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Ceratocystis pirilliformis, a new species from *Eucalyptus nitens* in Australia

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Abstract: Several species of *Ceratocystis* have been recorded on *Eucalyptus*. These include *C. fimbriata*, *C. eucalypti*, *C. moniliformis* and *C. moniliformopsis*. Of these, only *C. fimbriata* is known as a pathogen; it recently has been found causing serious wilt diseases in Uganda, Congo and Brazil. This study was undertaken to collect *Ceratocystis* species, including *C. eucalypti*, from artificially induced wounds on *Eucalyptus nitens* near Canberra in southeastern Australia. Trees were wounded in October 2000, and wounds were examined approximately one month later. Ascomata characteristic of a *Ceratocystis* species were found covering the wounds, and this fungus also was isolated from the wood using carrot baiting. This species of *Ceratocystis* has hat-shaped ascospores similar to those of *C. fimbriata*, but it differs from *C. fimbriata* and all other species of *Ceratocystis* in that it possesses ascomata with a pyriform base. Comparison of DNA sequences from the ITS and 5.8S rRNA operon confirmed that the fungus from *E. nitens* in Australia is unique, and we describe it here as a new species, *C. pirilliformis*.

Key words: Ascomycetes, *Ceratocystis fimbriata*, ITS rRNA, phylogeny, systematics

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INTRODUCTION

Species of *Ceratocystis* are well known for their association with insects and, in general, are vectored nonspecifically by flies (Diptera) and beetles (Coleoptera: Nitidulidae) (Crone and Bachelder 1961, Moller and DeVay 1968b, Hinds 1972, Juzwik and French 1983). These fungi require the host plant to be wounded to cause infections (Kile 1993) because fresh wounds attract sap-feeding insects that carry the fungi to these substrates. Sticky droplets of ascospores, produced at the apices of ostioles, adhere to the bodies of the insects. The insects then frequent wound sites on other plants and disperse ascospores from diseased to healthy hosts (Moller and DeVay 1968b, Upadhyay 1981). This insect-fungus relationship is facilitated by the fact that most species of *Ceratocystis* produce aromatics attractive to insects (Kile 1993).

Kile et al (1996) conducted a wounding study on *Eucalyptus* in Australia, and this resulted in the discovery of a new *Ceratocystis* species, *C. eucalypti* Z. Q. Yuan & Kile. This species is native to Australia, where it is found on *E. sieberi* L. Johnson and *E. globoidea* Blakely in Victoria and on *E. regnans* F. Muell. in Tasmania. *Ceratocystis eucalypti* appears to be a nonpathogenic colonist of fresh wounds on *Eucalyptus* (Kile et al 1996). The only other member of the genus known to infect living *Eucalyptus* is *C. fimbriata*, which has been reported to cause rapid wilting of trees in the Democratic Republic of Congo, Brazil (Roux et al 2000) and Uganda (Roux et al 2001). *Ceratocystis fimbriata* has been reported from Australia, although there are only two records of this fungus on rotting *Syngonium* in New South Wales, Queensland and Victoria (Walker et al 1988, Vogelzang and Scott 1991).

In an attempt to re-isolate *C. eucalypti* and other *Ceratocystis* species on *Eucalyptus* in Australia, an artificial wounding trial was conducted on planted *E. nitens* near Canberra and *E. globulus* near Cann River. *Ceratocystis eucalypti* was not isolated, but a morphologically distinctive species of *Ceratocystis* was abundant on the wounds. The aim of this investigation was to characterise this new species and identify its closest relatives, based on a comparison of rRNA sequence data.

MATERIALS AND METHODS

Fungal isolates.—Artificial wounds were made approximately 1.2 m above the ground on stems of 6-year-old *E. nitens* near Canberra, Australian Capital Territory (ACT), and on 8-year-old *E. globulus* near Cann River, Victoria, in southeastern Australia. A brick-cutting chisel was used to remove blocks of bark approximately 10 cm square from the stems and expose the cambium. Thereafter, a second wound approximately 2 cm deep was made at the center of the exposed cambium to expose the xylem. The wounds were made in early October 2000 on 10 trees at each site. Wounds were examined in early November 2000, and slices of wood from all trees were removed from the wound surfaces for laboratory examination.

Chips of wood, approximately 4 cm square and 5 mm thick, were incubated at 25 C in 9 cm diam Petri dishes containing moistened filter paper to induce the production of fungal structures. Duplicate isolations were made by wrapping pieces of wood tightly between two slices of surface-disinfected carrot (~5 mm thick) (Moller and DeVay 1968a). Carrot baits were incubated at 25 C for 10 d. Ascomata characteristic of *Ceratocystis* were found covering the wood chips and the carrot baits collected from five trees at the Canberra site. This fungus was not found on samples from Cann River. Cultures were obtained by transferring masses of ascospores from the apices of ascomatal necks onto 2% malt-extract agar (MEA, Biolab) and incubating them at 25 C. All cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Dried and living cultures of the holotype and paratypes are deposited in the Plant Pathology Herbarium of NSW Agriculture (DAR), Orange Agricultural Institute, Orange, New South Wales, Australia, and dried cultures are deposited with the National Collection of Fungi, Agricultural Research Council, Pretoria (PREM), South Africa.

Morphology.—The growth rates of three isolates (CMW6579, CMW6670 and CMW7569) were determined on 2% MEA. Isolates were cultured 2 wk at 25 C before growth-rate studies. Mycelial plugs were removed from the edges of actively growing cultures with a 4 mm cork borer, and a single plug was placed at the center of 60 mm Petri dishes containing 2% MEA. Four plates for each isolate were incubated at 15, 20, 25 and 30 C, respectively. Colony diameter was assessed after 12 d of incubation by making two measurements at right angles to each other for each culture. This resulted in eight measurements for each isolate at each temperature. Averages were computed for all growth measurements.

Morphological characteristics were described from cultures grown on 2% MEA. Fungal structures were mounted in lactophenol containing cotton blue. Fifty measurements for each taxonomically relevant structure were made, and corresponding ranges, averages and standard deviations calculated. Color descriptions were determined using the color charts of Rayner (1970).

DNA extraction.—Single drops containing ascospores from ascomata were transferred from sporulating cultures to 50 mL, 2% malt extract broth using sterile toothpicks. Cultures were incubated at 25 C for 2 wk to obtain thick mycelial mats. These were freeze-dried, crushed in liquid nitrogen and the DNA extracted according to the method described by Barnes et al (2001).

PCR amplification.—The two internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S gene of the ribosomal RNA operon were amplified using primers ITS1 and ITS4 (White et al 1990). Polymerase chain reaction (PCR) mixtures consisted of 200 nM of each primer, 200 μM of each dNTP, Expand High Fidelity PCR System enzyme mix (1.75 U) (Roche Molecular Biochemicals), 1× Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme) and 2–10 ng DNA. Reaction volumes were adjusted to 50 μL with Sabax water. The PCR program was set at 96 C for 2 min, followed by 10 cycles at 94 C for 20 s, 55 C for 48 s and 72 C for 45 s. A further 25 cycles were included with the annealing time altered to 40 s and a 5 s extension after each cycle. A final step of 10 min at 72 C completed the program. PCR amplicons were purified using the Magic PCR Preps, Purification System (Promega).

Sequencing.—PCR amplicons were sequenced with the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and the primers ITS1 and ITS4 were used. Sequence reactions were run on an ABI PRISM™ 377 Autosequencer (Applied Biosystems) and sequence electropherograms were analyzed with Sequence Navigator version 1.0.1 (Applied BioSystems).

The sequences obtained for the *Ceratocystis* species from *E. nitens* were compared to ITS sequences of *Ceratocystis* species obtained from GenBank (TABLE I). Sequences were aligned manually and analyzed using PAUP version 4.0* (Phylogenetic Analysis Using Parsimony * and other methods) (Swofford 1998). Gaps were treated as ''newstate'', and trees were obtained via stepwise addition of 1000 replicates. The Mulpar option was in effect. The heuristic search, based on parsimony with tree bisection reconnection, was used to obtain the phylogram. Confidence intervals using 1000 bootstrap replicates were calculated. Outgroup *C. moniliformis* was treated as a paraphyletic sister group with respect to the ingroup. All sequences derived in this study have been deposited in GenBank (TABLE I) and the sequence alignments in TreeBASE (5869).

RESULTS

DNA sequence comparisons.—Sequences of the ITS and 5.8S region of the ribosomal rRNA operon of three isolates of the *Ceratocystis* sp. from *E. nitens* were 647 bp in length. The aligned dataset included 573 characters, of which 82 ambiguously aligned characters were excluded in the analyses. Of the remaining 491 characters, 252 were constant and 190 parsimony informative. A single, most-parsimonious tree (FIG. 1) was produced with a tree length of 471.

The species of *Ceratocystis* included in the analyses were divided into two distinct clades. The *Ceratocystis*

TABLE I. Species of *Ceratocystis* used in ITS sequence comparisons

Taxon	Isolate no.[A]	Origin	Host	Genbank no.
C. albofundus Wingfield et al.	CMW2148	South Africa	*Acacia mearnsii*	AF264910
C. albofundus Wingfield et al.	CMW2475	South Africa	*Acacia mearnsii*	AF043605
C. coerulescens (Munch) Bakshi	CBS140.37	Germany	*Picea abies*	U75615
C. douglasii (Davidson) Wingfield & Harrington	CBS142.53	U.S.A.	*Pseudotsuga menziesii*	U75626
C. eucalypti Yuan & Kile	CMW3254	Australia	*Eucalyptus sieberi*	U75627
C. fimbriata Ellis & Halsted	CMW1547	Papua New Guinea	*Ipomoea batatas*	AF264904
C. fimbriata Ellis & Halsted	CMW2219	France	*Platanus* sp.	AF395679
C. fimbriata Ellis & Halsted	CMW2901	Canada	*Populus tremuloides*	AF395696
C. fimbriata Ellis & Halsted	CMW2911	U.S.A.	*Prunus* sp.	AF395693
C. fimbriata Ellis & Halsted	CMW7383	Uruguay	*Eucalyptus grandis*	AF453438
C. laricicola Redfern & Minter	CMW1016	Scotland	*Larix decidua*	U75622
C. moniliformis (Hedgecock) Moreau	CMW3782	South Africa	*Erythrina* sp.	AF043597
C. paradoxa (Dade) Moreau	CMW1546	New Zealand	*Musa* sp. × *paradisiaca*	U75630
C. pirilliformis Barnes & Wingfield	CMW6569[B]	Australia	*Eucalyptus nitens*	AF427104
C. pirilliformis Barnes & Wingfield	CMW6574[B]	Australia	*Eucalyptus nitens*	AF427106
C. pirilliformis Barnes & Wingfield	CMW6579[B]	Australia	*Eucalyptus nitens*	AF427105
C. resinifera Harrington & Wingfield	CMW3263	Norway	*Picea abies*	U75618
C. virescens (Davidson) Moreau	CMW0460	U.S.A.	*Quercus* sp.	AF043603

[A] CBS = Centraabureau voor Schimmelcultures.
[B] Isolates sequenced in this study.

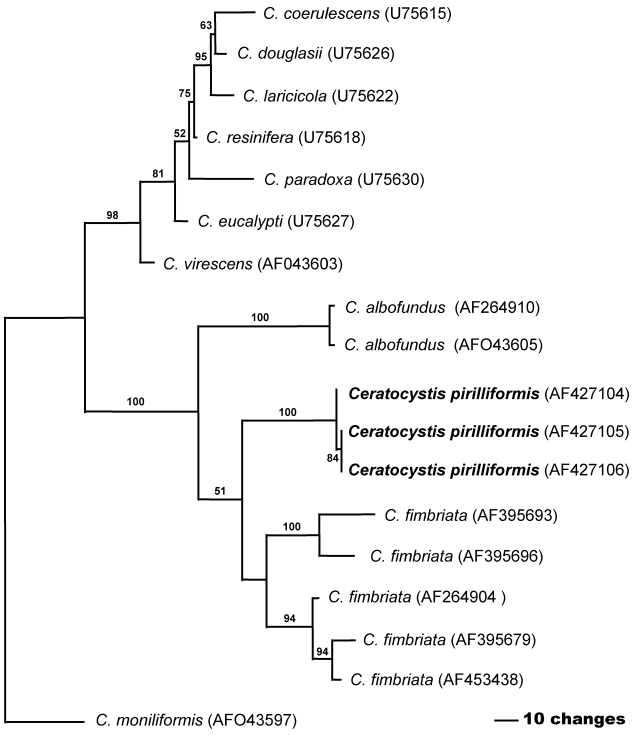

FIG. 1. Phylogram inferred from analysis of ITS 1, 5.8s and ITS 2 rDNA sequences (L = 471, CI = 0.745, RI = 0.848, g1 = −0.566). *Ceratocystis moniliformis* was used as outgroup. Bootstrap confidence values are indicated above the branches.

isolates from *E. nitens* in Australia formed a single, well-supported subclade (bootstrap support 100%) that was sister to *C. fimbriata* and *C. albofundus*. Based on sequence data, this species clearly represents a unique taxon.

TAXONOMY

Ceratocystis pirilliformis I. Barnes & M. J. Wingfield sp. nov. FIGS. 2 and 3

Coloniae tarde crescentiae, usque ad 22 mm diametro post 12 dies; effusae, mycelio aerio laete olivaceo-griseo, mycelio immerso griseo-olivaceo, pagina reversa coloniae griseo-olivacea. Ascomata nigra, venter pyriformis, basin versus globosa, 115.2–186.8(–205.5) μm diametro, parte superiori late papillata, 35.8–76.5(–86.8) × (23.9–42.4(–75.2) μm. Colla ascomatarum erecta vel curvata, basin versus nigra, apicem versus subhylainescens, parietibus laevis vel crenulatis, basin versus 18.8–32.7(–39.8) μm lata, apicem versus 11.9–21.0(–25.3) μm lata; 372.4–683.0(–777.5) μm longa, hyphis ostiolaribus inclusis. Ascosporae in guttulis in collis ascomatarum crescentes, unicellulares, hyalinae, e supra visae ellipticae, 4.3–5.7(–6.4) × 3.2–4.4(4.8) μm, vagina hyalina aspectu laterali petasiformi circumcincta, 2.9–5.2(–6.5) × 2.0–3.8(–5.4) μm, margine 4.7–6.4(–7.5) μm longa, diffluentes. Conidiophorae chalariformes, erectae, ramosae vel non ramosae, hyalinae, multiseptatae, laeves, 62–147(–216) μm longae, cellula terminali conidiogena inclusa. Cellulae conidiogenae tubulares, determinatae, cylindricae vel lageniformes, 31.9–63.6(–72.4) μm longae. Conidia enteroblastica phialidosa, formis duabus: i) doliiformia, basi

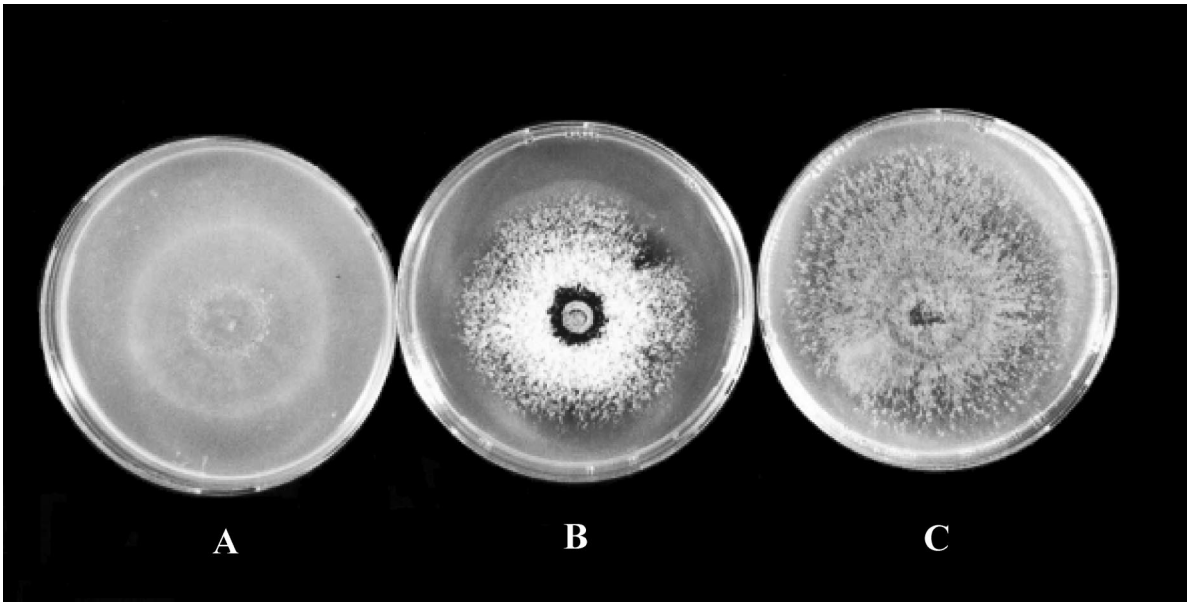

FIG. 2. Cultural characteristics of *Ceratocystis* species most closely related to *C. pirilliformis*. A) *C. albofundus* from *Acacia mearnsii* (CMW2475) in South Africa produces cultures that are cream-colored (21′′f). B) *C. pirilliformis* (CMW6579) has light gray aerial mycelium (21′′′′′d) with greenish to brownish submerged mycelium (21′′′′b). C) *C. fimbriata* from *Eucalyptus grandis* in Uruguay (CMW7383) produces cultures that are brownish (21′′K). All cultures were grown on 2% MEA at 25 C for ca 3 wk.

truncata, parietibus laevis, non septata, mononemata, hyalina, 4.1–5.6(–6.4) × 3.2–4.3(–4.8) μm, solitaria vel in catenis portata, vel ii) cylindrica, apicibus rotundatis, laevia, non septata, hyalina, 11.6–25.2(–33.0) × 2.4–4.0(–4.7) μm, in catenis portata.

Colonies slow growing with optimal growth at 25 C on 2% MEA, reaching 22 mm diam in 12 d. No growth below 15 C or above 30 C. Colonies effuse, aerial mycelium ''pale olivaceous grey'' (21′′′′′d), submerged mycelium ''grey olivaceous'' (21′′′′b). Reverse side of colony ''grey olivaceous'' (21′′′′b). Submerged mycelium darkening as the ascomata develop forming fine, radiating fibrils. *Ascomata* developing within 8 d and mature within 12 d, superficial or partly embedded in agar, black (7′′′′′k). Venter pyriform with basal part globose 115.2–186.8(–205.5) μm diam and upper part broadly papillate, 35.8–76.5(–86.8) × 23.9–42.4(–75.2) μm. *Ascomatal necks* erect, occasionally curved, black at bases becoming subhyaline toward the apex, smooth to crenulate, tapering slightly from 18.8–32.7(–39.8) μm wide at base to 11.9–21.0(–25.3) μm wide at apex, 372.4–683.0(–777.5) μm long including ostiolar hyphae. *Ostiolar hyphae* extending from the outer layer of the neck cells, hyaline, straight or flexuous, subulate, mostly convergent, non-septate. *Asci* evanescent, not seen. *Ascospores* accumulating in droplets at tips of ascomatal necks, single-celled, hyaline, elliptical in top view, 4.3–5.7(–6.4) × 3.2–4.4(–4.8) μm, surrounded by a hyaline sheath; sheath appearing hat-shaped in side view, 2.9–5.2(–6.5) × 2.0–3.8(–5.4) μm, dissolving at maturity. *Conidiophores,* thielaviopsis-like, erect, branched or unbranched, hyaline, multiseptate, smooth walled, 62–147(–216) μm long including the integrated, terminal conidiogenous cell. *Conidiogenous* cells tubular, determinate, cylindrical to lageniform, 31.9–63.6(–77.4) μm long. *Conidia* of two types emerging from different conidiogenous cells: i) barrel-shaped, doliiform with truncate bases, smooth-walled, non-septate, hyaline, 4.1–5.6(–6.4) × 3.2–4.3(–4.8) μm, borne in chains, ii) cylindrical with apices rounded, smooth, non-septate, hyaline, 11.6–25.2(–33.0) × 2.4–4(–4.7) μm, borne in chains. *Chlamydospores* oval, thick walled, smooth, isabelline (17′′i) to olivaceous (21′′m), 7.8–12(–13.0) × 5.4–8.3(–9.5) μm, embedded in agar, formed singly or in short chains.

HOLOTYPE: AUSTRALIA. ACT: Uriarra, near Canberra, isolated from wounds on *Eucalyptus nitens*, Nov 2000, *M. J. Wingfield* [PREM57323. ISOTYPE: DAR 75996 (culture CMW6579)].

PARATYPES: AUSTRALIA. ACT: Uriarra, near Canberra, isolated from wounds on *Eucalyptus nitens*, Nov 2000, *M. J. Wingfield* [PREM57322, DAR 75993 (culture CMW6569)]; same collecting data [PREM57320, DAR 75994 (culture CMW6574)]; same collecting data [PREM57325, DAR75997 (culture CMW6583)]; same collecting data [PREM57321 (culture CMW6566)];

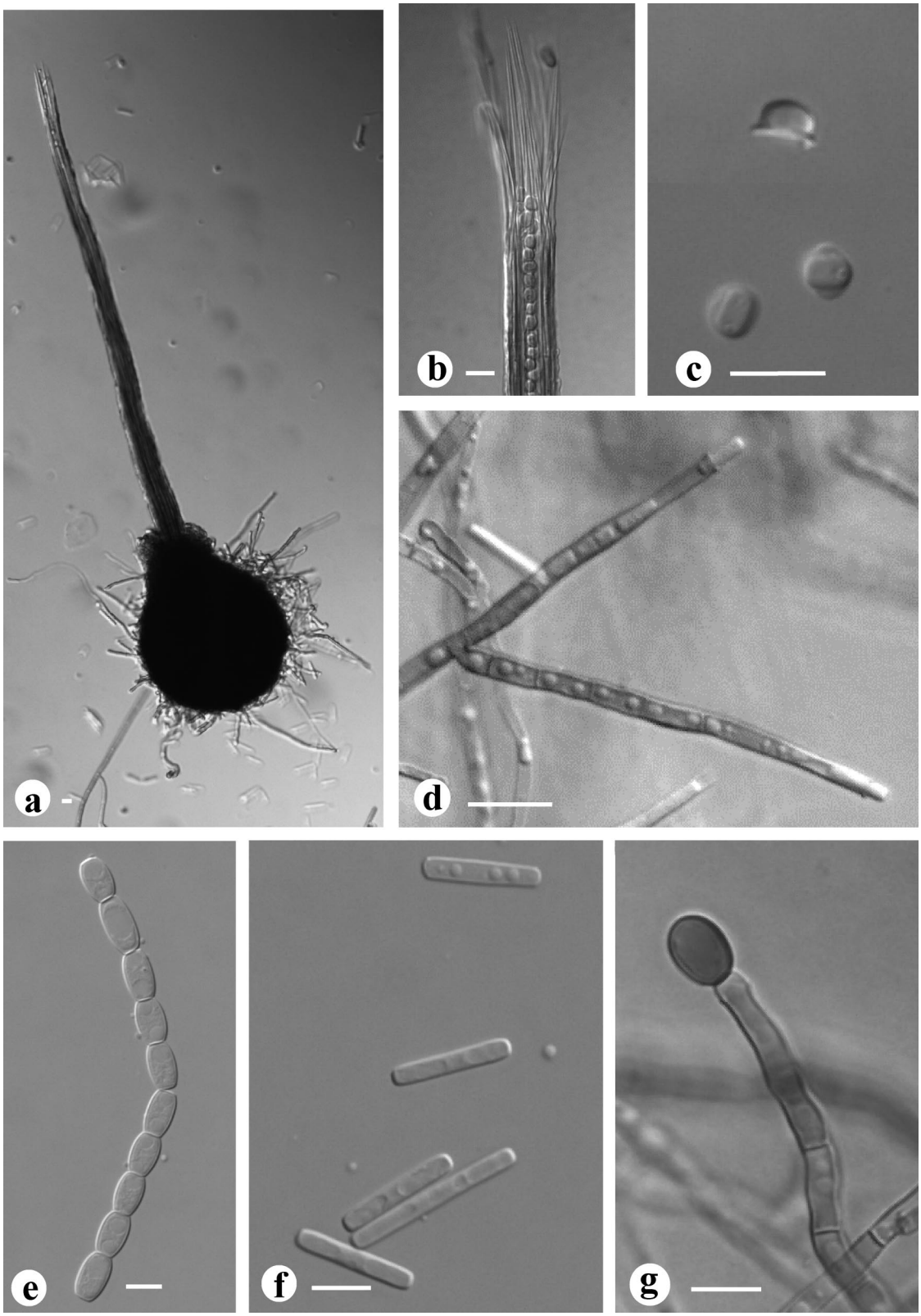

FIG. 3. *Ceratocystis pirilliformis* (PREM 57323). a. Ascomata with a pyriform venter. b. Ostiolar hyphae with ascospores emerging through the opening of the neck. c. Hat-shaped ascospores in side and top view. d. Conidiophores with cylindrical conidia being released from the conidiogenous cells. e. Barrel-shaped conidia. f. Cylindrical conidia. g. Chlamydospore. All scale bars = 10 μm.

same collecting data [PREM57324, DAR 75995 (culture CMW6577)].

Etymology. pirilliformis, Latin = shaped like a little pear; referring to the characteristic pear-shaped venter of the ascomata in this species.

DISCUSSION

Ceratocystis pirilliformis can be distinguished easily from all other species of *Ceratocystis*. Although, morphologically, it most resembles *C. fimbriata* and *C. albofundus*, it is distinctive in possessing ascomata with pyriform bases. Comparisons of ITS rRNA sequences support the treatment of *C. pirilliformis* as a new species. It is one of the five species of *Ceratocystis* known to occur on *Eucalyptus* and only the third isolated from living *Eucalyptus* tissue.

There is considerable overlap in the morphological characteristics of *Ceratocystis* species. The sizes of ascomata, ascospores and conidia of *C. pirilliformis*, for example, fall within the ranges observed for *C. fimbriata* and *C. albofundus* (Wingfield et al 1996). However, notable differences separate these three species. *Ceratocystis albofundus* has light-colored ascomatal bases, divergent ostiolar hyphae and lacks chlamydospores (Wingfield et al 1996). *Ceratocystis pirilliformis* and *C. fimbriata* are almost indistinguishable from each other. Both have dark ascomatal bases, convergent ostiolar hyphae and chlamydospores. The only obvious difference between these two species is the shape of the bases of their ascomata. *Ceratocystis fimbriata* has globose ascomatal bases, whereas the bases of *C. pirilliformis* are distinctly pyriform.

Respective culture morphologies of *C. fimbriata*, *C. albofundus*, and *C. pirilliformis* differ. Colonies of *C. albofundus* are pale, almost creamy, while *C. fimbriata* varies from greenish to brown. *Ceratocystis pirilliformis* forms colonies with a grayish aerial mycelium and a submerged mycelium that is green, sometimes turning brown in older cultures.

Ceratocystis pirilliformis and *C. eucalypti* both are found on *Eucalyptus* in southeastern Australia. *Ceratocystis eucalypti* probably is endemic to this area (Kile et al 1996), and we suspect that the same is true of *C. pirilliformis*. These species, however, are distinct morphologically. The most obvious difference is the shape of the ascospores; *C. eucalypti* has elongated, sheathed ascospores, whereas *C. pirilliformis* has hat-shaped, sheathed ascospores. Only five other species of *Ceratocystis* have hat-shaped ascospores, i.e., *C. moniliformis* (Hedgec.) C. Moreau, *C. moniliformopsis* Z.Q. Yuan & C. Mohammed, *C. acericola* Griffin, *C. fimbriata* and *C. albofundus*. *Ceratocystis moniliformis* and *C. moniliformopsis*, however, differ from other species with hat-shaped ascospores in that they have short conical spines on the ascomatal bases (Upadhyay 1981, Yuan and Mohammed 2002).

Phylogenetic comparisons, based on sequences of the ITS regions, strongly support the separation of species of *Ceratocystis* based on observed morphological differences. Species within *Ceratocystis* can be divided into two major groups. One of these includes *C. coerulescens* (Witthuhn et al 1999) and other species that do not have hat-shaped ascospores. *Ceratocystis eucalypti* is most closely allied to species in the *C. coerulescens* group, a relationship that confirms that ascospore shape is a reasonably strong indicator of phylogenetic relationships within *Ceratocystis*. The second major group in *Ceratocystis* is typified by *C. fimbriata*. It is not surprising, therefore, that *C. pirilliformis*, with a morphology similar to *C. fimbriata*, resides in a strongly resolved clade within this subgroup.

Eucalyptus nitens, also known as shining gum, occurs naturally in Australia, where it mainly is restricted to the southeast (Poynton 1979). Large artificial plantations have been established in Tasmania and, to a lesser extent, in New South Wales (Beadle 1999). The tree also is planted widely in South Africa for pulp production, and it increasingly is being used as a hybrid partner with other species to increase cold tolerance. Although we know little concerning the pathogenicity of *C. pirilliformis*, it certainly is able to cause wood discoloration. Given the economic importance of *E. nitens* and the fact that many species of *Ceratocystis* are pathogenic, it will be important to test this characteristic of *C. pirilliformis*.

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