Multi-gene genealogies and morphological data support *Diplodia cupressi* sp. nov., previously recognized as *D. pinea* f. sp. *cupressi*, as a distinct species

Artur Alves[1*], António Correia[1] and Alan J.L. Phillips[2]

[1]Centro de Estudos do Ambiente e do Mar, Departamento de Biologia, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[2]Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

Alves, A., Correia, A. and Phillips, A.J.L. (2006). Multi-gene genealogies and morphological data support *Diplodia cupressi* sp. nov., previously recognized as *D. pinea* f. sp. *cupressi*, as a distinct species. Fungal Diversity 23: 1-15.

The causal agent of Diplodia canker of cypress (*Cupressus* spp.) in the Mediterranean region was originally thought to represent a sub-population of the pine pathogen *Diplodia pinea* and was referred to as *D. pinea* f. sp. *cupressi*. In the USA a similar fungus causing canker and dieback of *Juniperus* spp. was referred to as *Diplodia mutila* (teleomorph: *Botryosphaeria stevensii*). The aim of this study was to characterise the cypress pathogen in terms of morphology and sequences of the ITS region, the β-tubulin and translation elongation factor 1-α genes. Phylogenetic analyses showed that the cypress canker pathogen resides in a clade together with other *Diplodia* species. It is, however, distinct from both *D. pinea* and *D. mutila* and more closely related to *Botryosphaeria tsugae*. The distinct phylogenetic position is supported by differences in conidial morphology and it is, therefore, described as *Diplodia cupressi* sp. nov.

Key words: *Botryosphaeriaceae*, *Cupressus*, *Diplodia*, ITS, phylogeny, taxonomy.

Introduction

A Diplodia canker of Italian cypress (*Cupressus sempervirens* L.) was described by Solel *et al*. (1987) in Israel. Since then the disease has been reported from other countries including Morocco (Frisullo and Graniti, 1990), Italy (Evidente *et al*., 1996), South Africa (Linde *et al*., 1997), Greece (Xenopoulos and Tsopelas, 2000), and Tunisia (Intini *et al*., 2005). Isolations made from bark and outer xylem rings of cankers yielded a fungus that according to Solel *et al*. (1987) was very closely related to *Diplodia pinea* (Desm.) J. Kickx f. (syn. *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & B. Sutton).

*Corresponding author: Artur Alves; e-mail: aalves@bio.ua.pt

They considered the cypress pathogen to represent a distinct subpopulation of the plurivorous species *D. pinea*, and named it *D. pinea* f. sp. *cupressi*. It differed from *D. pinea* by the smaller conidia, and the lack of ornamentation on the inner surface of the conidium wall. Also, unlike *D. pinea*, which is a common shoot blight and canker pathogen of *Pinus* species, the cypress isolates were unable to cause any disease symptoms in artificial inoculations of pine trees (Solel *et al*., 1987; Linde *et al*., 1997; Xenopoulos and Tsopelas, 2000).

Swart *et al*. (1993) challenged the close relationship of *D. pinea* and *D. pinea* f. sp. *cupressi* and showed that both fungi are considerably different in terms of morphological characters like conidium size and shape, growth rates on various culture media, and isozyme profiles. This led them to suggest that, in order to avoid further confusion, the cypress pathogen should be referred to as *Sphaeropsis* sp. until its taxonomy could be further elucidated. The observations of Swart *et al*. (1993) are further supported by molecular data, namely ITS and mt rDNA nucleotide sequence data (Zhou and Stanosz, 2001a, b) and ISSR fingerprinting (Zhou *et al*., 2001).

A fungus identified as *Botryosphaeria stevensii* Shoem. (anamorph *D. mutila* Fr.) was reported as the cause of a canker disease and dieback of *Juniperus* species in the United States (Tisserat *et al*., 1988; Flynn and Gleason, 1993; Stanosz and Moorman, 1997). Tisserat *et al*. (1988) observed the teleomorph and anamorph states of the fungus on *J. scopulorum* Sarg. in Kansas, and confirmed its pathogenicity by artificial inoculations of *J. scopulorum*, *J. virginiana* L., and *J. chinensis* L. Flynn and Gleason (1993) as well as Stanosz and Moorman (1997) reported only the anamorph in Pennsylvania and Iowa, respectively. On the basis of morphological and pathological data, as well as RAPD marker analysis, Stanosz *et al*. (1998) concluded that the cypress pathogen referred to as *D. pinea* f. sp. *cupressi* in Israel was the same species as the juniper pathogen identified as *B. stevensii* in the United States.

A close affinity between *D. mutila* and *D. pinea* f. sp. *cupressi* was noted by Swart *et al*. (1993), who showed that conidial dimensions of the two taxa were similar. However, Tisserat *et al*. (1988) reported differences between the juniper isolates and an isolate of *B. stevensii* obtained from *Malus pumila* Mill., as well as published descriptions of *B. stevensii*. Differences were noted in colony morphology, pigment production, radial growth rate and ability to rot apple fruit. Phylogenetic analysis of ITS and mt rDNA regions (Zhou and Stanosz, 2001a,b) and ISSR fingerprinting analysis (Zhou *et al*., 2001) showed that *B. stevensii* (*D. mutila*) isolates from juniper are closely related to the

cypress pathogen *D. pinea* f. sp. *cupressi* but considerably different from *B. stevensii* isolates from *Malus pumila*.

The purpose of this study was to clarify the taxonomy of the fungus causing disease on cypress and juniper. For this we studied the type specimen and authentic cultures of *D. pinea* f. sp. *cupressi* in terms of morphological characteristics and nucleotide sequence data of ITS, EF1-α, and β-tubulin regions.

Materials and methods

Isolates

Single-conidial or ascospore isolates were prepared according to the methods described by Alves *et al*. (2004). Additional isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Isolates were maintained on ½ strength Difco PDA.

Morphology

Morphological characters were determined from isolates sporulating on 2% water agar bearing autoclaved poplar twigs and incubated at 25°C under fluorescent light to induce sporulation. Structures were mounted in 100% lactic acid and digital images were recorded with a Leica DFC 320 camera on a Leica DMR HC microscope. Measurements were made with the Leica IM500 measurement module. From 50 measurements of each type of structure the mean, standard deviation and 95% confidence intervals were calculated. Dimensions are given as the range of dimensions with minimum and maximum dimensions in parentheses followed by mean and 95% confidence limits.

DNA isolation, PCR amplification and sequencing

The procedures described by Alves *et al*. (2004) were used to extract genomic DNA from fungal mycelium and to amplify part of the nuclear rRNA cluster using the primers ITS1 and ITS4 (White *et al*., 1990). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) and Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify part of the translation elongation factor 1-alpha (EF1-α) gene and part of the β-tubulin gene respectively. PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania) and PCR reaction mixtures were prepared according to Alves *et al*. (2004) and Phillips *et al*. (2006). The

amplification conditions for EF1- α and β -tubulin regions were as follows: initial denaturation of 5 min at 95ºC, followed by 30 cycles of 30 seconds at 94ºC, 45 seconds at 55ºC, and 90 seconds at 72ºC, and a final extension period of 10 min at 72ºC. In some cases where amplification of the EF1- α region was not accomplished, a second PCR was performed using 1 μL of the first PCR amplification as template.

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler. Cycle sequencing procedure was described by Alves *et al*. (2004).

The sequences were obtained with the ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA) and were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in the GenBank public database. Nucleotide sequences for all three DNA regions of additional *Botryosphaeria* species were taken from GenBank (Table 1).

Phylogenetic analyses

The ITS, EF1- α and β -tubulin sequences were aligned with ClustalX version 1.83 (Thompson *et al*., 1997), using pairwise alignment parameters of gap opening = 10, gap extension = 0.1, and multiple alignment parameters of gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25%. Alignments were checked and manual adjustments were made where necessary. Phylogenetic information contained in indels (insertions/deletions) was incorporated into the phylogenetic analyses using simple indel coding as implemented by GapCoder (Young and Healy, 2003).

Phylogenetic analyses of sequence data were done using PAUP*v. 4.0b10 (Swofford, 2003) for Maximum-parsimony (MP) and Neighbour-joining (NJ) analyses and Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck, 2003) for Bayesian analyses. The outgroup taxa selected for rooting the trees were *B. lutea* and *B. ribis*. Trees were visualized with TreeView (Page, 1996).

The kimura-2-parameter nucleotide substitution model (Kimura 1980) was used for distance analysis. All characters were unordered and of equal weight. Bootstrap values were obtained from 1000 NJ bootstrap replicates.

Maximum-parsimony analyses were performed using the heuristic search option with 1000 random taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and alignment gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis and Bull, 1993). Other measures used were tree length (TL) consistency index (CI), retention index (RI) and homoplasy index (HI).

Bayesian analyses employing a Markov Chain Monte Carlo method were performed with Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck, 2003). The general time-reversible model of evolution (Rodriguez *et al*., 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+Γ+G) was used. Four MCMC chains were run simultaneously, starting from random trees, for 1,000,000 generations. Trees were sampled every 100th generation for a total of 10,000 trees. The first 1000 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang, 1996) were determined from a majority-rule consensus tree generated with the remaining 9,000 trees. This analysis was done three times starting from different random trees to ensure that trees from the same tree space were being sampled during each analysis.

The validity of the incongruence length difference (ILD) test (= partition homogeneity test in PAUP*) for determining whether multiple data sets should be combined has been questioned (Cunningham, 1997; Barker and Lutzoni, 2002). In this study we adopted the method of assessing combinability of data sets by comparing highly supported clades among trees generated from different data sets to detect conflict. High support typically refers to bootstrap support values of ≥ 70% and Bayesian posterior probabilities ≥ 95% (Alfaro *et al*., 2003). If no conflict exists between the highly supported clades in trees generated from these different data sets, this suggests the genes share similar phylogenetic histories, and phylogenetic resolution and support could ultimately be increased by combining the data sets.

Results

Phylogenetic analyses

Approximately 550, 400 and 300 bases were determined for the ITS, β-tubulin and EF1-α genes, respectively, of the isolates (Table 1). New sequences were deposited in GenBank (Table 1) and the alignments in TreeBase

Table 1. Isolates studied.

Isolate number[1]	Identity	Host	Locality	Collector	ITS[2]	EF1-α	β-tubulin
CBS 110299	*B. lutea*	*Vitis vinifera*	Oeiras, Portugal	A.J.L. Phillips	*AY259091*	*AY573217*	DQ458848
CBS 115475	*B. ribis*	*Ribes* sp.	New York, USA	B. Slippers	*AY236935*	*AY236877*	AY236906
CBS 230.30	*B. stevensii*	*Phoenix dactylifera*	California, U.S.A.	L.L. Huillier	DQ458886	DQ458869	DQ458849
CBS 112553	*B. stevensii*	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259093*	*AY573219*	DQ458850
CBS 112554	*B. stevensii*	*Pyrus communis*	Monte da Caparica, Portugal	A.J.L. Phillips	*AY259095*	DQ458870	DQ458851
JL 375	*B. stevensii*	*Fraxinus excelsior*	Llanars, Catalonia, Spain	J. Luque	DQ458887	DQ458871	DQ458852
CMW 7060	*B. stevensii*	*Fraxinus excelsior*	Maarseveen, Netherlands	H.A. van der Aa	*AY236955*	*AY236904*	*AY236933*
CBS 112549	*B. corticola*	*Quercus suber*	Aveiro, Portugal	A. Alves	*AY259100*	*AY573227*	DQ458853
CBS 112547	*B. corticola*	*Quercus ilex*	Córdoba, Spain	M.E. Sanchez	*AY259110*	DQ458872	DQ458854
CBS 418.64	*B. tsugae*	*Tsuga heterophylla*	British Columbia, Canada	A. Funk	DQ458888	DQ458873	DQ458855
CBS 112555	*B. obtusa*	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259094*	*AY573220*	DQ458856
CBS 119049	*B. obtusa*	*Vitis* sp.	Italy	L. Mugnai	DQ458889	DQ458874	DQ458857
CBS 124.13	*B. rhodina*	Unknown	U.S.A.	J.J. Taubenhaus	DQ458890	DQ458875	DQ458858
CAA 006	*B. rhodina*	*Vitis vinifera*	California, USA	T.J. Michailides	DQ458891	DQ458876	DQ458859
CBS 115812	*L. gonubiensis*	*Syzygium cordatum*	Eastern Cape, South Africa	D. Pavlic	DQ458892	DQ458877	DQ458860
CBS 168.87	*D. cupressi*	*Cupressus sempervirens*	Bet Dagan, Israel	Z. Solel	DQ458893	DQ458878	DQ458861
CBS 261.85	*D. cupressi*	*Cupressus sempervirens*	Bet Dagan, Israel	Z. Solel	DQ458894	DQ458879	DQ458862
CBS 393.84	*D. pinea*	*Pinus nigra*	Putten, Netherlands	H.A. van der Aa	DQ458895	DQ458880	DQ458863
CBS 109725	*D. pinea*	*Pinus patula*	Habinsaran, South Africa	M.J. Wingfield	DQ458896	DQ458881	DQ458864
CBS 109727	*D. pinea*	*Pinus radiata*	Stellenbosch, South Africa	W.J. Swart	DQ458897	DQ458882	DQ458865
CBS 109943	*D. pinea*	*Pinus patula*	Indonesia	M.J. Wingfield	DQ458898	DQ458883	DQ458866
CBS 109944	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	DQ458899	DQ458884	DQ458867
CBS 113424	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	DQ458900	DQ458885	DQ458868

[1] Acronyms of culture collections: CAA – A. Alves, Universidade de Aveiro, Portugal; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW – M.J. Wingfield, FABI, University of Pretoria, South Africa; JL – J. Luque, IRTA, Spain. Isolates in bold are cultures ex-type.

[2] Sequence numbers in italics were retrieved from GenBank. All others were determined in the present study.

(S1542). Sequences of the three genes were aligned and analysed separately by maximum parsimony and Bayesian analysis, and the resulting trees were compared. No major conflicts were detected between single gene phylogenies indicating that the genes could be combined, thus resulting in increased phylogenetic resolution.

The sequence alignment of 23 isolates (Table 1), including the two outgroup species, consisted of 579 characters for the ITS region, 331 for the EF1-α gene and 413 for the β -tubulin gene, including alignment gaps. Indels were coded separately and added to the end of the alignment as characters 1326-1397. In the analyses, alignment gaps were treated as missing data.

The combined dataset consisted of 1397 characters, of which 1043 were constant and 83 variable characters were parsimony-uninformative. Maximum parsimony analysis of the remaining 271 parsimony-informative characters resulted in a single most parsimonious trees (TL = 518 steps, CI = 0.8205, HI = 0.1795, RI = 0.8818, RC = 0.7235). Bayesian and NJ analyses produced trees with the same topology as the MP tree. The Bayesian analysis was done three times and the resulting trees in each run were identical. The MP tree is shown in Fig. 1 with MP bootstrap supports above and posterior probabilities below the branches.

Phylogenetic analyses clearly separated the ingroup taxa into several clades which correspond to known species as well as two *D. pinea* morphotypes. The two species with *Lasiodiplodia* anamorphs (*B. rhodina* (Berk. & M.A. Curtis) Arx and *L. gonubiensis* Pavlic, Slippers & M.J. Wingf.) appear as a highly supported and distinct clade at the base of the ingroup. In phylogenetic terms *D. cupressi* is most closely related to *B. tsugae*, with both species forming a larger clade together with several isolates identified as *B. stevensii*.

Morphology

The two isolates received as *D. pinea* f. sp. *cupressi* (CBS 261.85 and CBS 168.87) sporulated well in culture. Conidia were of the type associated with *Diplodia mutila*. Thus, they were hyaline, thick-walled and aseptate with both ends rounded, or with a truncate base. Morphology in culture corresponded with the characteristics of a specimen lodged by Z. Solel in IMI 303475. These specimens and cultures differed from *D. pinea* in which the conidia are brown and larger. Conidia of the isolates from *Cupressus* are somewhat wider than typical for *D. mutila*. Since the isolates from *Cupressus* are phylogenetically and morphologically distinct, it is described here as a new species in *Diplodia*.

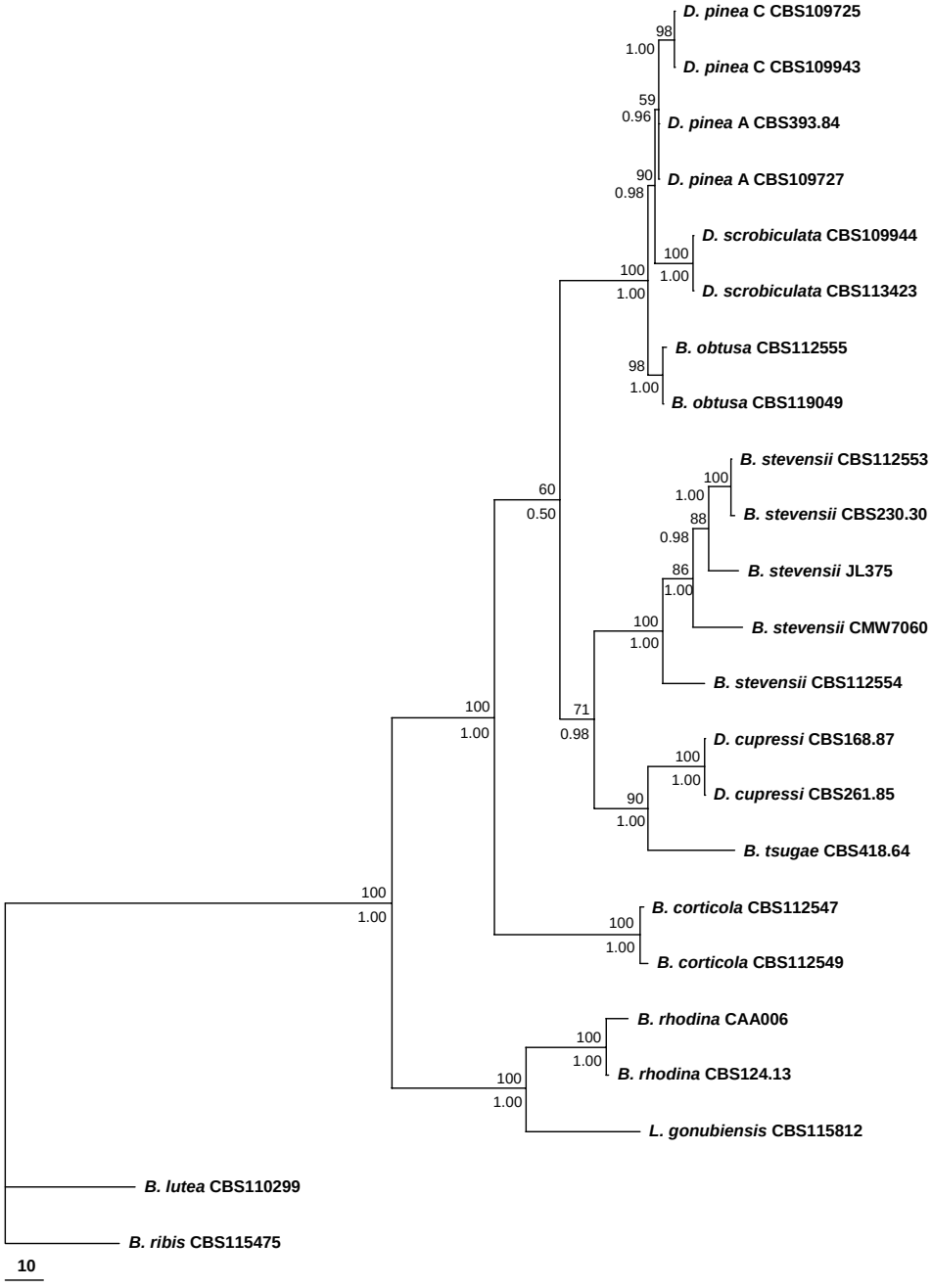


Fig. 1. Single most parsimonious tree resulting from combined ITS, EF1-α and β-tubulin sequence data. Bootstrap support values from 1000 replications are shown above the nodes with pooled posterior probabilities from three independent Bayesian analyses below the nodes. The tree was rooted to *B. lutea* and *B. ribis*. The bar represents 10 changes.

Diplodia cupressi A.J.L. Phillips & A. Alves, **sp. nov.** (Figs 2-10)
Mycobank: MB510137

Etymology: Named for the host genus it was first reported on, namely *Cupressus*.

Conidiomata in contextu hospitis inclusa, solitaria, stromatiformia, globosa, usque 300 µm diametro. *Cellulae conidiogenae* 12.5-20 × 4-4.5 µm, holoblasticae, hyalinae, subcylindricae, percurrenter cum 1-4 proliferationibus prolificentes, vel in plano eodem periclinaliter incrassatae. *Conidia* 23.5-28.5 × 13.5-15.0 µm, hyalinae, unicellulares, parietibus crassis, ovoidea, apicibus obtuse rotundato, in fundo obtuse rotundato, cum aetas colorescentia uno cum septo. *Microconidiophorae* cylindricae, hyalinae usque 10 µm longae, 2.5-3 µm latae. *Cellulae microconidiogenae* 10-14 × 2-2.5 µm, cylindricae, hyalinae, holoblasticae, phialidibus typicus periclinater spissescentibus. *Microconidia* hyalinae, unicellulares, 4-5 × 1.5 µm.

Conidiomata up to 300 µm diam., solitary, separate, uniloculate, dark brown to black, globose, ostiolate, wall composed of thick-walled textura angularis, becoming thin-walled and hyaline toward the inner region. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 12.5-20 × 4-4.5 µm, hyaline, smooth, holoblastic forming conidia at their tips, proliferating internally giving rise to periclinal thickenings or proliferating percurrently with 1-4 close or widely spaced annellations, formed from the inner wall of the pycnidium. *Conidia* (21.5–)23.5–28.5(–30.5) × (12.0–)13.5–15.0(–16.0) µm, 95% confidence limits = 24.4–25.4 × 13.9–14.5 µm, ($\overline{x}$ ± S.D. of 50 = 24.9 ± 1.9 × 14.2 ± 0.9 µm, L/W = 1.76 ± 0.18) thick-walled, wall up to 2 µm wide, ovoid with both ends rounded, aseptate, hyaline and remaining so for a long time, becoming brown and one-septate after discharge from the pycnidia. *Microconidiophores* hyaline, smooth, cylindrical, up to 10 µm long, 2.5-3 µm wide. *Microconidiogenous cells* discrete or integrated, hyaline, smooth, cylindrical, holoblastic or proliferating via determinate phialides with periclinal thickening, 10–14 × 2–2.5 µm. *Microconidia* hyaline, smooth, aseptate, rod-shaped with rounded ends, 4–5 × 1.5 µm.

Teleomorph: An unknown *Botryosphaeria* sp.

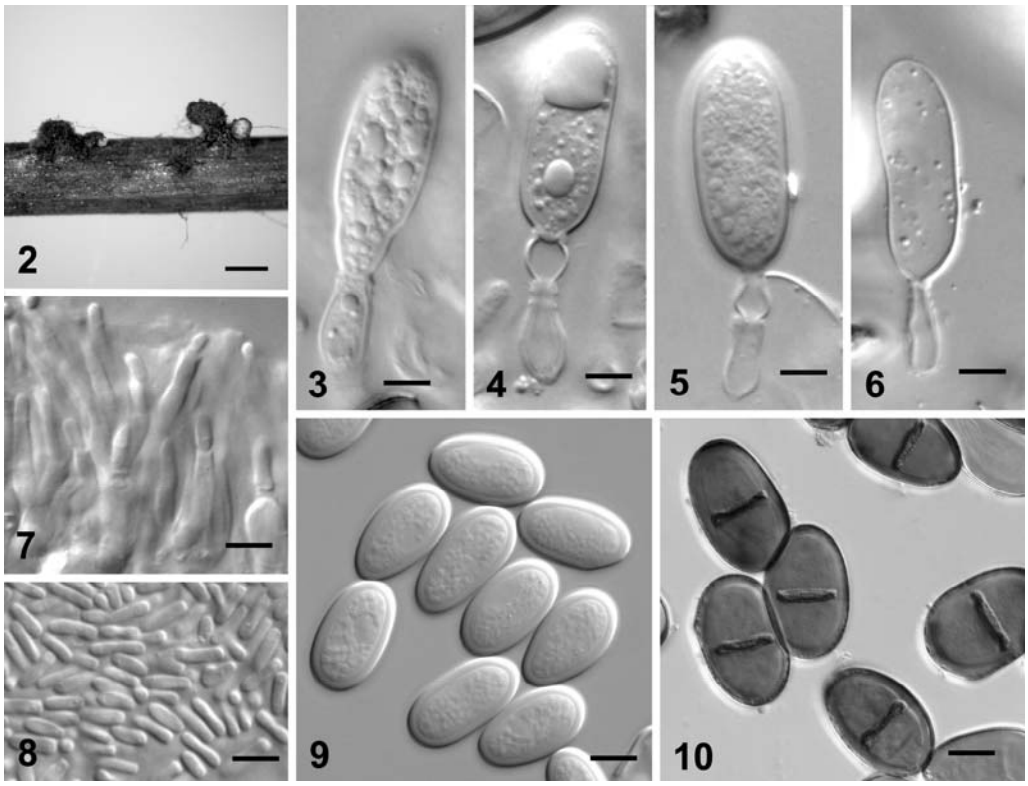
Habitat: On cankered stems of *Cupressus* and *Juniperus* species.

Known distribution: Greece, Israel, Italy, Morocco, South Africa, Tunisia, USA.

Material examined: ISRAEL, Bet Dagan, dried culture from cankered stems of *Cupressus sempervirens*, 1986, Z. Solel (HERB IMI 303475; **holotype**, culture ex-type CBS 168.87).

Discussion

The taxonomy of the fungal pathogen causing canker of cypress and juniper has been confused. Solel *et al*. (1987) considered it to be a forma specialis of the plurivorous pine pathogen *D. pinea* (= *S. sapinea*) and named it



Figs 2-10. *Diplodia cupressi* (from cultures ex-holotype). **2.** Conidiomata formed in culture on an autoclaved pine needle. **3-6.** Conidiogenous cells. **7.** Microconidiogenous cells. **8.** Microconidia. **9.** Hyaline, aseptate, thick-walled conidia. **10.** Brown, one-septate conidia. Bars: 2 = 500 μm; 3-10 = 10 μm.

D. pinea f. sp. *cupressi*, while in the United States it was identified as *B. stevensii* or its anamorph *D. mutila*.

In this paper we studied the type specimen and authentic cultures of *D. pinea* f. sp. *cupressi* in terms of morphological characteristics and nucleotide sequences of ITS, EF1-α, and β-tubulin regions. On the basis of the morphological and multigene sequence data we recognize the cypress and juniper canker pathogen as a new species and describe it as *D. cupressi* sp. nov.

Diplodia cupressi is clearly differentiated from *D. pinea* and *D. mutila* on the basis of morphological, cultural, and molecular characters. Conidia of *D. cupressi* are thick-walled, smooth, initially hyaline and aseptate and remain so for a long time, ultimately becoming one-septate and dark-walled after discharge from the pycnidia. In contrast, conidia of *D. pinea* become dark at an early stage of development and whilst within the pycnidial cavity. *Diplodia cupressi* differs from *D. pinea* not only in the stage at which the conidia

become pigmented, there are some other characters that differentiate the two species. Conidia of *D. pinea* become septate just before germination, while in *D. cupressi* septation does not seem to be associated with germination. Furthermore, conidia of *D. pinea* are larger than those of *D. cupressi* with average lengths exceeding 40 µm. As discussed previously by Swart *et al*. (1993) and Stanosz *et al*. (1998) and confirmed by the data presented in this study *D. cupressi* differs markedly from *D. pinea* and cannot be considered merely as a host specialized sub-population within this species.

The thick-walled conidia that remain hyaline for a long time are characteristic of *Diplodia* as typified by *D. mutila* (Alves *et al*., 2004, Phillips *et al*., 2005). In this respect, *D. cupressi* closely resembles *D. mutila*. However, the conidia of *D. cupressi* are wider than typical of *D. mutila* (Alves *et al*., 2004) and correspond well with those described by Solel *et al*. (1987) in the original description of this fungus. They also correspond to the description of the fungus Tisserat *et al*. (1988) referred to as *B. stevensii*. Since they are larger than in the type of *D. mutila* and in the anamorph associated with the type of *B. stevensii* (Alves *et al*., 2004) we conclude that this fungus is not *B. stevensii* (*D. mutila*). This is supported by previous observations from Tisserat *et al*. (1988) who reported that several differences existed between *D. cupressi* and isolates obtained from apple and identified as *B. stevensii*.

Phylogenetic analyses of separate and combined nucleotide sequence data from three genes (ITS, EF1-α, and β-tubulin) place *D. cupressi* within the Clade 1 of the *Botryosphaeriaceae* (Crous *et al*., 2006), but clearly separate it from all other *Diplodia* species. In phylogenetic terms *D. cupressi* is most closely related to *B. tsugae*, a species occurring on *Tsuga heterophylla* and known only from British Columbia (Funk, 1964), than to *B. stevensii*. Morphologically *D. cupressi* is easily separated from the anamorph of *B. tsugae*, which has much larger conidia (see Table 2).

Although no teleomorph has been described for this species it apparently does exist and is clearly a member of the genus *Botryosphaeria*. The teleomorph was reported by Tisserat *et al*. (1988) on dead branches of *J. scopulorum* and at the time identified as *B. stevensii*. However, no specimens of the teleomorph could be traced (Tisserat pers. comm.). Tisserat *et al*. (1988) established the connection between the teleomorphic and anamorphic states by culture of ascospores. According to these authors pseudothecia were immersed in the host, asci measured 120 × 16 µm; ascospores were hyaline, smooth, thick walled, elliptical to ovate, and 32-40(37) × 12-16(14) µm. Although dimensions of the asci fall within the range of the type of *B. stevensii*, the ascospores are much larger than were reported for the type of *B. stevensii*, which average 31.5

Table 2. *Botryosphaeria* and *Diplodia* species associated with gymnosperms.

Teleomorph	Anamorph	Conidia		References
		Colour/septation	Size (µm)	
B. laricis (Wehm.) Arx & E. Müll.	A form of *Macrophoma sapinea*	dark, aseptate	17.5-44.1 × 11.2-21	Smerlis (1970)
B. tsugae A. Funk	*Diplodia* sp.	hyaline, aseptate	36-41 × 18-22	Funk (1964)
Botryosphaeria sp.	*D. cupressi* A.J.L. Phillips & A. Alves	hyaline, aseptate	23.5-28.5 × 13.5-15	this work
"*B. stevensii*"	"*D. mutila*"	hyaline, aseptate	23-32 × 12-15	Tisserat *et al*. (1988)
Unknown	*D. pinea* (Desm.) J. Kickx f.	dark, aseptate	30-45 × 10-16	Punithalingam and Waterston (1970)
Unknown	*D. scrobiculata* J. de Wet *et al*.	dark, 1-3-septate	37.5-41.5 × 13-15.5	de Wet *et al*. (2003)
Unknown	*D. cyparissa* Cooke & Harkn.	hyaline, aseptate	20-22 × 9	Saccardo (1884)
Unknown	*D. juniperi* Westend.	dark, 1-septate	18-20 × 8-10	Saccardo (1884)
Unknown	*D. kansensis* Ellis & Everh.	dark, 1-septate	20-27 × 12-15	Saccardo (1895)
Unknown	*D. thujae* Sacc.	Unknown	20-25 × 10	Saccardo (1884)
Unknown	*D. thujae* G.H. Otth	dark, 1-septate	20 × 9	Saccardo (1895)
Unknown	*D. thujae* Westend.	dark, 1-septate	18-20 × 9-10	Saccardo (1899)
Unknown	*D. thujana* Peck & Clinton	dark	18-23	Saccardo (1884)
Unknown	*D. thyoidea* Cooke & Ellis	dark	25-28 × 12-13	Saccardo (1884)
Unknown	*D. virginiana* Cooke & Ravenel	dark, 1-septate	20-25 × 10	Saccardo (1884)
Unknown	*D. megalospora* Berk. & M.A. Curtis	Unknown	37 × 12	Saccardo (1884)
Unknown	*D. conigena* Desm.	dark, 1-septate	26-30 × 12-15	Saccardo (1884)

± 2.3 × 11.4 ± 0.9 µm (Alves *et al*., 2004). Also, ascospores of *B. stevensii* are fusiform, widest in the middle, both ends obtuse, hyaline, thin-walled, smooth, aseptate, rarely becoming pale brown and 1- or 2-septate with age. It is thus apparent that the ascomycete reported by Tisserat *et al*. (1988) represents a previously undescribed species for which no name is provided due to the lack of a suitable specimen. It can be distinguished from its closest phylogenetic relative, *B. tsugae*, whose asci and ascospores are larger.

Many *Botryosphaeria* and *Diplodia* species have been associated with Gymnosperms (Table 2). Cultures are lacking for most of these species thus making appropriate comparisons impossible. However, it can be seen from the data retrieved from the literature and summarized in Table 2 that none of the available names is suitable for *D. cupressi*.

Due to the close morphological resemblance *D. cupressi* has in the past been identified as *D. mutila* (Tisserat *et al*., 1988; Flynn and Gleason, 1993; Stanosz and Moorman, 1997). For this reason we included in this study several isolates obtained from different hosts and whose morphological features support their identification as *B. stevensii* or its anamorph *D. mutila*. These cultures differed phylogenetically from *D. cupressi* and grouped in a highly supported clade. Although closely related phylogenetically, some variation can be seen between these cultures in terms of nucleotide sequence from the three genes analysed (ITS, EF1-α, and β-tubulin). This variability falls within the range that can be found between different species in the genus *Botryosphaeria* and so these isolates probably represent a complex of cryptic species that needs to be resolved.

Acknowledgements

This work was financed by Fundação para a Ciência e a Tecnologia (FCT) under project Project POCTI/AGR/56140/2004 Artur Alves was supported by grant number SFRH/BD/10389/2002 from FCT. The curator of IMI is thanked for the loan of IMI 303475.

References

Alfaro, M.E., Zoller, S. and Lutzoni, F. (2003). Bayes or Bootstrap? A simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. Molecular Biology and Evolution 20: 255–266.

Alves, A., Correia, A., Luque, J. and Phillips, A.J.L. (2004). *Botryosphaeria corticola* sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph *Diplodia mutila*. Mycologia 96: 598-613.

Barker, F.K. and Lutzoni, F.M. (2002). The utility of the Incongruence Length Difference test. Systematic Biology 51: 625-637.

Carbone, I. and Kohn, L.M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553-556.

Crous, P.W., Slippers, B., Wingfield, M.J., Rheeder, J., Marasas, W.F.O., Phillips, A.J.L., Alves, A., Burgess, T., Barber, P. and Groenewald, J.Z. (2006). Phylogenetic lineages in the *Botryosphaeriaceae*. Studies in Mycology 55: 235-253.

Cunningham, C. W. (1997). Can three incongruence tests predict when data should be combined? Molecular Biology and Evolution 14: 733–740.

De Wet, J., Burgess, T., Slippers, B., Preisig, O., Wingfield, B.D. and Wingfield, M.J. (2003). Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. Mycological Research 107: 557–566.

Evidente, A., Sparapano, L., Motta, A., Giordano, F., Fierro, O. and Frisullo, S. (1996). A phytotoxic pimarane diterpene of *Sphaeropsis sapinea* f. sp. *cupressi*, the pathogen of a canker disease of cypress. Phytochemistry 42:1541-1546.

Flynn, P.H. and Gleason, M.L. (1993). Isolation of *Botryosphaeria stevensii*, cause of Botryosphaeria canker, from Rocky Mountain juniper in Iowa. Plant Disease 77: 210.

Frisullo, S. and Graniti, A. (1990). New records of Botryosphaeria and Diplodia cankers of cypress. In: *Proc. 8th Congress of the Mediterranean Phytopathological Union* (ed. A. Bouzouraa). Actes Editions, Rabat, Morocco: 431-432.

Funk, A. (1964). *Botryosphaeria tsugae* n. sp. causing dieback of western hemlock in British Columbia. Canadian Journal of Botany 42: 769-775.

Glass, N.L. and Donaldson, G.C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Applied and Environmental Microbiology 61: 1232–1330.

Hillis, D.M. and Bull, J.J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology 42: 182-192.

Intini, M., Panconesi, A. Ben Jamâa, M.L., Stanosz G. and Smith D. (2005). First report of Diplodia canker of cypress caused by *Diplodia pinea* f. sp. *cupressi* on Mediterranean cypress in Tunisia Plant Disease 89: 1246.

Kimura, M. (1980). A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111-120.

Linde, C., Kemp, G.H.J and Wingfield M.J. (1997). First report of Sphaeropsis canker of cypress in South Africa. European Journal of Forest Pathology 27:173-177.

Page, R.D. (1996). TreeView: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357-358.

Phillips, A.J.L., Alves, A., Correia, A. and Luque, J. (2005). Two new species of *Botryosphaeria* with brown, 1-septate ascospores and *Dothiorella* anamorphs. Mycologia 97: 513-529.

Phillips, A.J.L., Oudemans, P.V., Correia, A. and Alves, A., (2006). Characterisation and epitypification of *Botryosphaeria corticis*, the cause of blueberry cane canker. Fungal Diversity 21: 141-155.

Punithalingam, E. & Waterston, J.M. (1970). C.M.I. Descriptions of Plant Pathogenic Fungi and Bacteria No. 273. CAB International, Kew, England.

Rannala, B. and Yang, Z. (1996). Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. Journal of Molecular Evolution 43: 304-311.

Rodriguez, F., Oliver, J.F., Marin, A. and Medina, J.R. (1990). The general stochastic model of nucleotide substitutions. Journal of Theoretical Biology 142: 485-501.

Ronquist, F. and Huelsenbeck, J.P. (2003). MrBayes3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Saccardo, P.A. (1884). Sylloge fungorum omnium hucusque cognitorum III.

Saccardo, P.A. (1895). Sylloge fungorum omnium hucusque cognitorum XI.

Saccardo, P.A. (1899). Sylloge fungorum omnium hucusque cognitorum XIV.

Smerlis, E. (1970). *Botryosphaeria laricis* and its relationship to *Macrophoma sapinea*. Canadian Journal of Botany 48: 1899–1901.

Solel, Z., Madar, Z., Kimchi, M. and Golan, Y. (1987). Diplodia canker of cypress. Canadian Journal of Plant Pathology 9: 115-118.

Stanosz, G.R. and Moorman G.W. (1997). Branch dieback of savin juniper in Pennsylvania caused by Diplodia mutila. Plant Disease 81: 111.

Stanosz, G.R., Swart, W.J. and Smith D.R. (1998). Similarity between fungi identified as *Diplodia pinea* f. sp. *cupressi* in Israel and *Botryosphaeria stevensii* or *Diplodia mutila* on *Juniperus* in the United States. European Journal of Forest Pathology 28: 33-42.

Swart, W.J., Wingfield, M.J. and Grant W.S. (1993). Comparison of *Sphaeropsis sapinea* and *Sphaeropsis sapinea* f. sp. *cupressi*. Mycological Research 97: 1253-1260.

Swofford, D.L. (2003). *PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods) Version 4*. Sunderland, Massachusetts: Sinauer Associates.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876-4882.

Tisserat, N.A., Rossman, A.Y. and Nus A. (1988). A canker disease of Rocky Mountain juniper caused by *Botryosphaeria stevensii*. Plant Disease 72: 699-701.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols*: *A Guide to Methods and Applications* (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press San Diego, California: 315-322.

Xenopoulos S. and Tsopelas P. (2000). Sphaeropsis canker, a new disease of cypress in Greece. Forest Pathology 30: 121-126.

Young, N.D. and Healy, J. (2003). GapCoder automates the use of indel characters in phylogenetic analysis. BMC Bioinformatics 4: art. 6.

Zhou, S. and Stanosz, G.R. (2001a). Relationships among *Botryosphaeria* species and associated anamorphic fungi inferred from the analyses of ITS and 5.8S rDNA sequences. Mycologia 93: 516-527.

Zhou, S. and Stanosz G.R. (2001b). Primers for amplification of mt SSU rDNA, and a phylogenetic study of *Botryosphaeria* and associated anamorphic fungi. Mycological Research 105: 1033-1044.

Zhou, S., Smith, D.R. and Stanosz G.R. (2001). Differentiation of *Botryosphaeria* species and related anamorphic fungi using Inter Simple or Short Sequence Repeat (ISSR) fingerprinting. Mycological Research 105: 919-926.

(Received 28 March 2006; accepted 9 August 2006)