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Ceratocystis atrox sp. nov. associated with *Phoracantha acanthocera* infestations on *Eucalyptus grandis* in Australia

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Abstract. *Ceratocystis* spp. include important pathogens of trees as well as apparently saprophytic species. Four species have been recorded on *Eucalyptus grandis* in Australia, of which only one, *C. pirilliformis* Barnes and M.J. Wingf., is known to be pathogenic. A recent survey of pests and diseases of *Eucalyptus* trees in northern Queensland revealed a species of *Ceratocystis* associated with the tunnels made by the aggressive wood-boring insect *Phoracantha acanthocera* (Macleay) (Cerambicydae: Coleoptera). The aim of the present study was to identify the fungus based on morphological characteristics and comparisons of DNA sequence data for three gene regions. The fungus peripherally resembles *C. fimbriata* Ell. and Halst. but differs from this species most obviously by having much darker mycelium, longer ascomatal necks, segmented hyphae and an absence of aleuroconidia. Comparisons of combined sequence data confirmed that the *Ceratocystis* sp. from *P. acanthocera* represents an undescribed taxon, which is provided with the name *Ceratocystis atrox* sp. nov. *C. atrox* appears to have a close relationship with *P. acanthocera*, although its role in the biology of the insect is unknown and its pathogenicity has not been considered.

Additional keywords: bark beetles, Coleoptera : Scolytinae, sap stain.

Introduction

Species of *Ceratocystis* include some of the most important pathogens of trees in the world (Redfern *et al*. 1987; Christiansen and Solheim 1990; Kile 1993). They also include wound-infecting saprophytes, agents of sap stain and species of unknown ecology. The pathogenic species include two discrete groups. These include species that are either vectored by bark beetles (Coleoptera : Scolytinae) in a specific mutualistic relationship (Redfern *et al*. 1987; Christiansen and Solheim 1990; Marin *et al*. 2003) or those that infect wounds and are important wilt pathogens broadly treated as species of the *Ceratocystis fimbriata sensu lato* (*s.l.*) complex (Webster and Butler 1967; Kile 1993).

Recent studies have shown that species in the *C. fimbriata s.l.* complex represent a relatively large number of cryptic taxa (Wingfield *et al*. 1996; Barnes *et al*. 2003*a*; Van Wyk *et al*. 2004*b*; Baker-Engelbrecht and Harrington 2005; Johnson *et al*. 2005). Convincing evidence for the existence of these species has largely arisen from the application of the phylogenetic species concept and DNA sequence comparisons. There is also some evidence for host-specific taxa in this group (Baker-Engelbrecht and Harrington 2005) although overlapping of host ranges are also found (Marin *et al*. 2003).

Several *Ceratocystis* spp. have been recorded from *Eucalyptus* spp. in various parts of the world. The most important of these is *C. fimbriata s.l.*, which causes a serious vascular wilt disease of *Eucalyptus* spp. in Uruguay (Barnes *et al*. 2003*b*), Congo (Roux *et al*. 2000), Uganda (Roux *et al*. 2001) and is known in South Africa in the absence of an associated disease (Roux *et al*. 2004). In Australia, where most *Eucalyptus* spp. are native, four species of *Ceratocystis* have been found on these trees. They include *C. moniliformis* Hedgc., *C. moniliformopsis* Yuan and Mohammed (Yuan and Mohammed 2002), *C. eucalypti* Yuan and Kile (Kile *et al*. 1996) and *C. pirilliformis* (Barnes *et al*. 2003*a*). Of these, *C. pirilliformis* has been shown to be pathogenic and this has only been on greenhouse grown trees in South Africa, where the fungus also occurs on *Eucalyptus* (Roux *et al*. 2004).

During a recent survey of *Eucalyptus* pests and diseases in northern Queensland (Qld), a *Ceratocystis* sp. was found sporulating in the tunnels of the aggressive wood-boring insect *Phoracantha acanthocera* (Cerambicydae: Coleoptera; syn. *Tryphocaria acanthocera*) (Wang *et al*. 1999), commonly known as the bulls-eye borer. This insect is native to Australia and can cause serious damage to *Eucalyptus* spp. grown in plantations (Phillips 1993*a*, 1993*b*) and in regrowth forests (Abbott *et al*. 1991; Farr *et al*. 2000). The aim of the present study was to identify the unknown *Ceratocystis* sp. that occurs in association with *P. acanthocera*.

Methods

Isolates

Tunnels of *P. acanthocera* in 7-year-old *E. grandis* trees growing in a plantation west of Cairns, Qld were examined

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(Fig. 1). Wood associated with the tunnels had very distinct vascular staining (Fig. 1) and fungi were commonly found sporulating on the surface of the discoloured wood. The most common of these fungi had ascomata with globose bases and long necks, resembling species of *Ceratocystis* and *Ophiostoma*.

Fig. 1. Disease symptoms and damage caused by *Phoracantha acanthocera* on *Eucalyptus grandis* trees in Australia: (*A*) cracking bark, (*B*) damage caused by larvae and (*C*, *D*) fungal staining associated with insect tunnels and *Ceratocystis atrox*.

Samples representing the inner surface of tunnels of *P. acanthocera* were collected from five trees and transferred to the laboratory for further study. Spore droplets from the apices of perithecia were transferred to 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with streptomycin sulfate (0.001 g/vol, Sigma, Steinheim, Germany) and incubated at 25°C. In addition, a selective carrot baiting technique (Moller and DeVay 1968) was used to obtain isolates of *Ceratocystis*.

All isolates from the tunnels of *P. acanthocera* were purified on 2% MEA. They were subsequently stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa and representative isolates have been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Representative cultures were dried on glycerol and deposited with the National Herbarium of South Africa (PREM).

Morphology

Morphological characters were described from 2-week-old cultures grown on 2% MEA. Fungal structures were mounted on glass slides in lactophenol and examined under a Zeiss Axio Vision microscope (Carl Zeiss Ltd, Germany). Fifty measurements were made for each taxonomically relevant structure. Ranges, averages and standard deviations (s.d.) were determined for each of these structures. The measurements are presented as: [(minimum−) mean minus s.d. − mean plus s.d.(−maximum)]. Colours of cultures were defined based on the mycological colour charts of Rayner (1970). Growth studies were performed on the type of the species as well as a paratype, by placing a 5-mm plug from an actively growing culture (2 weeks old) in the centre of a 90-mm 2% MEA Petri dish. The plates were incubated at five different temperatures ranging from 5–35°C (with 5°C intervals). Measurements were made after 1 week. This study was then repeated.

DNA isolation, PCR reactions and sequence analysis

DNA of four isolates was extracted as described by Van Wyk *et al*. (2006). PCR reactions for internal transcribed spacer regions (ITS) 1 and 2, including the 5.8 S rDNA region, the β-tubulin region and the transcription elongation factor-1α (EF-1α) region were prepared. The primers used to amplify the DNA for these three regions were those of White *et al*. (1990), Glass and Donaldson (1995) and Jacobs *et al*. (2004), respectively.

PCR reaction mixtures, for all three gene regions, consisted of 10 × FastStart Taq DNA polymerase PCR Buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 200 µM of each dNTP, FastStart *Taq* enzyme (2 U) (Roche Diagnostics, Mannheim, Germany), 200 nM of the forward and reverse primers, and 2–10 ng DNA. Reaction volumes were adjusted to 50 µL with sterile water. The PCR program was set for 4 min at 95°C for initial denaturation of the double stranded DNA. This was followed by 10 cycles consisting of a denaturation step at 95°C for 40 s, an annealing step for 40 s at 55°C and an elongation step for 45 s at 70°C. Subsequently, 30 cycles of 94°C for 20 s, 55°C for 40 s with a 5 s extension step after each cycle and 70°C for 45 s, were performed. A final step of 10 min at 72°C completed the program. Amplification of the DNA for the three gene regions was confirmed under UV illumination using 2% agarose (Roche Diagnostics) gel electrophoresis in the presence of ethidium bromide. After amplification, amplicons were purified using 6% Sephadex G-50 columns (Sigma).

PCR amplicons were sequenced in both directions using the ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, CA), with the same primers as those used for DNA amplication. Sequencing reactions were run on an ABI PRISM 3100 Autosequencer (Applied BioSystems) and sequences were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems). Sequences were manually aligned with known species of *Ceratocystis* obtained from GenBank and analysed using PAUP version 4.0b10* (Swofford 2002). To determine whether the sequences for the three gene regions could be combined in one dataset, a partition homogeneity test (Swofford 2002) was conducted. Gaps were treated as a fifth character and trees were obtained via stepwise addition of 1000 replicates with the Mulpar option in effect. The heuristic search option based on parsimony with stepwise addition was used to obtain the phylogram. Confidence intervals using 1000 bootstrap replicates were calculated. *C. virescens* (Davidson) Moreau was designated as the out-group taxon. All sequences derived from this study were deposited in GenBank (Table 1).

Results

Isolates

Isolations from ascomata in the tunnels of *P. acanthocera* yielded cultures of a fungus that had a very distinct dark green colour. The perithecia exuded hat-shaped ascospores in sticky spore drops, typical of many species of *Ceratocystis* and *Ophiostoma*. Cultures of the fungus had a distinct *Thielaviopsis* anamorph that is specific to ophiostomatoid fungi residing in *Ceratocystis*.

Four isolates of a *Ceratocystis* sp. (CMW 19383/CBS 120517, CMW 19385/CBS 12051, CMW 19387/CBS 120519, CMW 19389/CBS 120225) were collected from four of the five *E. grandis* trees sampled. Based on their very dark green colour, the isolates were distinct from all known species of this genus. Chlamydospores were also absent in this fungus. Two types of conidiophores were found. The more common of these had long conidiogenous cells and others were shorter with wider apices. Both cylindrical and barrel-shaped conidia were present. The optimum growth range for these isolates was 20–25°C. No growth was observed at 5, 10 and 35°C. At 15°C the isolates grew ∼12 mm in 7 days. At 20, 25 and 30°C the isolates grew ∼26, 33 and 17 mm, respectively.

DNA isolation, PCR reactions and sequence analysis

DNA sequencing yielded amplicons of ∼500 bp for both the ITS and β-tubulin gene regions and amplicons of ∼800 bp were obtained for the EF-1α. Partition homogeneity tests showed that the data could be combined ($P = 0.05$). Two most parsimonious trees were obtained, one of which was selected for representation (Fig. 2). This tree had a length of 1472 bp, the total amount of characters were 1913, with 1150 of these characters being constant, 301 characters being parsimony uninformative and 462 characters being parsimony informative, with CI = 0.7554, HI = 0.2446, RI = 0.8216 and RC = 0.6207.

Table 1. *Ceratocystis* isolates used in this study

Species	Isolate no.	GenBank accession number	Host	Geographical origin	Collector(s)
C. albifundus	CMW 4068	DQ520638 EF070429 EF070400	*Acacia mearnsii*	RSA	J. Roux
C. albifundus	CMW 5329	AF388947 DQ371649 EF070401	*Acacia mearnsii*	Uganda	J. Roux
C. cacaofunesta	CMW 15051 CBS 152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Costa Rica	A. J. Hansen
C. cacaofunesta	CMW 14809 CBS 115169	DQ520637 EF070428 EF070399	*Theobroma cacao*	Ecuador	C. Suarez
C. fimbriata	CMW 15049 CBS 141.37	DQ520629 EF070442 EF070394	*Ipomoea batatas*	USA	C. F. Andrus
C. fimbriata	CMW 1547	AF264904 EF070443 EF070395	*Ipomoea batatas*	Papua New Guinea	E. C. H. McKenzie
C. pirilliformis	CMW 6569	AF427104 DQ371652 AY528982	*Eucalyptus nitens*	Australia	M. J. Wingfield
C. pirilliformis	CMW 6579	AF427105 DQ371653 AY528983	*Eucalyptus nitens*	Australia	M. J. Wingfield
C. platani	CMW 14802 CBS 115162	DQ520630 EF070425 EF070396	*Platanus occidentalis*	USA	T. C. Harrington
C. platani	CMW23918	EF070426 EF070397	*Platanus* sp.	Greece	M. J. Wingfield
C. polychroma	CMW 11424 CBS 115778	AY528970 AY528966 AY528978	*Syzygium aromaticum*	Indonesia	M. J. Wingfield
C. polychroma	CMW 11436 CBS 115777	AY528971 AY528967 AY528979	*Syzygium aromaticum*	Indonesia	M. J. Wingfield
C. atrox	CMW 19383 CBS 120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia	M. J. Wingfield
C. atrox	CMW 19385 CBS 120518	EF070415 EF070431 EF070403	*Eucalyptus grandis*	Australia	M. J. Wingfield
C. atrox	CMW 19387 CBS 120519	EF070416 EF070432 EF070404	*Eucalyptus grandis*	Australia	M. J. Wingfield
C. atrox	CMW 19389 CBS 120225	EF070417 EF070433 EF070405	*Eucalyptus grandis*	Australia	M. J. Wingfield
C. populicola	CMW 14789 CBS 119.78	EF070418 EF070434 EF070406	*Populus* sp.	Poland	J. Gremmen
C. populicola	CMW 14819 CBS 114725	EF070419 EF070435 EF070407	*Populus* sp.	USA	T. Hinds
C. caraye	CMW 14793 CBS 114716	EF070424 EF070439 EF070412	*Carya cordiformis*	USA	J. Johnson
C. caraye	CMW 14808 CBS 115168	EF070423 EF070440 EF070411	*Carya ovata*	USA	J. Johnson
C. smalleyi	CMW 14800 CBS 114724	EF070420 EF070436 EF070408	*Carya cordiformis*	USA	G. Smalley
C. variospora	CMW 20935 CBS 114715	EF070421 EF070437 EF070409	*Quercus alba*	USA	J. Johnson
C. variospora	CMW 20936 CBS 114714	EF070422 EF070438 EF070410	*Quercus robur*	USA	J. Johnson
C. virescens	CMW 11164	DQ520639 EF070441 EF070413	*Fagus americanum*	USA	D. Houston

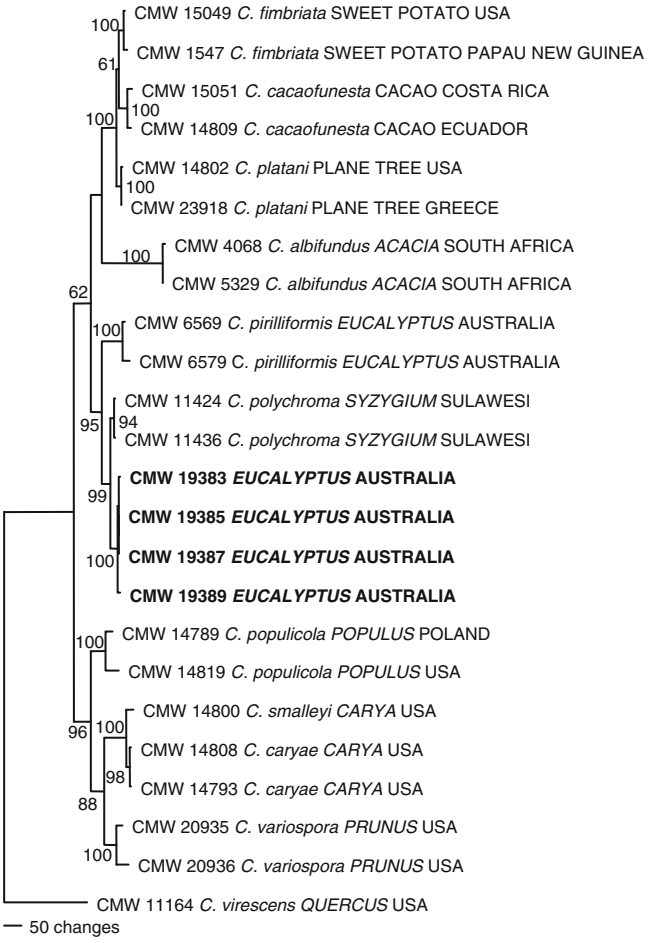

Fig. 2. Phylogenetic tree based on the combined regions of the internal transcribed spacer regions, β-tubulin and transcription elongation factor-1α for *Ceratocystis atrox* and other species in the *C. fimbriata* species complex. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated on the branches. *C. virescens* is used as the out-group taxon.

In the phylogenetic tree, *C. fimbriata sensu stricto* (*s.s.*), *C. platani* Engelbrecht and Harrington, *C. cacaofunesta* (Walter) Engelbrecht and Harrington, *C. pirilliformis*, *C. polychroma* M. van Wyk and M. J. Wingfield, *C. albifundus* M. J. Wingf., De Beer and M. J. Morris, *C. caryae* J. A. Johnson and Harrington, *C. smalleyi* J. A. Johnson and Harrington, *C. variospora* (Davids.) C. Moreau and *C. populicola* J. A. Johnson and Harrington all formed distinct clades, supported by high bootstrap values. The four isolates of the morphologically distinct *Ceratocystis* sp. from the tunnels of *P. acanthocera* on *E. grandis* in Australia formed a separate and distinct clade (Fig. 2).

Taxonomy

Based on morphological characteristics and DNA sequence comparisons for three gene regions, the *Ceratocystis* sp. considered in this study clearly represents a unique taxon. It is thus described as follows:

Ceratocystis atrox M. van Wyk and M.J. Wingf. **sp. nov. (Fig. 3)**

Anamorph: *Thielaviopsis*.

Etymology: Name refers to the dark-coloured cultures of the fungus from the Latin word *atrocis* meaning dark, fierce, fearsome.

Coloniae atro-olivaceae, hyphae laeves segmentatae, 3–4 µm latae. Bases ascomatum atrobrunneae vel nigrae, globosae, (120–)140–80(–222) µm diametro. Colla ascomatum basin *v.* atrobrunnea, (21–)26–34(–40) µm lata, apicem *v.* pallescentia, (13–)14–16(–19) µm lata, (277–)313–401(–451) µm longa. Hyphae ostiolares divergentes, hyalinae, (18–)20–26(–28) µm longae. Asci non visi. Ascosporae in massa rotundata alba vel luteo-bubalina in apicibus collorum ascomatum crescunt, lateralitre visa cucullatae vel pileatae, non septatae, hyalinae, vaginatae, 3–4 µm longae, 4–6 µm latae.

Anamorpha Thielaviopsis: conidiophorae biformes, in mycelio singuli, conidiophorum primarium hyalinum, (78–)87–151(–218) µm longum, basi 5–7(–13) µm, apice 4–8(–9) µm latum, conidiophorum secondarium hyalinum, phialido primario brevius, (39–)43–57(–66) µm longum, basi 5–7(–9) µm, apice 4–6(–7) µm latum. Evolutio conidiorum phialidica per formatione parietum annularium, conidia biformia, singula vel concatenata, primaria (9–)11–15(–17) µm longa, 3–5 µm lata, secondaria (7–)8–12(–14) µm longa, (5–)6–8(–9) µm lata. Chlamydosporae desunt.

Colonies on 2% MEA dark-olive (21″m) in colour. *Hyphae* smooth and segmented, 3–4 µm wide. *Ascomatal bases* dark brown to black, globose, (120–)140–180(–222) µm in diameter. *Ascomatal necks* dark brown at base becoming lighter towards apex, (21–)26–34(–40) µm wide at base of neck, (13–)14–16(–19) µm wide at tip of neck, (277–)313–401(–451) µm in length. *Ostiolar hyphae* divergent, hyaline, (18–)20–26(–28) µm in length. *Asci* not observed. *Ascospores* accumulate in a round, white to yellow (yellow-buff 19d) mass at the apices of the ascomatal necks, cucullate (hat-shaped) in side view, aseptate, hyaline, invested in sheath, 3–4 µm in length by 4–6 µm in width.

Thielaviopsis anamorph: conidiophores of two types occurring singly on mycelium, primary *conidiophores* hyaline, (78–)87–151(–218) µm in length, 5–7(–13) µm wide at base, 4–8(–9) µm wide at tip, secondary conidiophores hyaline, shorter than primary phialide, (39–)43–57(–66) µm in length, 5–7(–9) µm wide at base, 4–6(–7) µm wide at tip. Phialidic *conidium* development through ring wall building, *conidia* of two types formed singly or in chains, primary conidia (9–)11–15(–17) µm in length, 3–5 µm wide, secondary conidia (7–)8–12(–14) µm in length, (5–)6–8(–9) µm wide. *Chlamydospores* not present. Optimum growth range is between 20–25°C.

Specimens examined: **Australia**, Queensland, isolated from tunnels of *Phoracantha acanthocera* in *Eucalyptus grandis* trees, *M.J. Wingfield*, **holotype** Herb. *PREM 59012; culture ex-type CMW 19385 = CBS 120518*. Aug. 2005. **Australia**, Queensland, isolated from tunnels of *Phoracantha acanthocera* in *Eucalyptus grandis* trees, *M.J. Wingfield*, **paratype** *PREM 59013; culture ex-paratype CMW 19383 = CBS 120517*, Aug. 2005.

Discussion

Results of this study have led to the discovery of a new species of *Ceratocystis* in Australia. This fungus, which has been given

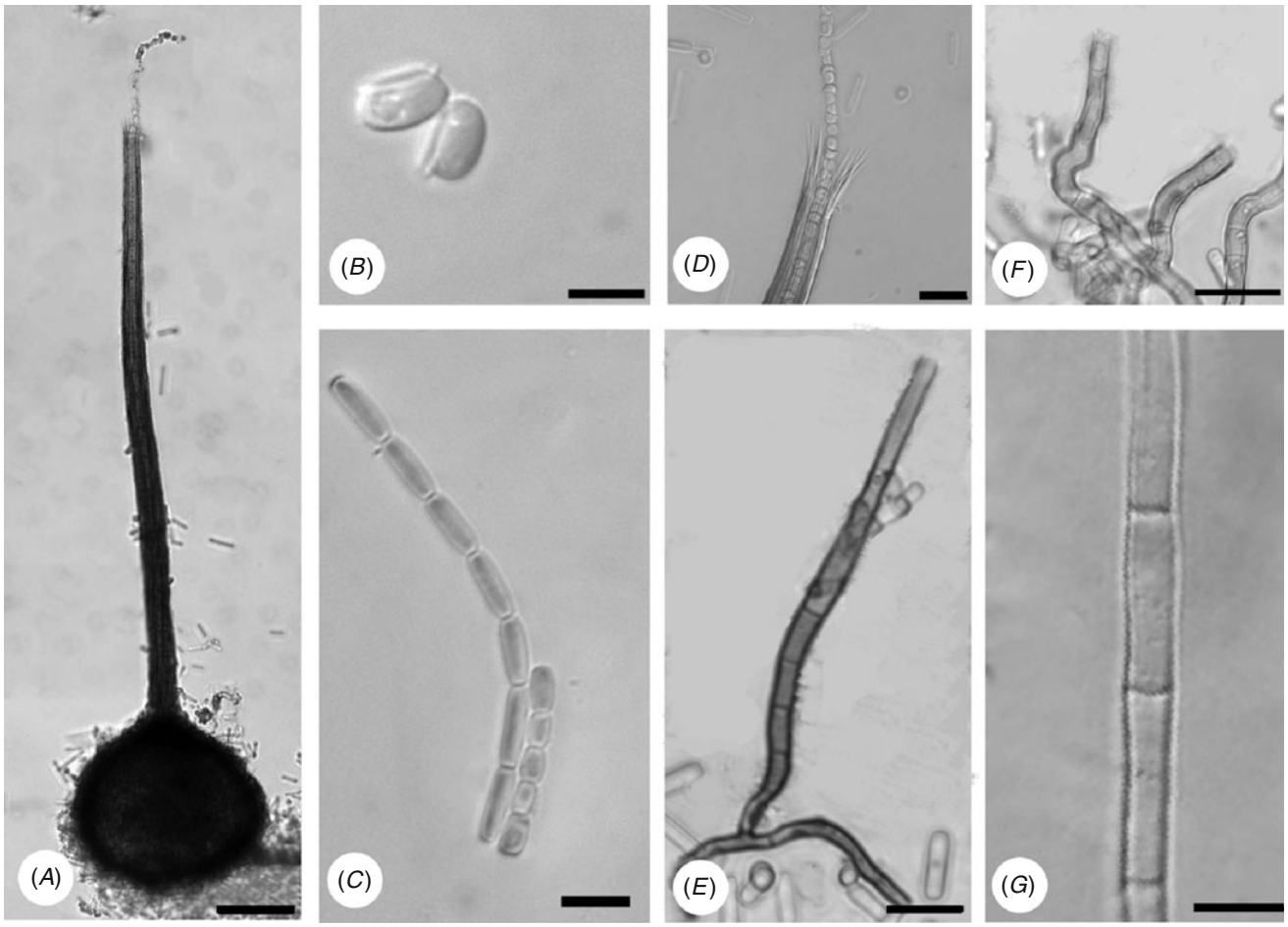

Fig. 3. Morphological characteristics of *Ceratocystis atrox*: (*A*) globose ascomata, (*B*) hat-shaped ascospores, (*C*) cylindrical (left) and barrel (right) conidia in chains, (*D*) divergent ostiolar hyphae with emerging hat-shaped ascospores, (*E*) primary phialidic conidium, (*F*) secondary conidium, (*G*) segmented hyphae. Scale bars (in μm): $A = 20$, $B = 20$, $C = 10$, $D = 5$, $E = 10$, $F = 5$, $G = 5$.

the name *C. atrox*, together with *C. pirilliformis*, *C. eucalypti*, *C. moniliformis* and *C. moniliformopsis*, is the fifth species to have been described from *Eucalyptus* spp. in the country. *C. atrox* is also the first *Ceratocystis* sp. to have been found associated with the tunnels of a wood-boring insect in Australia.

C. atrox has very obvious hat-shaped ascospores and a *Thielaviopsis* anamorph, which places it in either the *C. moniliformis s.l.* or *C. fimbriata s.l.* complex (Baker-Engelbrecht and Harrington 2005; Van Wyk *et al*. 2005). The absence of spines on the bases of the perithecia and the absence of a disk-like shape at the base of the perithecial necks makes it typical of species residing in the latter group. *C. atrox* can easily be distinguished from all other species in the *C. fimbriata* species complex based on various morphological characteristics. One of the most obvious of these is its very dark green colour in culture. It is also unique among species of *Ceratocystis* in the *C. fimbriata s.l.* species complex based on its segmented hyphae, short ascomatal necks and the fact that no chlamydospores are found in this species.

Based on cultural characteristics, *C. atrox* differs from all other species of the genus found in Australia. *C. moniliformis* has white to grey coloured cultures, *C. moniliformopsis* cultures are cream to brown coloured (Van Wyk *et al*. 2004*a*) and both species produce a very fruity aroma. *C. pirilliformis* has an olivacious green colour (Barnes *et al*. 2003*a*) and bears some similarity to *C. atrox*, although the cultures of the latter species are much darker. *C. eucalypti* has a dark greenish grey to black colour (Kile *et al*. 1996). When *C. atrox* is compared with the other *Ceratocystis* spp. residing in the same phylogenetic clade, it is also clearly distinct. In this regard, *C. fimbriata s.s.*, *C. polychroma*, *C. cacaofunesta* and *C. platani* all have relatively dark-coloured cultures but *C. atrox* is a considerable darker green colour than any of them.

All five of these species of *Ceratocystis* occurring on *Eucalyptus* in Australia share common morphological characteristics. These include the formation of hat-shaped ascospores and divergent ostiolar hyphae. They can, however, be distinguished based on the shapes of the ascomatal bases. The ascomatal bases of *C. pirilliformis* are pear-shaped (Barnes *et al*. 2003*a*) compared with the globose bases of the remaining four species. *C. moniliformis* and *C. moniliformopsis* both have conical spines on their bases. *C. atrox* has segmented hyphae that are not seen in any of the other four species as they all have non-segmented hyphae.

DNA sequence comparisons for three gene regions have shown that *C. atrox* resides in the *C. fimbriata* species complex, yet in a discrete clade separate from all other taxa in this group. Comparisons with *C. fimbriata s.s.*, *C. cacaofunesta*, *C. platani*, *C. pirilliformis*, *C. polychroma*, *C. caryae*, *C. populicola*, *C. smalleyii*, *C. variospora* and *C. albifundus* and using *C. virescens* as the monophyletic sister out-group indicate that the closest relative of *C. atrox* is *C. polychroma*. This is a species thought to be native to Sulawesi (Indonesia), which is associated with a severe die-back disease on clove trees (*Syzygium aromaticum*) (Van Wyk *et al*. 2004*b*). It is interesting that *C. polychroma* and *C. atrox* share similar ecological habitats associated with cerambycid beetles.

Nothing is known regarding the ecology of the association between *C. atrox* and *P. acanthocera*. The fungus was consistently found in tunnels of the insect on all trees examined and this implies a close relationship between the two organisms. The very clear discolouration of the wood associated with the tunnels and the fungus growing in them, implies that the fungus is able to penetrate the wood deeply and that it is more than a surface inhabitant in the tunnels. It is possible that it plays a role in excluding other fungi such as moulds from this niche, as has been suggested for the ophiostomatoid fungi occurring in the infructesences of *Protea* spp. in South Africa (Marais *et al*. 1998). *C. atrox* may also be a mild pathogen, contributing to the development of its insect associate, but it is unlikely to be highly pathogenic, as trees infested with *P. acanthocera* were never found to be dying.

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