *Cupressus sempervirens* in Greece and Cyprus, of which those from Greece had been identified as *D. pinea* f.sp *cupressi* based only on morphology (Xenopoulos and 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 I). Sequence data for various Botryosphaeriaceae not generated in this study were obtained from GenBank (TABLE I). All isolates were accessed from the Culture Collection (CMW) of the Tree Protection Cooperative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates from this study also have 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 C in constant light. Single conidial isolates were generated by breaking pycnidia that were formed on the pine needles, spreading the conidia and letting them germinate. A single, germinating conidium was transferred and 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.

Fruiting structures were sectioned by hand and mounted in clear lactic acid for morphological characterization. Morphological observations were made and images were recorded with 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 C. Color descriptions of cultures, mycelium and conidia were made according to Rayner (1970).

DNA extractions.—DNA was extracted (Raeder and Broda 1985) from the freeze-dried mycelium of the 11 single conidial isolates (TABLE I). Isolates were grown in 500 µL 2% malt extract (ME) (Biolab Diagnostics, Midrand, South Africa) broth in 1.5 mL Eppendorf tubes, incubated at 25 C 1 wk before DNA extraction. The broth was 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 and Kohn 1999) gene was amplified for 11 diplodia-like isolates (TABLE I) with primers and conditions as described by de Wet et al (2000, 2003). The ITS regions of the rDNA operon (White et al 1990) for four of these isolates (TABLE I) also were amplified, while those of the rest were obtained from a study by de Wet et al (2008). PCR products were viewed on a 1% agarose gel containing ethidium bromide with UV illumination. The PCR products were purified with the Roche High Pure PCR product purification kit (Roche Diagnostics, Germany). Both DNA strands were sequenced with the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing kit and an ABI PRISM® 3100 DNA sequencer (Applied Biosystems, Foster City, California). All reactions were done with protocols recommended by the manufacturers. All tsequence data were processed with Sequence Navigator 1.0.1 (Perkin Elmer) and aligned with MAFFT 5 (Katoh et al 2005).

Phylogenetic analyses.—ITS and EF-1α sequence data were combined after a partition homogeneity test 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 dataset. 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 datasets was determined with PAUP* and compared with critical values (Hillis and 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. Neighbor joining distance analysis was done in PAUP* with the most appropriate model of DNA substitution as determined with MODELTEST 3.5 (Posada and Crandall 1998). Bayesian analysis with MrBayes 3.1.2 (Ronquist and 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 500 000 generations, and trees were sampled every 100 generations. The first 700 of 5001 trees generated were discarded as burn-in. 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.—A total of 260 bp of the EF-1α gene were amplified and sequenced for 11 diplodia-like isolates (TABLE I). For four of these isolates 540 bp of the rDNA operon including the ITS1, ITS2 and 5.8S subunit also were amplified and sequenced (TABLE I). GenBank sequences of 26 isolates, representing *Diplodia*, *Lasiodiplodia*, *Dothiorella*, *Botryosphaeria* and *Neofusicoccum*, were added for comparative purposes (TABLE I). A partition homogeneity test showed that no significant conflict exists between the phylogenies of the rDNA and EF-1α sequences ($P = 0.1$). 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 dataset contained 808 characters of which 327 characters were constant, 71 were variable and 410 were variable, parsimony informative characters. The dataset 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 comprised seven subclades, 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 subclades 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 genus *Dothiorella* also was provided when the two datasets were analyzed separately as well as when the most variable and ambiguously aligned regions in the datasets were excluded (bases 77–144 in the ITS dataset and bases 1–30 and 153–230 of the EF-1α dataset).

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 this description is provided:

Dothiorella casuarini J. de Wet, Slippers & M.J. Wingfield anam. sp. nov. FIGS. 2–7
MB510856

Etymology. 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 parietum pycnidiorum proxime portatae, holoblasticae, hyalinae, subcylindricae, in plano eodem in concretionibus periclinalibus proliferantes, raro percurrente proliferantes bis vel ter indistincte annulatae. Conidia 22–38 × 8–13.5 μm (mediocriter 27.1 × 10.8 μm), primo non septata hyalina subcylindrica, dum etiam in pycnidio brunnescentia vel atrobrunnescentia, uniseptata raro 2–3 septata, ellipsoidea vel ovoidea, raro anguste ellipsoidea, apice late rotundata, basi truncata.

Cultures smooth to fluffy, pale greenish gray to greenish gray from above, becoming lighter or white around the edges, light bluish of sky gray 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, subcylindrical, 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 (av. 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.

TABLE I. *Diplodia* and *Dothiorella* isolates included in this study as well as other members of the Botryosphaeriaceae used for comparative purposes

Isolates[a]	Species	Origin	Host	Reference/collector[b]	GenBank accession numbers[c]	
					ITS	EF-1α
CMW19954	*D. cupressi*	Greece	*Cupressus sempervirens*	P. Tsopelas (SH-1/ CBS120691)	**DQ846775**	**DQ875334**
CMW19955	*D. cupressi*	Greece	*Cupressus sempervirens*	P. Tsopelas (SH-2/ CBS120692)	**DQ846776**	**DQ875335**
CMW19956	*D. cupressi*	Cyprus	*Cupressus sempervirens*	P. Tsopelas (SH-4/ CBS120693)	**DQ846777**	**DQ875336**
CMW19957	*D. cupressi*	Cyprus	*Cupressus sempervirens*	P. Tsopelas (SH-7/ CBS121027)	**DQ846779**	**DQ875338**
CMW4854	*Dothiorella casuarini*	Australia	*Casuarina* sp.	MJ. Wingfield	EF107752	**EF107758**
CMW4855 (CBS120688)	*Dothiorella casuarini*	Australia	*Casuarina* sp.	MJ. Wingfield	DQ846773	**DQ875331**
CMW4856 (CBS120689)	*Dothiorella casuarini*	Australia	*Casuarina* sp.	MJ. Wingfield	DQ846772	**DQ875332**
CMW4857 (CBS120690)	*Dothiorella casuarini*	Australia	*Casuarina* sp.	MJ. Wingfield	DQ846774	**DQ875333**
CMW4858	*Dothiorella casuarini*	Australia	*Casuarina* sp.	MJ. Wingfield	EF107753	**EF107759**
CMW1182	*D. cupressi*	Israel	*C. sempervirens*	W. Swart (Swart et al 1993)	EU220433	**EU220487**
CMW1183	*D. cupressi*	Israel	*C. sempervirens*	W. Swart (Swart et al 1993)	EU220434	**EU220488**
CBS168.87	*D. cupressi*	Israel	*C. sempervirens*	Alves et al 2006	DQ458893	DQ458878
CMW190	*D. pinea* (A)	United States	*Pinus resinosa*	Palmer et al 1987, De Wet et al 2000 and 2003	AY253290	AY624251
CMW4876	*D. pinea* (C)	Indonesia	*P. patula*	De Wet et al 2000 and 2003	AY253294	AY624252
CMW5870	*D. scrobiculata*	California	*P. radiata*	De Wet et al 2003	AY623704	AY624254
CMW4900	*D. scrobiculata*	Mexico	*P. greggii*	De Wet et al 2003	AY623705	AY624255
CMW189	*D. scrobiculata*	USA	*P. banksiana*	Palmer et al 1987, De Wet et al 2000 and 2003	AY253292	AY624253
CMW8230	*D. seriata*	Canada	*Picea glauca*	De Wet et al 2003	AY972104	DQ280418
CMW8232	*D. seriata*	South Africa	*Malus domestica*	De Wet et al 2003	AY972105	DQ280419
CMW9074	*Lasiodiplodia theobromae*	Mexico	*Pinus* sp.	Slippers et al 2004a	AY236952	AY236901
CMW10130	*L. theobromae*	Uganda	*Vitex doniana*	Slippers et al 2004a	AY236951	AY236900
CMW7060	*D. mutila*	Netherlands	*Fraxinus excelsior*	Slippers et al 2004a	AY236955	AY236904
CMW7776	*D. mutila*	Italy	Fraxinus excelsior	Slippers et al 2004a	AY972106	DQ280420
CMW7999	*B. dothidea*	Switzerland	*Ostrya* sp.	Slippers et al 2004a	AY236948	AY236897
CMW8000	*B. dothidea*	Switzerland	*Prunus* sp.	Slippers et al 2004a	AY236949	AY236898
CBS110574	*D. porosum*		*Vitis vinifera*	Van Niekerk et al 2004	AY343378	AY343340
CBS110496	*D. porosum*	South Africa	*Vitis vinifera*	Van Niekerk et al 2004	AY343379	AY343339
IMI63581b	*Do. sarmentorum*	England	*Ulmus* sp.	Phillips et al 2005	AY573212	AY573223
CBS115038	*Do. sarmentorum*	Netherlands	*M. pumila*	Phillips et al 2005	AY573206	AY573235
CBS115041	*Do. iberica*	Spain	*Quercus ilex*	Phillips et al 2005	AY573202	AY573222
CBS115035	*Do. iberica*	Spain	*Quercus ilex*	Phillips et al 2005	AY573213	AY573228
CBS121117	*Do. viticola*	South Africa	*V. vinifera*	Damm et al 2007	EF445361	EF445394
CBS121118	*Do. viticola*	South Africa	*V. vinifera*	Damm et al 2007	EF445360	EF445393
CMW7772	*Neofusicoccum ribis*	New York, USA	*Ribes* sp.	Slippers et al 2004a	AY236935	AY236877
CMW7773	*Neofusicoccum ribis*	New York, USA	*Ribes* sp.	Slippers et al 2004a	AY236936	AY236878

Table I. Continued

Isolates[a]	Species	Origin	Host	Reference/collector[b]	GenBank accession numbers[c] ITS	EF-1α
BOT24	*N. eucalyptorum*	South Africa	*Eucalyptus* sp.	Smith et al 2001	AF283686	AY339264
BOT16	*N. eucalyptorum*	South Africa	*Eucalyptus* sp.	Smith et al 2001	AF283687	AY236892
CMW9076	*N. luteum*	New Zealand	*M. domestica*	Slippers et al 2004b	AY236946	AY339265
CMW10310	*N. luteum*	Portugal	*V. vinifera*	Slippers et al 2004b	AY339259	AY339267

[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 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 boldface were generated in the present study while the remainder were obtained from GenBank.

Known host. *Casuarina* sp.

Known geographical range. Canberra, Australia.

Cultures examined. AUSTRALIA: Canberra: Cotter River. On *Casuarina* sp., 2000, M.J. Wingfield (CMW4855/CBS120688) in Herb. PREM59650 (HOLOTYPE). AUSTRALIA: Canberra: Cotter River. On *Casuarina* sp., 2000, M.J. Wingfield (CMW4856/CBS120689, CMW4857/CBS120690, CMW4854, CMW4858) all in Herb. PREM59651, PREM59652, PREM59649, PREM59653 (PARATYPES).

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 confirmed by DNA sequence data 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 because 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 spp. 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 frequently are observed but have not been reported in other *Dothiorella* spp. Furthermore *Do. casuarini* has obvious smooth to fluffy gray-green cultures with typical irregular, rosette-like borders.

No teleomorph structures have been observed for *Do. casuarini*. This is not unusual because sexual states are typically less common in the Botryosphaeriaceae than anamorph states. The known teleomorphs of other *Dothiorella* sp. were 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 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 described by Alves et al (2006) and was known previously 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 and Tsopelas 2000) and Tunisia (Intini and 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).

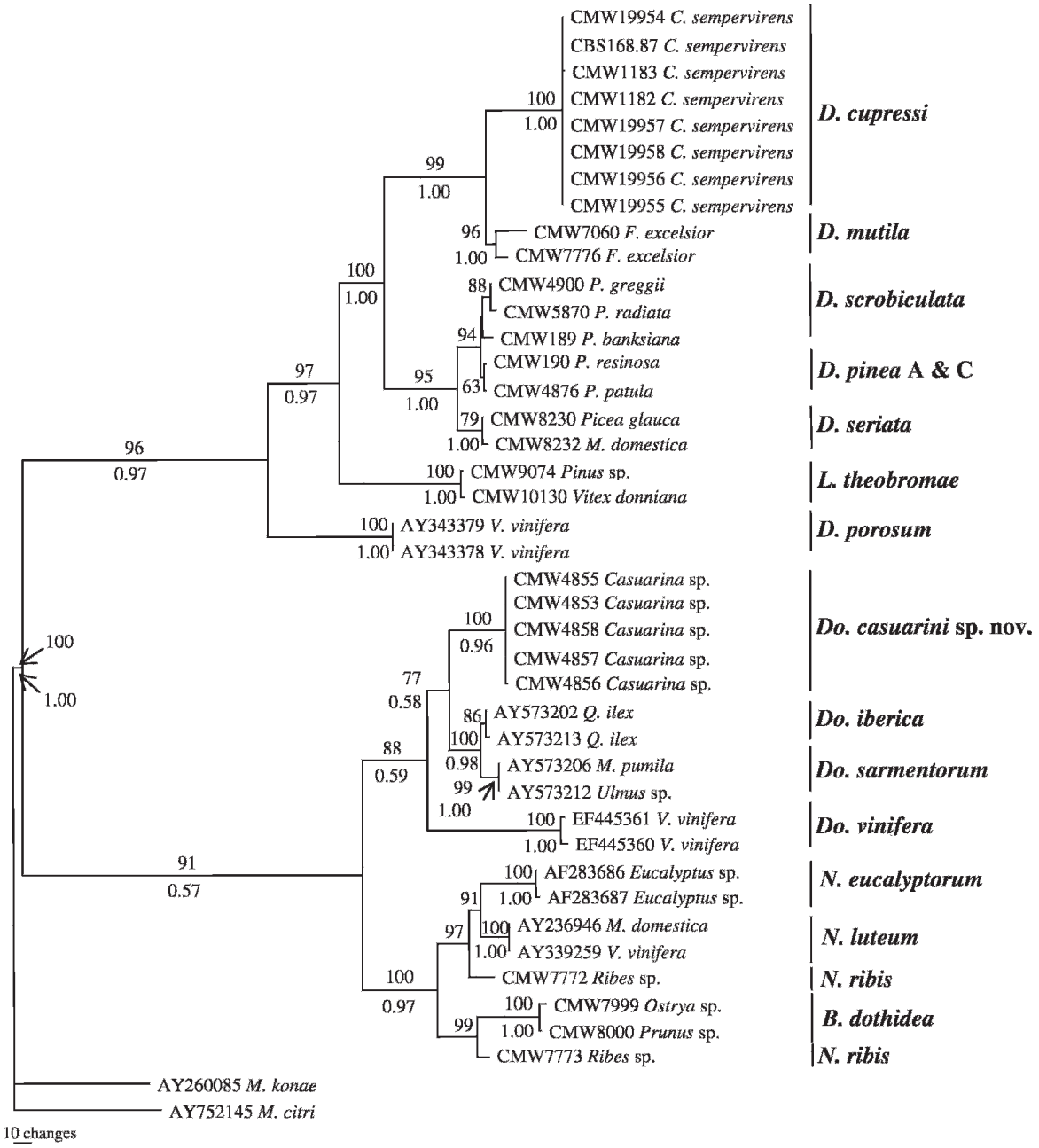
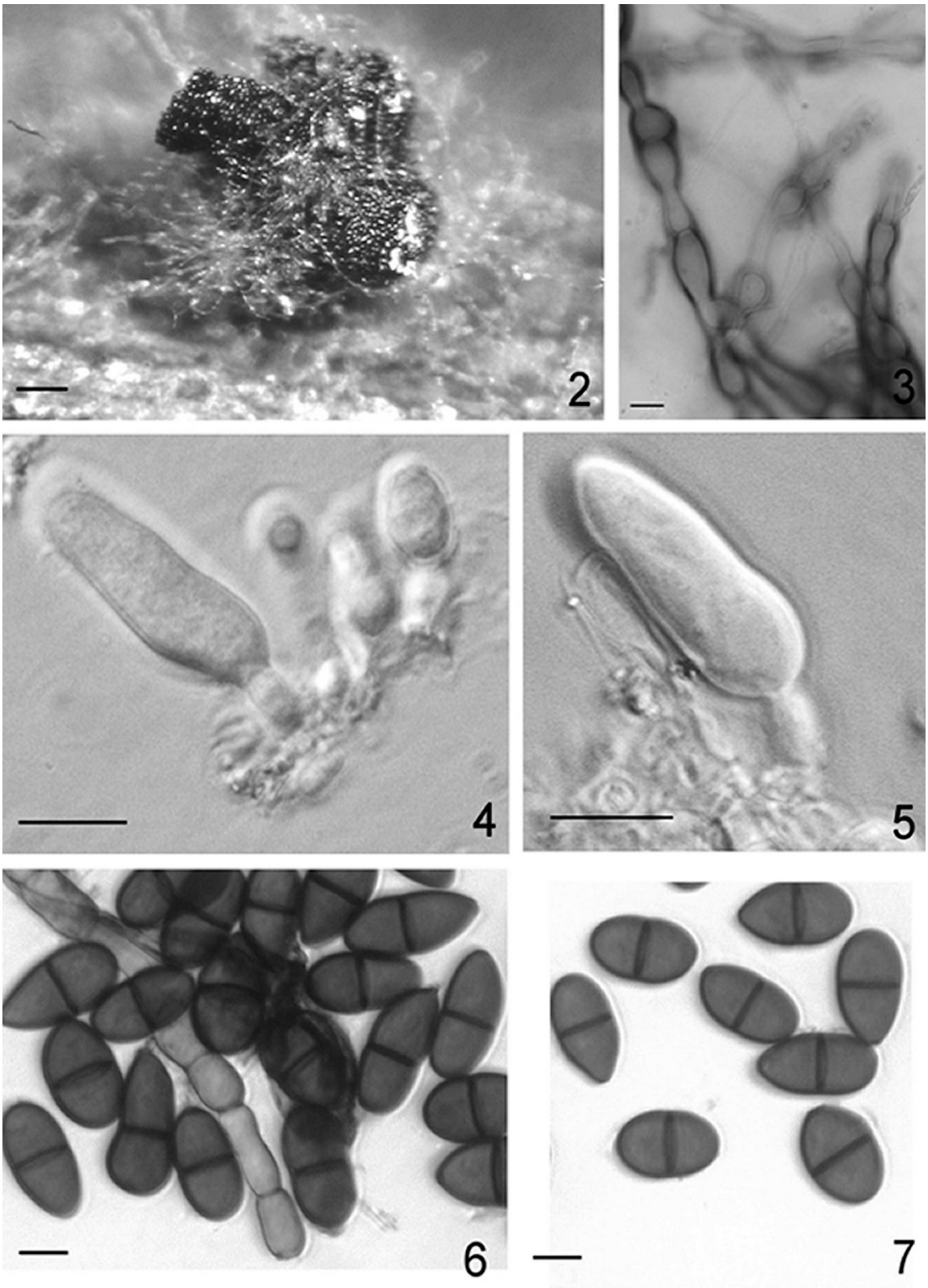


FIG. 1. Maximum parsimony phylogram constructed from the sequences of the rDNA operon (ITS regions and 5.8S ribosomal subunit) and partial elongation factor 1 alpha (EF-1α) based on strict heuristic searches with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse 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 with MrBayes 3.1.2 with the Tamura-Nei model and a gamma distribution shape parameter (TrN+G). The bar represents 10 changes.

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*. Of interest *D. pinea* also is 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), instead of co-evolution with the hosts, most likely contributed to the speciation of the taxa. These results also support a study by de Wet et al (2008) in which we showed that species of *Diplodia*



Figs. 2–7. *Dothiorella casuarini* sp. nov. 2. Pycnidium formed on a sterile pine needle in culture on water agar. 3. Pigmented chlamydospore-like hyphal cells in chains. 4–5. Conidiogenous cells and immature developing conidia. 6–7. Mature, septate, dark conidia. Bars = 10 µm.

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 almost all from angiosperms, which is the likely ancestral host group of the Botryosphaeriaceae (de Wet et al 2008).

Diplodia, *Lasiodiplodia* and *Dothiorella* all are morphologically similar members of the Botryosphaeriaceae. These genera 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 however could easily be assigned to these genera with 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* however is more closely related to morphologically distinct genera such as *Neofusicoccum* and *Botryosphaeria*. The latter taxa have conidia that are mostly hyaline and fusoid and only rarely become pigmented, thus are 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 among 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 morphology or host association does not necessarily reflect the evolutionary history of the Botryosphaeriaceae genera. 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.

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