Ceratocystis species, including two new species associated with nitidulid beetles on eucalypt in Australia

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Abstract The genus *Ceratocystis* includes important fungal pathogens of trees, including *Eucalyptus* spp. Ironically, very little is known regarding the diversity or biology of *Ceratocystis* species on *Eucalyptus* species in Australia, where most of these trees are native. The aim of this study was to survey for *Ceratocystis* spp., and their possible insect associates on eucalypts in Australia and thus, to establish a foundation of knowledge regarding these fungi on the continent. Collections were made in three states of Australia from wounds on trees, as well as from nitidulid beetles associated with these wounds. *Ceratocystis* spp. were identified based on morphology and multigene sequence comparisons. Of the 54 isolates obtained, two previously unknown species of *Ceratocystis* were found and these are described here as *C. corymbiicola* sp. nov. and *C. tyalla* sp. nov. Furthermore, the distribution of *C. pirilliformis* is expanded to include *Eucalyptus* spp. in Tasmania.

Keywords *Brachypeplus*, *Carpophilus*, *Corymbia*, *Eucalyptus*, fungal diversity, plantation forestry.

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Introduction

The genus *Ceratocystis* includes important insect-associated pathogens of agricultural and forestry crops, worldwide (Kile 1993; Roux and Wingfield 2009). Disease symptoms associated with infection by these fungi include stem cankers, root and fruit rot, wood stain and vascular wilt. The type species of the genus, *C. fimbriata* Ellis & Halsted, is best known as the causal agent of sweet potato black rot disease (Halsted 1890; Halsted and Fairchild 1891). However, in the past two decades *Ceratocystis* species have emerged as important threats to plantation forestry trees, causing diseases of *Eucalyptus* species, *Acacia mearnsii* de Wild and *A. mangium* Wild trees in non-native plantation situations (Roux & Wingfield 2009; Tarigan et al. 2010). Important examples include *C. fimbriata sensu lato* (*s.l.*) reported to cause wilt and death of *Eucalyptus* spp. in the Republic of Congo, Uganda, Uruguay and Brazil (Barnes et al. 2003a; Roux et al. 1999; 2000; 2001), and *C. albifundus* De Beer, Wingfield & Morris that causes a serious wilt disease of plantation-grown *A. mearnsii* trees in South Africa

(Morris et al. 1993; Wingfield et al. 1996; Roux and Wingfield 2009) and *C. acaciivora* Tarigan & M. van Wyk the cause of wilt and death of *A. mangium* in Indonesia (Tarigan et al. 2010).

The eucalypts include more than 700 tree species distributed in the genera, *Eucalyptus* L'Her. *Corymbia* K.D. Hill & L.A.S. Johnson and *Angophora* Cav. (Hill and Johnson 1995). Many of these trees are grown as non-natives in commercial plantations and woodlots, covering more than 20 million hectares in more than 60 countries (Iglesias-Trabado and Wilstermann 2008). In Australia, numerous indigenous fungi are found on these trees and none have been reported to cause major disease in native forest situations (Keane et al. 2000; Park et al. 2000; Carnegie et al. 2007). In contrast, where eucalypts are planted as non-natives in plantations, there have been multiple reports of diseases of these trees (Keane et al. 2000; Wingfield 2003; Wingfield et al. 2008). These include disease caused by, or associated with *Ceratocystis* species (Roux and Wingfield 2009).

A number of *Ceratocystis* spp. have been reported from Australia infecting wounds on *Eucalyptus* trees. These include *C. eucalypti* Z.Q. Yuan & Kile that causes vascular stain on *E. regnans* F. Muell., *E. sieberi* L.A.S. Johnson and *E. globoidea* Blakely (Kile et al. 1996), *C. pirilliformis* I. Barnes & M.J. Wingf. causing sap-stain on *Eucalyptus* species (Barnes et al. 2003b; Kamgan Nkuekam et al. 2009), *C. moniliformopsis* Yuan & Mohammed infecting *E. obliqua* L'Her (Yuan and Mohammed 2002), *C. moniliformis* (Hedgcock) Moreau from *E. grandis* (Hill) Maiden (Fouche et al. 2007) and *C. atrox* M. Van Wyk & M.J. Wingfield found in the galleries of *Phoracantha acanthocera* Macleay (Coleoptera: Cerambycidae) infesting *E. grandis* (Van Wyk et al. 2007). These reports have all been from very limited studies. Based on these and the recent descriptions of numerous previously undescribed species of *Ceratocystis* from *Eucalyptus* elsewhere in the world, it is likely that many more species await discovery on eucalypts in Australia. A recent study on *C. pirilliformis*, using polymorphic simple sequence repeat (SSR) markers, for example, suggests that this fungus was introduced to South Africa (Kamgan Nkuekam et al. 2009) and it might be native to Australia (Barnes et al. 2003b; Kamgan Nkuekam et al. 2009) where it was first discovered.

Ceratocystis species commonly have associations with insects, particularly bark beetles (Furniss et al. 1990; Harrington and Wingfield 1998; Redfern et al. 1987; Wingfield et al. 1997) and nitidulid beetles (Cease and Juzwik 2001; Moller and Devay 1968). These associations have been broadly classified into "specific" associations, such as that of the conifer pathogens, *C. polonica* (Siemaszko) C. Moreau, *C. laricicola* Redfern & Minter and *C. rufipenni* Wingfield, Harrington & Solheim that are consistently vectored by *Ips typographus* L., *Ips cembrae* Heer and *Dendroctonus rufipenni* Kirby, respectively (Furniss et al. 1990; Harrington and Wingfield 1998; Redfern et al. 1987; Wingfield et al. 1997). Alternatively, the association of *Ceratocystis* species with nitidulid insects and drosophilid flies are termed "non-specific" or loose, such as in the case of *C. fagacearum* (Bretz) Hunt and *C. fimbriata s.l.* (Cease and Juzwik 2001; Moller and Devay 1968). There have been no studies regarding insect associates of *Ceratocystis* species in Australia although it is likely that most of these fungi on eucalypts are vectored by nitidulids and flies.

This study aimed to increase the base of knowledge pertaining to *Ceratocystis* species on native eucalypts in Australia. The intention is to provide an improved baseline for quarantine procedures and to predict threats of *Ceratocystis* disease outbreaks, globally. Wounds on eucalypt trees in the eastern part of Australia were thus investigated for the presence of

these fungi and their nitidulid insect associates. Both morphological characteristics and multigene DNA sequence data were used to identify all fungi collected and their potential pathogenicity to eucalypt trees was investigated in greenhouse inoculation studies.

Materials and Methods

Source of samples and isolations

Collections of *Ceratocystis* species from *Eucalyptus* species and *Corymbia* species in Australia were made over a six-week period between March and April 2008. Because *Ceratocystis* species require wounds for infection, and previous studies have shown that stem wounds are commonly infected with these fungi (Kile et al. 1996; Barnes et al. 2003b; Roux et al. 2004; Kamgan Nkuekam et al. 2009). Surveys were focused on wounds resulting from harvesting as well as stem boring insects. Plantations and forests in Tasmania, New South Wales and the southern part of Queensland were visited. In Tasmania, plantations and forests near Burnie, Tarraleah and Geevestown were surveyed. In New South Wales (NSW) commercial plantations and native eucalypt forests between Sydney in the south and up to the border with Queensland were included. Some key areas sampled in NSW included Pine Creek State Forest, Wattagan State Forest, Wedding Bells State Forest, Crab-tree plantation, Ingalba State Forest and other localities around these main areas. In the state of Queensland, samples were collected from *Eucalyptus* trees growing around Brisbane.

Samples were collected from stumps of felled trees in all areas surveyed in Tasmania. In NSW wounds included stumps of felled trees, galleries of cossid moths (Lepidoptera: Cossidae) and cerambycid beetles (Coleoptera: Cerambycidae), as well as excavations made on the trees by parrots scavenging for larvae of insects infesting the trees. In Queensland, samples were collected from artificially induced wounds and from wounds made by birds feeding on wood-boring larvae. Pieces of bark or wood were collected from wounds and stored in separate brown paper bags for each tree, after they had been examined with a 20X magnification lens for the presence of *Ceratocystis* fruiting structures. All the samples were placed in separate brown paper bags for each tree or stump and transported to the laboratory in plastic bags that also served to conserve moisture and to induce sporulation. Samples from individual trees were maintained separately in brown bags and samples from each locality were grouped separately into plastic bags.

To obtain information on some of the possible insect associates of *Ceratocystis* species in Australia, Nitidulidae were collected from wounds on eucalypt trees bearing fungal mats. Insects were collected using an aspirator (Fergusson 1982) and transferred to separate cylindrical glass containers containing a piece of tissue paper for each tree sampled. In the laboratory, insects were inactivated by cooling the glass containers in a box containing ice. The insects were grouped according to morphological characteristics using an Axiocam stereo microscope (Carl Zeiss Ltd., Germany). Representatives of each insect group were preserved in 70% ethanol prior to identification, by Dr. Andrew Cline, Senior Insect Biosystematist, Plant Pest Diagnostics Center, California Department of Food & Agriculture, United State of America.

Isolation and purification of fungi from wood samples followed the same protocols described by Kamgan Nkuekam et al. (2008a). Nitidulid beetles not retained for species identification were used to determine the presence of *Ceratocystis* species on their bodies. This was done by squashing individual insects between two slices of carrot (Moller and Devay 1968) and incubating them for five days at 25ºC. Fungi were isolated by transferring fruiting bodies (spore masses, mycelium, ascomata) growing on the carrots to 2% malt extract agar (MEA: 20 g/l malt extract and 15 g/l agar, Biolab, Midrand, South Africa and 1000 ml sterile deionised water) containing 0.05 g/l of streptomycin sulphate (SIGMA-ALDRICH, Steinheim, Germany).

Isolates were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and representative cultures have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Dried specimens of representative isolates were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Morphological characterization

Ceratocystis isolates were grouped into morphotypes, based on their morphological features in pure culture on 2% MEA. Cultures were incubated at 25ºC until sporulation and then grouped into morphotypes based on colour (Rayner 1970) and macro-morphology. Fruiting structures including ascomata and ascospores, phialides and conidia from selected isolates representing each morphotype were mounted in 80% lactic acid on microscope slides and studied using a Zeiss Axiocam light microscope. Fifty measurements of all characteristic morphological features were made for isolates chosen as the types of new species and ten measurements were made for additional isolates. Measurements were noted as (minimum -) mean minus st. dev. - mean plus st. dev. (- maximum).

Scanning electron microscopy was used to observe conidia and conidiophores of the fungi. For this purpose, specimens were prepared following protocols described by Grobbelaar et al. (2009). Finally the specimens were critical point dried (Bio-Rad E3000, Watford, England), then mounted and coated with gold in a sputter coater and examined using a JEOL JSM-840 scanning electron microscope.

Growth in culture

One isolate of each of the purported new species found in this study was used for growth studies in culture. Disks of agar (9 mm diam.) bearing mycelium of the test isolates were transferred from the actively growing margins of seven-day-old cultures and placed upside down at the centres of 90 mm Petri dishes containing 2% MEA. The plates were incubated in the dark for 10 days at temperatures ranging from 5ºC to 35ºC at 5 degree intervals. Five replicate plates were used for each isolate at each temperature considered. Two diameter measurements, perpendicular to each other, were taken daily for each colony and the averages of ten diameter measurements for each temperature were computed (Kamgan Nukekam et al. 2008a; b).

DNA sequence comparisons

All isolates of each morpho-group of the *Ceratocystis* spp. collected in this study were selected for DNA sequence comparisons. Single spore drops collected from the apices of ascomata or conidiophores in pure cultures were grown on 2% MEA for 7-10 days. Mycelium was scraped from the surface of the actively growing cultures and then transferred to 1.5 ml Eppendorf tubes using a sterile hypodermic needle. DNA was extracted using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions.

The internal transcribed spacer regions (ITS1, ITS2) and 5.8S gene of the ribosomal RNA operon were amplified on an Eppendorf Mastercycler (Merck, Hamburg, Germany) using primers ITS1 (3'-TCCGTAGGTGAACCTGCGG-5') and ITS4 (3'-TCCTCCGCTTATTGATATGC-5') (White et al. 1990). Part of the β-tubulin gene (BT1) and the transcription elongation factor-1α gene (TEF) were also amplified using the primers βt1a (5'-TTCCCCCGTCTCCACTTCTTCATG-3') and βt1b (5'-GACGAGATCGTTCATGTTGAACTC-3') (Glass and Donaldson 1995), EF1F (5'-TGCGGTGGTATCGACAAGCGT-3') and EF2R (5'-AGCATGTTGTCGCCGTTGAAG-3') (Jacobs et al. 2004), respectively.

The PCR reaction mixtures as well as the thermal cycling used in this study were the same as described previously (Kamgan Nkuekam et al. 2008a). An aliquot of 5 µl of the PCR products were stained with GelRed^{TM} Nucleic Acid Gel stain (Biotium, Hayward, CA, USA), separated on a 1% agarose gel and visualized under UV light. PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich), following the manufacturer's instructions. Subsequently, the concentrations of the purified PCR products were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, USA). Sequencing reactions were performed using the Big Dye cycle sequencing kit with Amplitaq DNA polymerase, FS (Perkin-Elmer, Warrington, UK) following the manufacturer's protocol on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequencing PCRs were prepared as described by Kamgan Nkuekam et al. (2008a) and both DNA strands were sequenced.

A preliminary identity for the *Ceratocystis* isolates was obtained by performing a similarity search (standard nucleotide BLAST) against the GenBank database (http://www.ncbi.nlm.nih.gov). Sequences of both strands for each isolate were examined visually and combined using the programme Sequence Navigator. Sequences were then aligned automatically using Mafft ver.5.851 (Katoh et al. 2002) and analyzed using PAUP 4.0b10 (Swofford 1998). Additional sequences of related *Ceratocystis* species were obtained from the GenBank database. PAUP 4.0b10 was used to construct phylogenetic trees from the distance matrices by pair-wise alignment of the sequences, using the maximum parsimony method (Swofford 1998). Confidence levels of the phylogenies were estimated with the bootstrap method (Felsenstein 1985).

Bayesian analyses was performed with MrBayes V3.1 (Ronquist and Heuelsenbeck 2003), based on Markov Chain Monte Carlo (MCMC) methods. The best-fit model of evolution was determined using MrModeltest V2.2 (Nylander 2004) and included for each gene partition in MrBayes. Four simultaneous MCMCs were run for 1 000 000 generations and trees were sampled every 100th generation. The burn-in procedure in MrBayes V3.1 was used to discard trees that formed before the

point of convergence, and the posterior probability in the majority rule concensus trees were calculated by MCMC sampling in MrBayes V3.1, using the best-fit model of evolution.

Pathogenicity tests

Pathogenicity tests were conducted in a quarantine greenhouse using *Eucalyptus grandis* clone TAG5. Five strains of each of two *Ceratocystis* species obtained in this study were used in the inoculation experiment. Ten trees, approximately two-years-old (~1 cm diameter), were inoculated with each test strain and five trees of the same age were inoculated with a sterile agar disc to serve as controls. Test strains included isolates (CMW28917, CMW28920, CMW28925, CMW28928, CMW28932) belonging to the *C. moniliformis s.l.* complex, and isolates (CMW29120, CMW29275, CMW29349, CMW29354, CMW29546) belonging to the *C. fimbriata s.l.* complex (Table 1). Greenhouse conditions included an average temperature of 25ºC and natural day/night conditions of about 13 hours daylight and 11 hours darkness. Inoculations were done using the same technique as described before by Kamgan Nkuekam et al. (2008a). Six weeks (42 days) after inoculation, the lengths of lesions, including the original wound on the bark surface as well as in the cambium of each tree were measured. Results were then analyzed using the GLM procedure in SAS/STAT (SAS Institute Inc. 1999). Re-isolations were made from the lesions to confirm that they had resulted from the effects of the test fungi.

Results

Source of samples and isolations

A wide variety of *Ceratocystis* isolates arose from collections in New South Wales, Queensland and Tasmania. Samples from which fungi were isolated were from harvesting and stem boring insect wounds on two eucalypt genera (*Eucalyptus* and *Corymbia*) spanning nine different species. These trees species included *E. nitens* Deane & Maiden, *E. globulus* Labill, *E. pilularis* Sm., *E. saligna* Sm., *E. dunnii* Maiden, *E. grandis* Hill ex Maiden, *E. grandis x camaldulensis*, *E. tereticornis* and *C. variegata* (F.Muell.) K.D.Hill & L.A.S.Johnson (Table 1). A total of 54 isolates were obtained from wounds on 200 trees sampled across the three states of Australia. Apart from discoloration of the wood around the wounds no other diseases symptoms were observed on the trees.

Nitidulidae were found only at Ingalba State forest in New South Wales. A limited number (30) of insects were collected from *E. pilularis* trees. These insects represented two groups based on morphology and included *Brachypeplus binotatus* Murray (13 insects) and *Brachypeplus planus* Erichson (10 insects). The remaining specimens were characteristic of *Carpophilus* species (7 insects) but could not be identified to species level. *Ceratocystis* spp. were isolated from four nitidulid beetles using the carrot baiting technique.

Morphological identification

Ceratocystis spp. collected in this study could broadly be assigned to three morphological groups based on colony morphology and the type of fruiting bodies produced on MEA (Table 1). Morpho-group A included species resembling

those in the *C. moniliformis s.l.* species complex. This group, consisting of 18 cultures, was characterized by fast growing isolates with a strong fruity (banana) odor, ascomata exuding sticky spore drops containing hat-shaped ascospores typical of *Ceratocystis* species, echinulate perithecial bases and ascomatal necks having disciform bases. These isolates covered the entire surface of the 60mm Petri dishes within three days at 25ºC.

Morpho-group B isolates (Table 1) included species resembling those in the *C. fimbriata s.l.* species complex. There were 33 isolates in this group and they were slow growing and had a granular appearance. Limited numbers of ascomata were produced in culture. These isolates had no spines on the ascomatal bases, they lacked disc-like bases on the ascomatal necks, and produced hat-shaped ascospores. Isolates in this morpho-group could be further placed in two sub-groups based on morphological characteristics. One of these sub-groups from NSW, where it was collected from both insects and trees, was lighter coloured and ascospore drops were produced abundantly. This was in contrast to isolates that originated from *Eucaltyptus* trees in Tasmania, and which were recognized as representing *C. pirilliformis* based on morphology, as described by Barnes et al. (2003b).

DNA sequence comparisons

All isolates of *Ceratocystis* in morpho-group A (*C. moniliformis s.l.*) were sequenced and selected isolates (CMW21598, CMW28917, CMW28920, CMW28925, CMW28928, CMW28932) (Table 2) including representatives from each host was used in phylogenetic analyses (Fig. 1). All isolates generated amplicons of about 600, 550 and 850 bps for the ITS, BT1 and TEF gene regions, respectively. Partition homogeneity tests using 1000 replicates for sequence data of these three gene regions resulted in a P-value of 0.283, suggesting that the data from the three gene regions could be combined. Comparison of these isolates with those of previously published species in GenBank by analyses of the combined data sets in PAUP resulted in a total of 1130 characters including gaps, with 960 constant characters, 5 variable characters (parsimony-uninformative) and 165 parsimony informative characters.

Phylogenetic analysis using parsimony and the heuristic search options resulted in 419 best trees with a consistency index (CI) and retention index (RI) value of 0.670 and 0.892, respectively. Isolates from Australia formed a well-resolved clade (Fig. 1), supported by a bootstrap value of 98%, separate from any of the described species in the *C. moniliformis s.l.* species complex, suggesting that they represent an undescribed species. The closest phylogenetic neighbor of these isolates was *C. moniliformis*.

In separate analyses, using both parsimony and Bayesian computations across the ITS, BT1 and TEF gene regions respectively, only representatives of Morpho-group A and their most closely related phylogenetic neighbors were considered to confirm their species delimitation. In addition, the multilocus nucleotide polymorphisms showing differences among these taxa, as well as the number of fixed base pair differences across the three gene regions, were computed. In these analyses, representatives of Morpho-group A formed a well resolved clade clearly separated from its sister clades (Supplementary Material, Table 3) (Fig. 3A, 3B, 3C). This separation was supported by a number of polymorphic nucleotide sites found across the three gene regions (Supplementary Material, Table 4.1, 4.2). A number of fixed base pair

differences were also found (Supplementary Material, Table 5, 6, 7) across the three gene regions considered and these were consistent with results based on concatenated analyses and morphology.

Isolates (CMW29120, CMW29275, CMW29354, CMW29349, CMW29546, CMW29549) in Morpho-group B1, and Morpho-group B2 (CMW29111, CMW29112, CMW29119, CMW29355) generated amplicons of about 600, 550 and 850 bps for parts of the ITS, BT1 and TEF gene regions, respectively. Partition homogeneity tests using 1000 replicates for sequence data of these three gene regions resulted in a P-value of 0.273, suggesting that the data from the three gene regions could be combined. Comparison of these isolates with those from GenBank and automatic alignment using Mafft, followed by analyses in PAUP, resulted in a total of 1289 characters including gaps, with 630 constant characters, 166 variable characters (parsimony-uninformative) and 493 parsimony informative characters. Phylogenetic analysis using parsimony and the heuristic search option resulted in 734 best trees with a consistency index (CI) and retention index (RI) value of 0.692 and 0.889, respectively. Isolates resolved into two different clades within the larger *C. fimbriata s.l* group. Isolates representing Morpho-group B1 formed a well-resolved clade (Fig. 2), supported by a bootstrap value of 85%, separate from any of the described species in the *C. fimbriata s.l.* species complex. The closest phylogenetic neighbor of these isolates was *C. atrox* and *C. polychroma*. Isolates of morphogroup B2 clustered with strains of *C. pirilliformis*, consistent with results based on morphological identification.

Bayesian analyses for representatives of Morpho-group B1 and Morpho-group B2 and their most closely related phylogenetic neighbors showed that representatives of morpho-group B1 formed a well resolved clade, clearly separated from its sister clades *C. atrox* and *C. polychroma*, based on the ITS, BT1 and TEF gene regions (Fig. 4A, 4B, 4C). This separation was supported by a number of fixed base pair differences found (Supplementary Material, Table 8.1, 8.2, 9, 10, 11) and these were consistent with results based on concatenated analyses. Analyses of the TEF sequence data did not provide separation between these species. Isolates in morphogroup B2 grouped with strains of *C. pirilliformis*, consistent with results based on maximum parsimony analysis (Fig. 2, 4A, 4B, 4C).

Taxonomy

Based on morphological studies and multigene sequence phylogenies, two *Ceratocystis* spp. from Australia emerged as distinct taxa, clearly separated from other related *Ceratocystis* reference strains. These included one species residing in the *C. moniliformis s.l.* group and one in the *C. fimbriata s.l.* complex. The following descriptions are provided for them.

Ceratocystis tyalla Kamgan-Nkuek. & Jol. Roux sp. nov. **(**Fig.5) MB519980

Etymology: The name is derived to the aboriginal name for eucalypts in Australia.

Coloniae mellicolores in MEA in 3 diebus in 30°C ad 36.17 mm crescentes. Bases ascomatum brunneae vel nigrae, globosae vel obpyriformes, spinis indumentoque hypharum fuscis. Colla ascomatum atrobrunnea hyphis ostiolaribus divergentibus. Bases collorum basin versus ornamentis disciformibus. Asci evanescentes. Ascosporae pileiformes hyalinae non septatae, vaginis vestitae. Anamorpha *Thielaviopsis* conidiophoris phialidicis hyalinis tubulosis, colliculis visibilibus

(1.5-) 2.5-6.0 (-8.5) µm. Conidia hyalina non septata biformia; oblonga extremis obtusis (5.0-) 6.5-8.5 (-9.5) x (1.5-) 2.0-2.5 (-3.0) µm, bacilliformiaque basibus rotundatis (8.5-) 9.0-11.0 (-12.0) x (1.5-) 2.0-2.5 (-3.5) µm.

Colonies honey (19''b) coloured on MEA, reverse honey (19''b) coloured, almost brown. Colony diameters reaching 36.17mm in 3 days on MEA at 30ºC. Optimal growth at 30ºC, growth at 35ºC with colony diameters reaching 33.8mm in 3 days. No growth at 5ºC. Mycelium forming thick mats on agar with some white aerial mycelia. Hyphae septate, not constricted at septa. *Ascomata* scattered over the surface of the colonies or embedded in mycelium. *Ascomatal* bases brown to black, globose to obpyriform (124.5-) 143.0-175.5 (-195.5) µm long and (116.5-) 136.0-167.0 (-177.5) µm wide, with dark conical spines, (5.5-) 7.5-12.5 (-18.0) µm long and hyphal hair. *Ascomatal* necks dark brown (428.5-) 466.5-607.5 (-772.5) µm long, middle of necks (17.5-) 19.0-22.5 (-24.0) µm wide, tips of necks (9.0-) 11.0-13.5 (-14.5) µm wide, producing sticky and hyaline spore drops at the tips of divergent *ostiolar hyphae*, (14.5-) 18.0-24.5 (-28.5) µm long. Neck bases ornamented with disc-shapes, (37.0-) 43.0-52.5 (-59.5) µm wide at bases. *Asci* rarely seen, evanescent, deliquescing early in the development. *Ascospores* hat-shaped, hyaline, aseptate, invested in sheaths (3.5-) 4.0-4.5 (-5.0) x (2.0-) 2.0-2.5 (-3.0) µm, accumulating in round, straw yellow (21'd) spore drops, becoming creamy with age.

Anamorph: *Thielaviopsis*. *Conidiophores* singly on mycelium, phialidic, hyaline, tubular (15.2-) 17.9-27.3 (-35.7) x (1.8-) 2-2.9 (-3.9) µm; colarettes visible (1.5-) 2.5-6.0 (-8.5) µm. *Conidia* hyaline, aseptate, two types, oblong with obtuse ends (5.0-) 6.5-8.5 (-9.5) x (1.5-) 2.0-2.5 (-3.0) µm and bacilliform with rounded bases (8.5-) 9.0-11.0 (-12.0) x (1.5-) 2.0-2.5 (-3.5) µm. Chlamydospores (aleurioconidium) not observed.

Specimens examined: Australia, New South Wales, Marsden State Forest, isolated from cut stump of *Eucalyptus dunnii*, 19/03/2008, G. Kamgan Nkuekam and A. J. Carnegie, holotype PREM60434, living culture CMW28932, CBS128703

Additional specimens: Australia, New South Wales, Cairncross State Forest, isolated from *E. pilularis* stumps, 18/03/2008, G. Kamgan Nkuekam and A. J. Carnegie, paratype, living culture CMW28925/PREM60436/CBS127211, Wattagans State Forest, isolated from *E. saligna* stumps, 17/03/2008, G. Kamgan Nkuekam and A. J. Carnegie, CMW28928/PREM60435/CBS128342, Pine Creek State Forest, isolated from *E. grandis* stumps, 18/03/2008, G. Kamgan Nkuekam and A. J. Carnegie, CMW28917, CMW28920.

Ceratocystis corymbiicola Kamgan-Nkuek. & Jol. Roux sp. nov. (Fig.6) MB519979.

Etymology: The name reflects the host Corymbia.

Coloniae olivaceo-bubalinae in MEA in 10 diebus in 30°C ad 20 mm crescentes. Bases ascomatum nigrae, globosae sine spinis nec ornamentis. Colla ascomatum nigra hyphis ostiolaribus divergentibus. Asci evanescentes. Ascosporae pileiformes, vaginis vestitae, non septatae. Anamorpha *Thielaviopsis* conidiophoris phialidicis tubulosis hyalinis, sine colliculis. Conidia biformia; bacilliformia basibus rotundatis (11.0-) 15.0-21.5 (-27.5) x (3.0-) 3.5-4.5 (-5.5) µm, bacilliformiaque extremis obtusis (7.5-) 8.5-12.0 (-14.5) x (3.5-) 4.0-5.5 (-6.5) µm. Chlamydosporae (aleurioconidium) ovoideae laeves singulae terminales, iuventute hyalinae, maturitate nigrescentes (8.5-) 11.0-12.0 (-16.5) x (6.5-) 8.0-11.0 (-16.5) µm.

Colonies olivaceous Buff (21'''d) on MEA, reverse smoke grey (21''''d). Colony diameters reaching 20 mm in 10 days on MEA at 30ºC. Optimal growth at 30ºC, no growth at 10ºC. Colony surfaces scattered with black ascomata. Mycelium immersed and superficial, with white-grey aerial mycelia. Hyphae septate, not constricted at septa. *Ascomatal* bases black, globose (159-) 189-241 (-290) µm long and (160.5-) 185.0-237.5 (-272.5) µm wide. Spines or ornamentations absent. *Ascomatal* necks black (603.0-) 755.0-1009.0 (-1097.5) µm long, bottom of necks smooth (43.0-) 55.0-70.5(-77.0) µm wide, middle of necks (20.0-) 27.5-34.5 (-38.5) µm wide, tips of necks (12.5-) 15.5-19.5 (-22.0) µm wide. *Ostiolar hyphae* present, divergent (22.5-) 42.0-58.5 (-67.5) µm long. *Asci* evanescent. *Ascospores* hat-shaped, invested in sheaths, aseptate (4.5-) 5.0-5.5 (-6.0) µm long and (2.5-) 3.0-3.5 (-4.0) µm wide. Ascospores accumulating in round or disk-shaped, Buff (19''d) colored spore drops.

Anamorph: *Thielaviopsis*. *Conidiophores* occurring singly, phialidic (17.0-) 38.5-83.0 (-109.0) x (2.0-) 3.0-5.0 (-5.5) µm, tubular with thin bases making them almost constricted at septa, hyaline, colarettes absent. Two types of *Conidia* produced; bacilliform-shaped conidia with round bases (11.0-) 15.0-21.5 (-27.5) x (3.0-) 3.5-4.5 (-5.5) µm, and bacilliform-shaped conidia with obtuse ends (7.5-) 8.5-12.0 (-14.5) x (3.5-) 4.0-5.5 (-6.5) µm, often produced in chains. *Chlamydospores* (aleurioconidium) ovoid, smooth, formed singly, terminal, hyaline when young, becoming black when mature (8.5-) 11.0-12.0 (-16.5) x (6.5-) 8.0-11.0 (-16.5) µm.

Specimens examined: Australia, New South Wales, Wedding Bells State Forest, isolated from cut stumps of *Corymbia variegata*, 19/03/2008, G. Kamgan Nkuekam and A. J. Carnegie, holotype PREM60431, living culture CMW29120/CBS127215.

Additional specimens: Australia, New South Wales, Ingalba State Forest, isolated from *Brachypeplus planus* infesting *Eucalyptus pilularis* stumps, 18/03/2008, G. Kamgan Nkuekam and A. J. Carnegie, paratype, living culture CMW29354/PREM60432/CBS127217. Isolated from *E. pilularis* stumps CMW29349/PREM60433/CBS127216. Wattagans State Forest, New South Wales. Isolated from *E. saligna* stumps, 17/03/2008, CMW29275/PREM60442, Ingalba State Forest, isolated from *Carpophylus* sp. infesting *Eucalyptus pilularis* tree, 18/03/2008, CMW29546, Dyraba State Forest, isolated from *E. grandis x camaldulensis* stumps, 20/03/2008, CMW29549.

Pathogenicity tests

Six weeks after inoculation, *E. grandis* trees were assessed for disease development based on the length of lesions on the bark or at the cambial surfaces. *Ceratocystis tyalla* and *C. corymbiicola* produced very small lesions on both the bark (Fig. 7) and the cambial surfaces (Fig. 8). Trees showed no signs of disease, and re-isolation did not yield cultures of either *C. tyalla* or *C. corymbiicola*. Significant differences ($P<0.0001$) in lesion lengths were, however, found between *C. tyalla* and *C. corymbiicola* when compared to the control inoculations (Fig. 7, 8).

Discussion

This study reports on the most extensive survey of *Ceratocystis* species on eucalypts ever conducted in their country of origin. Three species of *Ceratocystis* were collected in Australia from either wounds on eucalypt trees or from nitidulid beetles collected from wounds on trees. Two of these are previously undescribed fungal species for which the names *C. tyalla* and *C. corymbiicola* have been provided. Furthermore, the host and geographic range of *C. pirilliformis*, previously

known from Australia (Barnes et al. 2003b, Kamgan Nkuekam et al. 2009) have been expanded to include *Eucalyptus* trees in Tasmania.

Three *Ceratocystis* spp., two in the *C. fimbriata s.l.* species complex and one in the *C. moniliformis s.l.* species complex emerged from this study. These include two species described for the first time in this study and *C. pirilliformis*. *Ceratocystis tyalla* grouped in a unique sub-clade, most closely related to *C. moniliformis* in phylogenetic analyses. Similar to *C. moniliformis* and other fungi in the *C. moniliformis s.l.* complex, it grows rapidly in culture, produces hat-shaped ascospores and has short conical spines on the ascomatal bases. *Ceratocystis tyalla* could, however, be distinguished from *C. moniliformis*, and other related fungi, by the fact that it has a disc-like ascomatal neck base, which is less well defined than in other species of the group. Phylogenetic inference based on three gene regions also clearly distinguished this taxon from its closest relatives.

Ceratocystis corymbiicola collected from eucalypts in Australia, grouped in the *C. fimbriata s.l.* complex. It was most closely related to *C. atrox* and *C. polychroma* in both the combined ITS, BT and TEF data sets as well as when these gene regions were analysed separately. Bootstrap support for the unique clade containing *C. corymbiicola* was, however, low in the concatenated tree. Morphological differences could, however, be detected to support the description of *C. corymbiicola* as a unique species, distinct from *C. atrox*, a species also described from Australia (Van Wyk et al. 2007). *C. corymbiicola* produces chlamydospores structures that have not been found in *C. atrox*. In addition, *C. corymbiicola* produces two morphological forms of bacilliform conidia and only one type of phialide, while *C. atrox* produces cylindrical and barrel-shaped conidia, and two morphologically distinct forms of phialides (Van Wyk et al. 2007). The conidia of *C. corymbiicola* have swellings, even when young and this feature also make the species easy to distinguish from other.

Inoculation studies with *C. tyalla* and *C. corymbiicola* under greenhouse conditions suggest that these fungi are not serious pathogens of *Eucalyptus* trees. Neither of them gave rise to significant lesions or any other disease symptoms of young *E. grandis* trees. This is not surprising for *C. tyalla* since species in the *C. moniliformis s.l.* group include only non-pathogens (Davidson 1935, Kamgan Nkuekam et al. 2008b, Van Wyk et al. 2006). Although *C. corymbiicola* resides in a group of *Ceratocystis* species known to include important plant pathogens, it appears not to have the potential to cause disease of *Eucalyptus*, at least under natural situation.

Both previously undescribed *Ceratocystis* spp. collected in this study could have much wider distributions and host ranges as found. The discovery of these fungi infecting different eucalypt genera spanning several different species suggests that eucalypts are probably their primary hosts. Furthermore, isolating *C. corymbiicola* from nitidulid beetles suggests that these insects could be potential vectors of the fungus in Australia, similar to what is known for these fungi from other continents.

It was not surprising to find *C. pirilliformis* infecting wounds of *Eucalyptus* trees in Australia. This fungus was first described from wounds on *E. nitens* in Australia (Barnes et al. 2003b) and has subsequently also been found on *Eucalyptus* species in South Africa (Roux et al. 2004; Kamgan Nkuekam et al. 2009). Results of this study expand the host and geographic range of *C. pirilliformis*. It is suspected that the fungus is native to Australia, based on its wide distribution (Tasmania to Queensland) in the country and the fact that it appears not to cause disease on eucalypts (Barnes et al. 2003b;

Kamgan Nkuekam et al. 2009). A recent population diversity study of *C. pirilliformis* collected in South Africa showed a low level of diversity for isolates in South Africa and suggested high population diversity for the few isolates available from Australia (Kamgan Nkuekam et al. 2009). However, larger populations of *C. pirilliformis* from Australia are required to fully elucidate the origin of this fungus.

Brachypeplus binotatus and *B. planus*, the two nitidulid species identified in this study in association with *Ceratocystis* species, have been reported from Australia previously. Numerous other species of Nitidulidae including *Carpophilus* species are also known to occur in Australia in various localities and habitats (Masters 2009). However, nitidulid beetles have not previously been found in association with *Ceratocystis* species in Australia. The results suggest that these insects are probably involved in overland spread of these fungi and further study of this association is likely to yield interesting insights into the biology of *Ceratocystis* species and their vectors in that country.

Acknowledgements

We thank the DST/NRF Center of Excellence in Tree Health Biotechnology (CTHB), National Research Foundation of South Africa (NRF), the THRIP Initiative of the Department of Trade and Industry (THRIP/DST), members of the Tree Protection Co-operative Programme (TPCP) and the University of Pretoria for funding and the facilities to undertake this study. We are also most grateful to the CSIRO Division of Forestry in Hobart (Tasmania), the New South Wales Department of Primary Industry and Fisheries in Sydney and the Queensland Department of Primary Industry and Fisheries in Brisbane for hosting G. Kamgan Nkuekam and providing the opportunity to collect specimens in Australia. Prof. Goeneveld and Dr. Van der Linde from the Department of Statistics are thanked for their assistance with the statistical analyses. Dr. Andrew Cline from the USA is thanked for identifying insects collected in this study. We further thank Dr. Hugh Glen who provided the Latin diagnosis and suggested the names for the new taxa.

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Table 1 List of *Ceratocystis* isolates and their morphogroups collected during surveys in Eastern and Southern Australia.

Morphogroups	States	Area	Hosts	Isolate Number	Fungal species	Coordinates	Collectors
A	NSW	Marsden State Forest	*E. dunnii* stumps	28930, 28931, 28932, 28933	*C. tyalla*	S30º 14, 503' E152º 38, 745'	G.NK. Kamgan & A.J. Carnegie
	"	Wattagans State Forest	*E. saligna* stumps	28927, 28928,28929	"	S33º 02, 738' E151º 19, 402'	"
	"	Cairncross State Forest	*E. pilularis* trees	28923, 28924, 28925, 28926	"	S31º 21, 275' E152º 45, 671'	"
	"	Pine Creek State Forest	*E. grandis* stumps	28920, 28921, 28922	"	S31º 21, 361' E152º 45, 009'	"
	"	Pine Creek State Forest	*E. grandis* stumps	28917, 28918, 28919	"	S30º 23, 545' E152º 56, 975'	"
	QLD	Brisbane	*E. tereticornis*	21598	"	NA	J. Roux & G.S. Pegg
B1	QLD	Brisbane	*E. tereticornis*	21599, 21600, 21601, 21602	*C. corymbiicola*	NA	"
	NSW	Wedding bells State Forest	*C. variegata*	29120, 29121, 29122, 29123, 29124, 29347, 29348, 29428	"	S30º 03, 498' E153º 10, 266'	G.NK. Kamgan & A.J. Carnegie
	"	Wattagans State Forest	*E. saligna*	29275, 29276, 29434	"	S33º 02, 738' E151º 19,	"

							402’	
		“	Dyraba State Forest	*E. grandis x camaldulensis* trees	29549	“	S28º 48, 522’ E152º 50, 370’	“
		“	Nabiac	*E. grandis* trees	29344, 29345, 29346	“	S32º 05, 411’ E152º 21, 838’	“
		“	KEW	*E. grandis x camaldulensis* trees	29547	“	S31º 36, 954’ E152º 44, 096’	“
		“	Ingalba State Forest	*E. pilularis* stumps	29349	“	S30º 47, 970’ E152º 51, 706’	“
		“	Ingalba State Forest	*Carpophilus* sp. infesting *E. pilularis* tree	29353, 29546	“	S30º 47, 970’ E152º 51, 706’	“
		“	Ingalba State Forest	*Brachypeplus planus* infesting *E. pilularis* tree	29354	“	S30º 47, 970’ E152º 51, 706’	“
B2	NSW	Crab tree plantation	*E. grandis* trees, infested by giant wood moths and damaged by cocketou bird	29355	*C. pirilliformis*	S30º 08, 345’ E153º 06, 123’	“
	Tasmania	Burnie	*E. nitens* stumps	29111, 29112, 29113, 29114	“	S41º 10, 185’ E145º 45, 441’	G.NK. Kamgan & Caroline Mohammed
		“	Burnie	*E. globulus* stumps	29119	“	S41º 08, 783’ E145º 48,	“

	"	Tarraleah	*E. nitens* stumps	29092, 29093, 29094	"	435' S11º 15, 846' E024º 19, 115'	"

Table 2 List of *Ceratocystis* isolates and their GenBank accession numbers sequenced in this study (*) or used for DNA sequence comparisons.

Isolates designation	**Isolate number**	**Genbank**	*Gene regions*	*Other numbers*	*Hosts*	**Collectors**	**Origin**
C. acaciivora	CMW22562	EU588655	ITS	NA	*Acacia mangium*	M. Tarigan	Indonesia
		EU588635	BT	"	"	"	"
		EU588645	EF	"	"	"	"
	CMW22563	EU588656	ITS	NA	"	"	"
		EU588636	BT	"	"	"	"
		EU588646	EF	"	"	"	"
C. albifundus	CMW4068	DQ520638	ITS	NA	*Acacia mearnsii*	J. Roux	South Africa
		EF070429	BT	"	"	"	"
		EF070400	EF	"	"	"	"
	CMW5329	AF388947	ITS	NA	*A. mearnsii*	J. Roux	Uganda
		DQ371649	BT	"	"	"	"
		EF070401	EF	"	"	"	"
C. atrox	CMW19383	EF070414	ITS	CBS120517	*E. grandis*	M.J. Wingfield	Australia
		EF070430	BT	"	"	"	"
		EF070402	EF	"	"	"	"
	CMW19385	EF070415	ITS	CBS120518	*E. grandis*	M.J. Wingfield	Australia
		EF070431	BT	"	"	"	"
		EF070403	EF	"	"	"	"
C. bhutanensis	CMW8399	AY528959	ITS	CBS115772, BH 8/8	*Picea spinulosa*	T. Kirisits & D.B. Chhetri	Bhutan
		AY528964	BT	"	"	"	"
		AY528954	EF	"	"	"	"
	CMW8215	AY528958	ITS	CBS114290, PREM57805	*P. spinulosa*	T. Kirisits & D.B. Chhetri	Bhutan
		AY528963	BT	"	"	"	"
		AY528953	EF	"	"	"	"
C. caryae	CMW14793	EF070424	ITS	CBS114716	*Carya cordiformis*	J. Johnson	USA
		EF070439	BT	"	"	"	"
		EF070412	EF	"	"	"	"
	CMW14808	EF070423	ITS	CBS115168	*C. ovata*	"	USA
		EF070440	BT	"	"	"	"
		EF070411	EF	"	"	"	"
C. colombiana	CMW5751	AY177233	ITS	CBS121792	*Coffea arabica*	M. Marin	Colombia

		AY177225	BT	"	"	"	"
		EU241493	EF	"	"	"	"
	CMW5761	AY177234	ITS	CBS121791	"	B. Castro	"
		AY177224	BT	"	"	"	"
		EU241492	EF	"	"	"	"
C. corymbiicola	*CMW29120	HM071902	ITS	CBS127215	*C. variegata*	G.NK. Kamgan	"
		HM071914	BT	"	"	"	"
		HQ236453	EF	"	"	"	"
	*CMW29354	HM071907	ITS	CBS127217	*B. planus*	"	"
		HM071919	BT	"	"	"	"
		HQ236455	EF	"	"	"	"
	*CMW29349	HM071905	ITS	CBS127216	*E. pilularis*	"	"
		HM071917	BT	"	"	"	"
		HQ236456	EF	"	"	"	"
	*CMW29275	HM071903	ITS	NA	*E. saligna*	"	"
		HM071915	BT	NA	"	"	"
		HQ236454	EF	NA	"	"	"
	*CMW29546	HM071904	ITS	NA	*Carpophilus* sp.	"	"
		HM071916	BT	NA	"	"	"
		HQ236457	EF	NA	"	"	"
	*CMW29549	HM071906	ITS	NA	*E. grandis x camaldulensis*	"	"
		HM071918	BT	NA	"	"	"
		HQ236458	EF	NA	"	"	"
C. fimbriata	CMW1547	AF264904	ITS	NA	*Ipomoea batatas*	E.C.H. McKenzie	Papua N. Guinea
		EF070443	BT	"	"	"	"
		EF070395	EF	"	"	"	"
	CMW15049	DQ520629	ITS	CBS141.37	*I. batatas*	C.F. Andrus	USA
		EF070442	BT	"	"	"	"
		EF070394	EF	"	"	"	"
C. fimbriatomima	CMW24174	EF190963	ITS	CBS121786	*Eucalyptus* sp.	M.J. Wingfield	Venezuela
		EF190951	BT	"	"	"	"
		EF190957	EF	"	"	"	"
	CMW24176	EF190964	ITS	CBS121787	*Eucalyptus* sp.	"	"
		EF190952	BT	"	"	"	"
		EF190958	EF	"	"	"	"
C. inquinans	CMW21106	EU588587	ITS	NA	*A.mangium*	M. Tarigan	Indonesia

		EU588666	BT	"	"	"	"
		EU588674	EF	"	"	"	"
	CMW21107	EU588588	ITS	"	"	"	"
		EU588667	BT	"	"	"	"
		EU588675	EF	"	"	"	"
C. larium	CMW25436	EU881908	ITS	CBS122607	*Styrax benzoin*	M.J. Wingfield	Indonesia
		EU881896	BT		"	"	"
		EU881902	EF		"	"	"
	CMW25437	EU881909	ITS	NA	"	"	"
		EU881897	BT		"	"	"
		EU881903	EF		"	"	"
C. manginecans	CMW13851	AY953383	ITS	"	*Mangifera indica*	M. Deadman	Oman
		EF433308	BT	"		"	"
		EF433317	EF	"		"	"
	CMW13852	AY953384	ITS	"	*Hypocryphalus mangifera*	"	"
		EF433309	BT	"		"	"
		EF433318	EF	"		"	"
C. microbasis	CMW21115	EU588592	ITS	"	*A.mangium*	M. Tarigan	Indonesia
		EU588671	BT	"	"	"	"
		EU588679	EF	"	"	"	"
	CMW21117	EU588593	ITS	"	"	"	"
		EU588672	BT	"	"	"	"
		EU588680	EF	"	"	"	"
C. moniliformis	CMW9590	AY431101	ITS	CBS116452	*E. grandis*	J. Roux	South Africa
		AY528985	BT	"	"	"	"
		AY529006	EF	"	"	"	"
	CMW8379	AY529005	ITS	NA	*Cassia fistula*	M.J. Wingfield	Bhutan
		AY528995	BT	"	"	"	"
		AY529016	EF	"	"	"	"
C. moniliformopsis	CMW10214	AY528999	ITS	CBS115792, ORB 33	*E. sieberi*	M.J. Dudzinski	Australia
		AY528988	BT	"	"	"	"
		AY529009	EF	"	"	"	"
	CMW9986	AY528998	ITS	CBS109441	*E. obliqua*	Z.Q. Yuan	Australia
		AY528987	BT	"	"	"	"
		AY529008	EF	"	"	"	"

C. neglecta	CMW17808	EF127990	ITS	CBS121789	*Eucalyptus* sp.	M.J. Wingfield	Colombia
		EU881898	BT			"	"
		EU881904	EF			"	"
	CMW18194	EF127991	ITS	CBS121017	*Eucalyptus* sp.	"	"
		EU881899	BT			"	"
		EU881905	EF			"	"
C. oblonga	CMW23802	EU245020	ITS	CBS122820	*A. mearnsii*	R.N. Heath	South Africa
		EU244992	BT	"	"	"	"
		EU244952	EF	"	"	"	"
	CMW23803	EU245019	ITS	CBS122291	*A. mearnsii*	R.N. Heath	South Africa
		EU244991	BT	"	"	"	"
		EU244951	EF	"	"	"	"
C. obpyriformis	CMW23807	EU245004	ITS	CBS122608	*A. mearnsii*	R.N. Heath	South Africa
		EU244976	BT	"	"	"	"
		EU244936	EF	"	"	"	"
	CMW23808	EU245003	ITS	CBS122511	"	"	"
		EU244975	BT	"	"	"	"
		EU244935	EF	"	"	"	"
C. omanensis	CMW11048	DQ074742	ITS	CBS115780, PREM57815	*Mangifera indica*	AO. Al-Adawi	Oman
		DQ074732	BT	"	"	"	"
		DQ074737	EF	"	"	"	"
	CMW11046	DQ074739	ITS	CBS118112, PREM57814	*M. indica*	AO. Al-Adawi	Oman
		DQ074729	BT	"	"	"	"
		DQ074734	EF	"	"	"	"
C. papillata	CMW8850	AY233866	ITS	CBS121794	*Citrus x tangelo*	M.J. Wingfield	Colombia
		AY233875	BT			"	"
		EU241485	EF			"	"
	CMW8856	AY233867	ITS	CBS121793	*Citrus lemon*	"	"
		AY233874	BT			"	"
		EU241484	EF			"	"
C. pirilliformis	CMW6569	AF427104	ITS	PREM57322, DAR75993	*E. nitens*	M.J. Wingfield	Australia
		DQ371652	BT	"	"	"	"
		AY528982	EF	"	"	"	"
	CMW6579	AF427105	ITS	PREM57323, DAR75996	*E. nitens*	M.J. Wingfield	Australia

		DQ371653	BT	"	"	"	"
		AY528983	EF	"	"	"	"
	*CMW29111	NA	ITS	NA	*E. nitens*	G.NK. Kamgan	"
		NA	BT	NA	"	"	"
		NA	EF	NA	"	"	"
	*CMW29112	NA	ITS	NA	*E. nitens*	"	"
		NA	BT	NA	"	"	"
		NA	EF	NA	"	"	"
	*CMW29119	NA	ITS	NA	*E. globulus*	"	"
		NA	BT	NA	"	"	"
		NA	EF	NA	"	"	"
	*CMW29355	NA	ITS	NA	*E. grandis*	"	"
		NA	BT	NA	"	"	"
		NA	EF	NA	"	"	"
C. platani	CMW14802	DQ520630	ITS	CBS115162	*Platanus occidentalis*	T.C. Harrington	USA
		EF070425	BT	"	"	"	"
		EF070396	EF	"	"	"	"
	CMW23918	EU426554	ITS	NA	"	M.J. Wingfield	Greece
		EU426555	BT	"	"	"	"
		EU426556	EF	"	"	"	"
C. polychroma	CMW11424	AY528970	ITS	CBS115778, PREM57818	*Syzygium aromaticum*	E.C.Y. Liew & M.J. Wingfield	Indonesia
		AY528966	BT	"	"	"	"
		AY528978	EF	"	"	"	"
	CMW11436	AY528971	ITS	CBS115777, PREM57819	*S. aromaticum*	E.C.Y. Liew & M.J. Wingfield	Indonesia
		AY528967	BT	"	"	"	"
		AY528979	EF	"	"	"	"
C. polyconidia	CMW23809	EU245006	ITS	CBS122289	*A. mearnsii*	R.N. Heath	South Africa
		EU244978	BT	"	"	"	"
		EU244938	EF	"	"	"	"
	CMW23818	EU245007	ITS	CBS122290	"	"	"
		EU244979	BT	"	"	"	"
		EU244939	EF	"	"	"	"
C. populicola	CMW14789	EF070418	ITS	CBS119.78	*Populus* sp.	J. Gremmen	Poland
		EF070434	BT	"	"	"	"
		EF070406	EF	"	"	"	"

	CMW14819	EF070419	ITS	CBS114725	*Populus* sp.	T. Hinds	USA
		EF070435	BT	"	"	"	"
		EF070407	EF	"	"	"	"
C. savannae	CMW17300	EF408551	ITS	CBS121151	*Acacia nigrescens*	G.NK. Kamgan & J. Roux	South Africa
		EF408565	BT	"	"	"	"
		EF408572	EF	"	"	"	"
	CMW17297	EF408552	ITS	CBS121021	*Combretum zeyheri*	G.NK. Kamgan & J. Roux	South Africa
		EF408566	BT	"	"	"	"
		EF408573	EF	"	"	"	"
C. smalleyi	CMW14800	EF070420	ITS	CBS114724	*Carya cordiformis*	G. Smalley	USA
		EF070436	BT	"	"	"	"
		EF070408	EF	"	"	"	"
C. tanganyicensis	CMW15992	EU244999	ITS	CBS122293	*A. mearnsii*	R.N. Heath & J. Roux	Tanzania
		EU244971	BT	"	"	"	"
		EU244931	EF	"	"	"	"
	CMW15999	EU244998	ITS	CBS122294	"	"	"
		EU244970	BT	"	"	"	"
		EU244939	EF	"	"	"	"
C. tribiliformis	CMW13015	AY529004	ITS	CBS115949	*Pinus mercusii*	M.J. Wingfield	Indonesia
		AY528994	BT	"	"	"	"
		AY529015	EF	"	"	"	"
	CMW13013	AY529003	ITS	CBS115866	*P. mercusii*	M.J. Wingfield	Indonesia
		AY528993	BT	"	"	"	"
		AY529014	EF	"	"	"	"
C. tsitsikammensis	CMW14276	EF408555	ITS	CBS121018	*Rapanea melanophloeos*	G.NK. Kamgan & J. Roux	South Africa
		EF408569	BT	"	"	"	"
		EF408576	EF	"	"	"	"
	CMW14278	EF408556	ITS	CBS121019	*R. melanophloeos*	G.NK. Kamgan & J. Roux	South Africa
		EF408570	BT	"	"	"	"
		EF408577	EF	"	"	"	"
C. tyalla	*CMW28932	HM071900	ITS	CBS128703	*E. dunnii*	G.NK. Kamgan	Australia
		HM071913	BT	"	"	"	"

		HQ236452	EF	"	"	"	"
	*CMW28928	HM071898	ITS	CBS128342	*E. saligna*	"	"
		HM071912	BT	"	"	"	"
		HQ236451	EF	"	"	"	"
	*CMW28925	HM071897	ITS	CBS127211	*E. pilularis*	"	"
		HM071911	BT	"	"	"	"
		HQ236450	EF	"	"	"	"
	*CMW21598	HM071901	ITS	NA	*E. tereticornis*	"	"
		HM071908	BT	NA	"	"	"
		HQ236447	EF	NA	"	"	"
	*CMW28917	HM071899	ITS	NA	*E. grandis*	"	"
		HM071909	BT	NA	"	"	"
		HQ236448	EF	NA	"	"	"
	*CMW28920	HM071896	ITS	NA	*E. grandis*	"	"
		HM071910	BT	NA	"	"	"
		HQ236449	EF	NA	"	"	"
C. variospora	CMW20935	EF070421	ITS	CBS114715	*Quercus alba*	J. Johnson	USA
		EF070437	BT	"	"	"	"
		EF070409	EF	"	"	"	"
	CMW20936	EF070422	ITS	CBS114714	*Q. robur*	D. Houston	USA
		EF070438	BT	"	"	"	"
		EF070410	EF	"	"	"	"
C. virescens	CMW3276	DQ061281	ITS	NA	*Quercus* sp.	T. Hinds	USA
		AY528990	BT	"	"	"	"
		AY529011	EF	"	"	"	"
C. zombamontana	CMW15235	EU245002	ITS	CBS122297	*Eucalyptus* spp.	R.N. Heath & J. Roux	Malawi
		EU244974	BT	"	"	"	"
		EU244934	EF	"	"	"	"
	CMW15236	EU245000	ITS	CBS122296	"	"	"
		EU244972	BT	"	"	"	"
		EU244932	EF	"	"	"	"

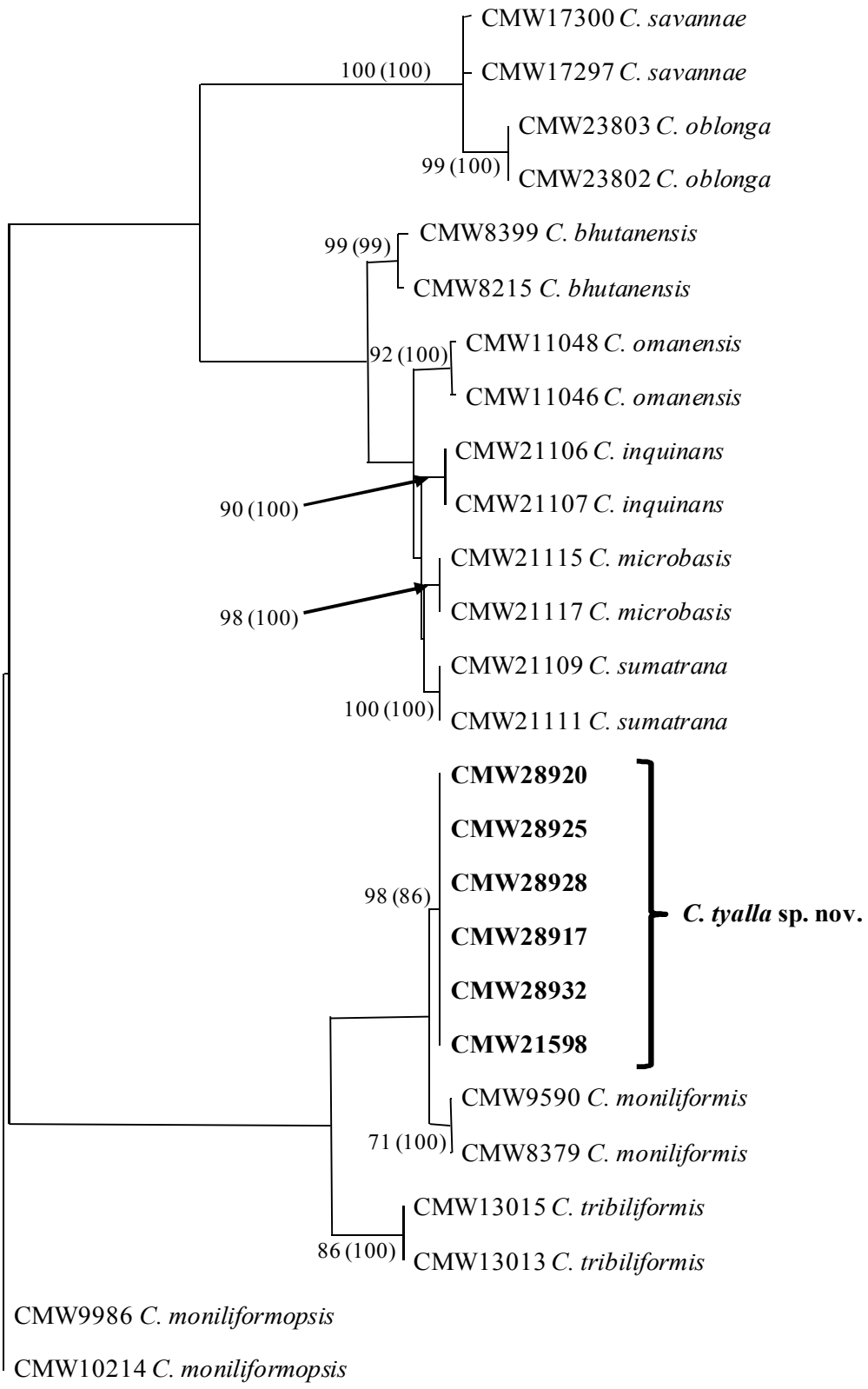

Fig. 1 Phylogenetic tree produced from a heuristic search of the combined ITS, BT and TEF sequence data, showing the relationship between *C. tyalla* sp. nov. from *Eucalyptus* trees in Australia and other *Ceratocystis* spp. resembling *C. moniliformis*. *C. virescens* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated next to each clade with posterior probability values in brackets.

Fig. 2 Phylogenetic tree produced from a heuristic search of the combined ITS, BT and TEF sequence data, showing the relationship between *C. corymbiicola* sp. nov. from eucalypt trees in Australia and other *Ceratocystis* spp. resembling *C. fimbriata*. *C. virescens* was used as out-group taxon. Bootstrap values were derived from 1000 replicates and are indicated next to each clade with the posterior probability values in brackets.

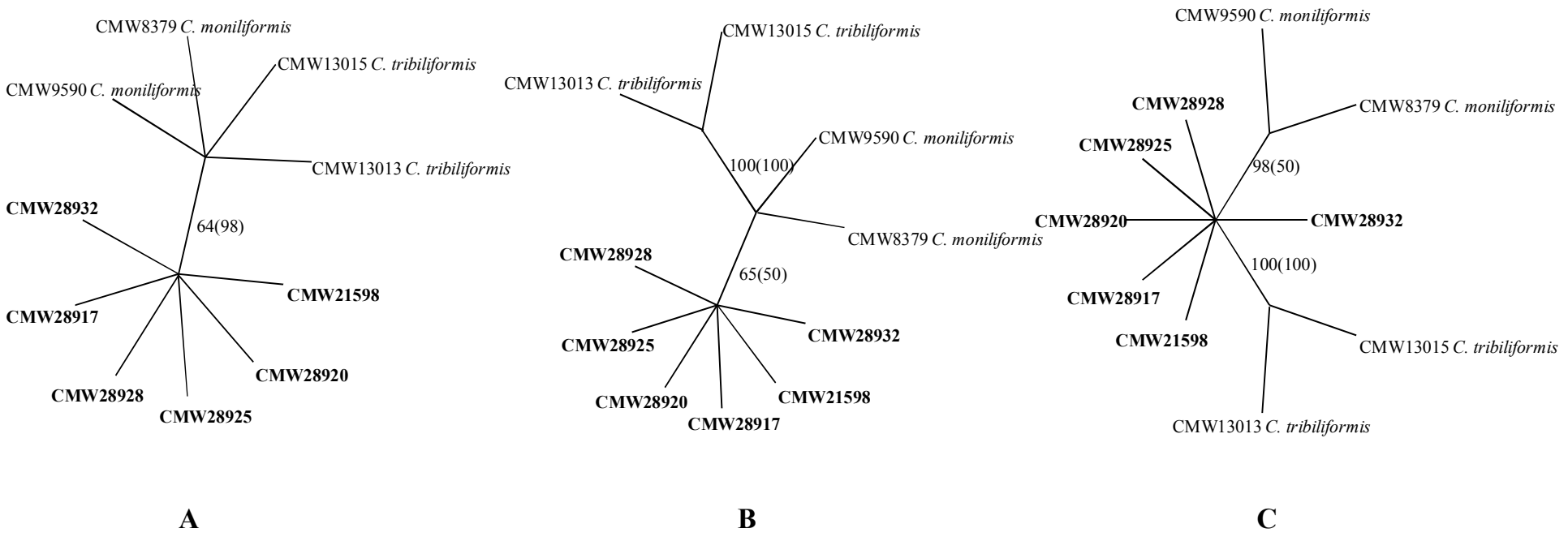

Fig. 3 Unrooted maximum parsimony tree produced from a heuristic search of the ITS (A) BT (B) and TEF (C) sequence data respectively, showing the relationship between *C. tyalla* sp. nov. (isolates in bold font type) from *Eucalyptus* trees in Australia and its most closely related neighbor in the *C. moniliformis s.l.* species complex. Bootstrap values were derived from 1000 replicates and are indicated next to each clade with Bayesian posterior probability values in brackets.

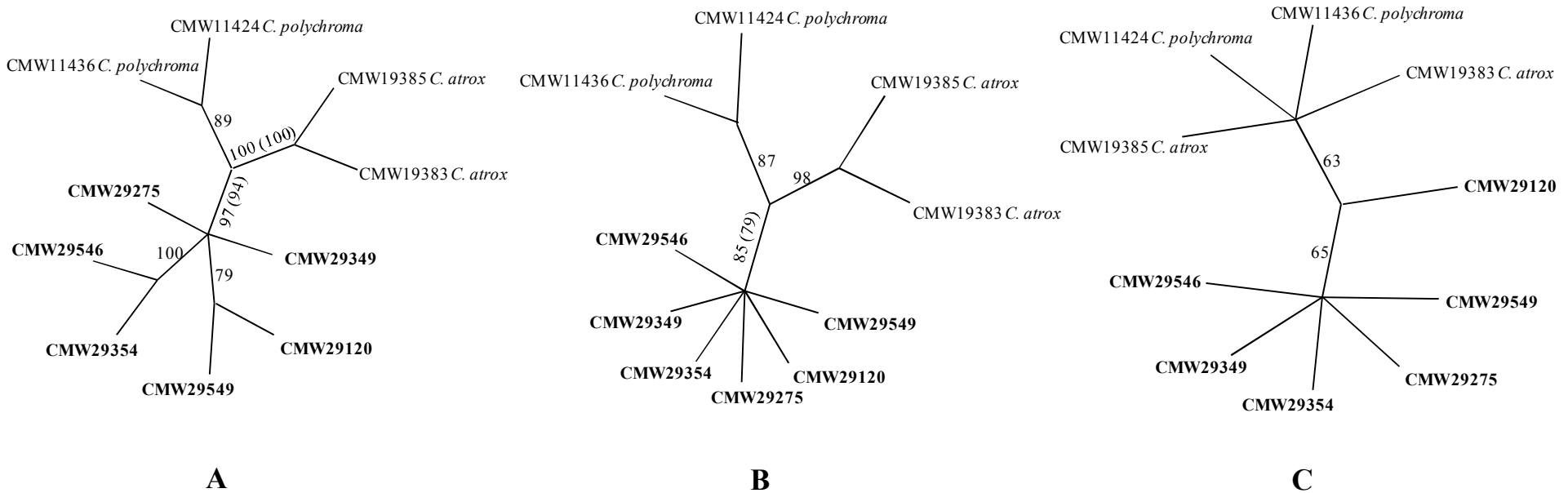

Fig. 4 Unrooted maximum parsimony tree produced from a heuristic search of the ITS (A) BT (B) and TEF (C) sequence data respectively, showing the relationship between *C. corymbiicola* sp. nov. (isolates in bold font type) from *Eucalyptus* trees in Australia and its most closely related neighbor in the *C. fimbriata s.l.* species complex. All isolates sequenced are in bold type. Bootstrap values were derived from 1000 replicates and are indicated next to each clade with Bayesian posterior probability values in brackets.

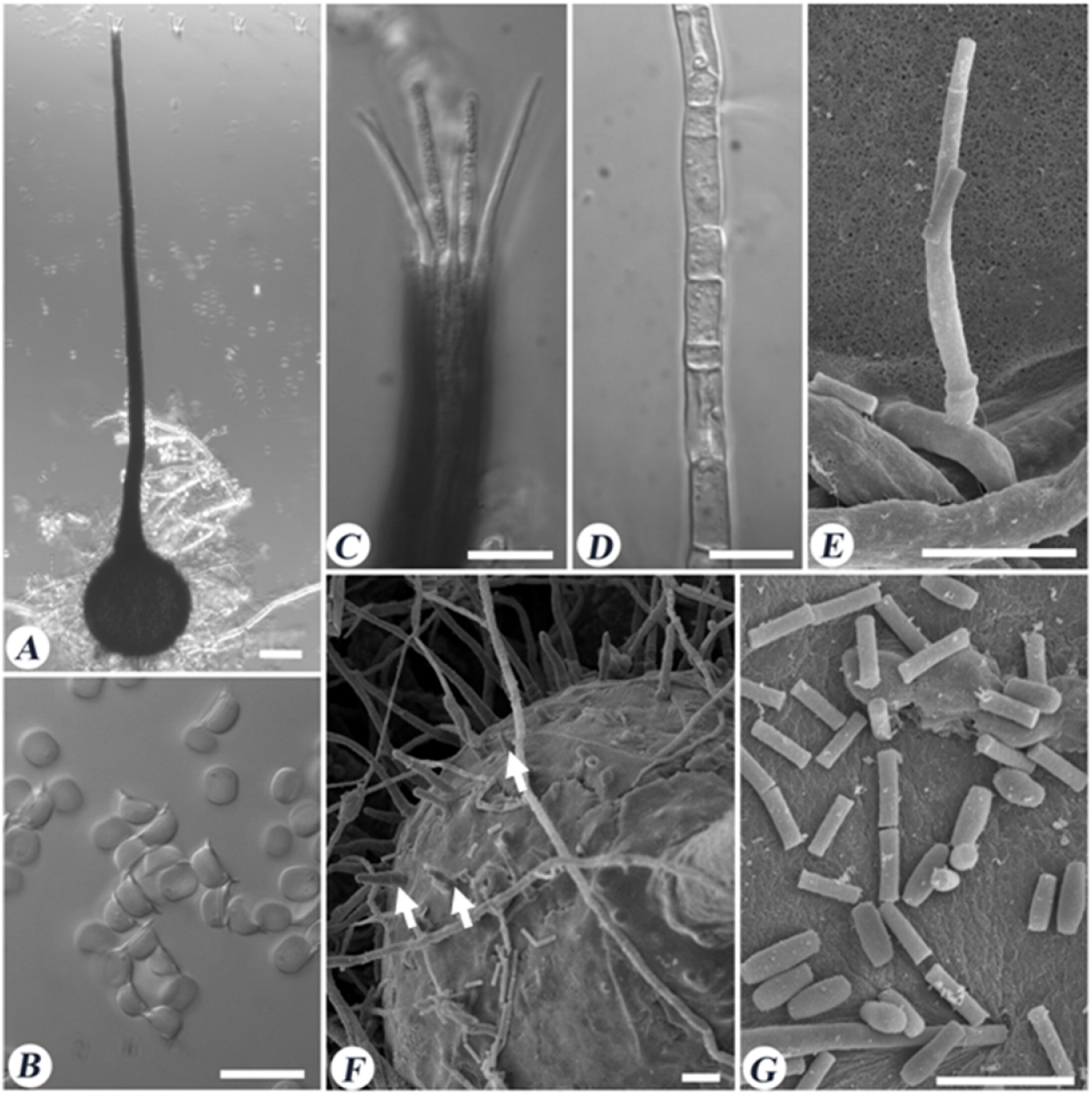

Fig. 5 Morphological characteristics of *Ceratocystis tyalla sp. nov*. A) Globose ascomatal base (scale bar = 50 µm), B) Hat-shaped ascospores (scale bar = 10 µm) C) Divergent ostiolar hyphae (scale bar = 10 µm), D) Septate hyphae (scale bar = 10 µm) E) Phialidic conidiogenous cell with emerging conidia (scale bar = 10 µm), F) Ascomatal base with conical spines (scale bar = 10 µm), G) Oblong conidia with obtuse end and bacilliform shaped conidia (scale bar = 10 µm).

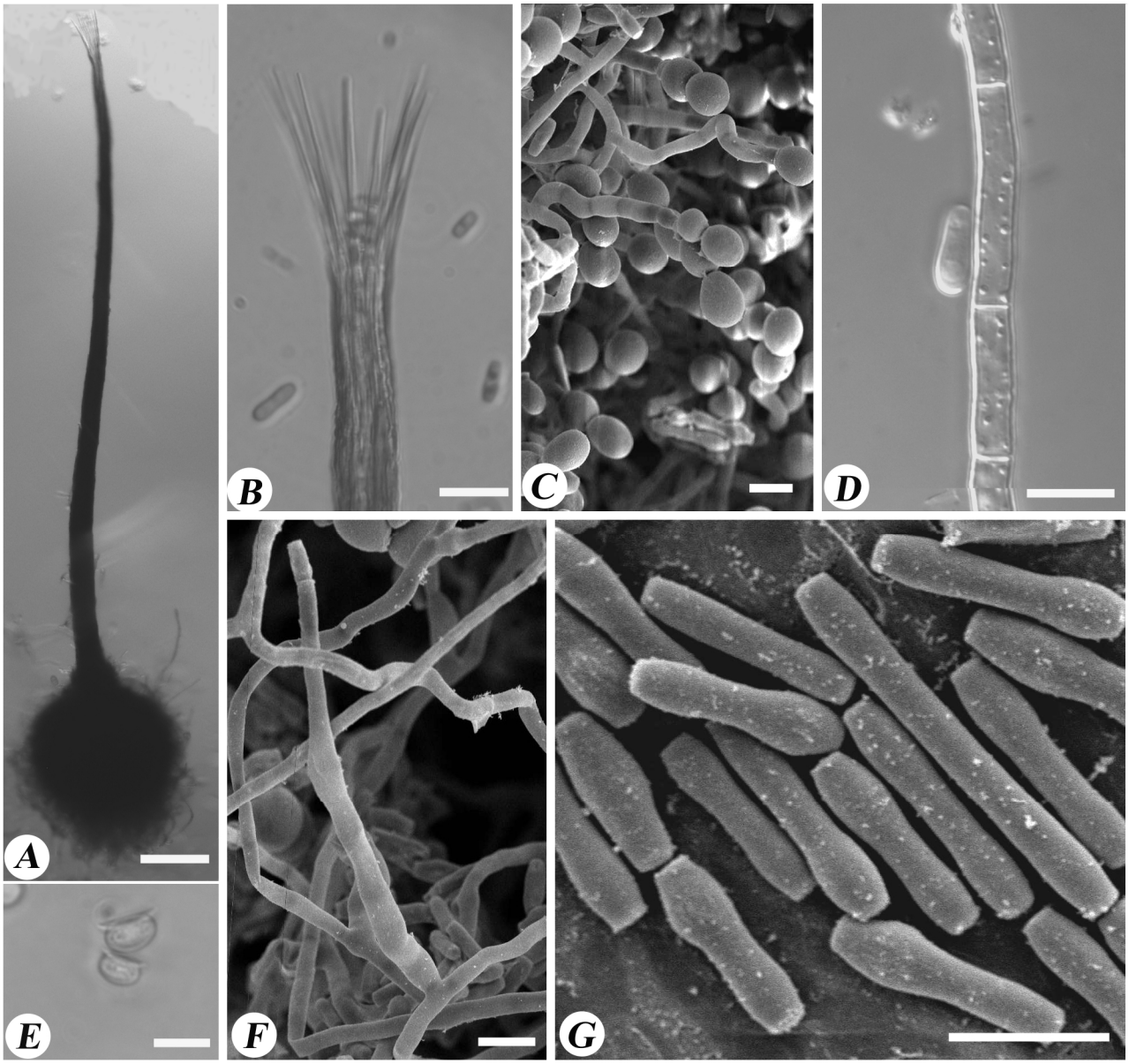

Fig. 6 Morphological characteristics of *Ceratocystis corymbiicola sp. nov*. A) Globose ascomatal base (scale bar = 100 µm), B) Divergent ostiolar hyphae (scale bar = 20 µm), C) Ovoid chlamydospores (scale bar = 10 µm), D) Septate hyphae (scale bar = 10 µm), E), Hat-shaped ascospores in side view (scale bar = 10 µm), F) Phialidic conidiogenous cell with emerging bacilliform conidia (scale bar = 10 µm), G) Bacilliform shaped conidia with obtuse end and bacilliform shaped conidia with round bases (scale bar = 10 µm).

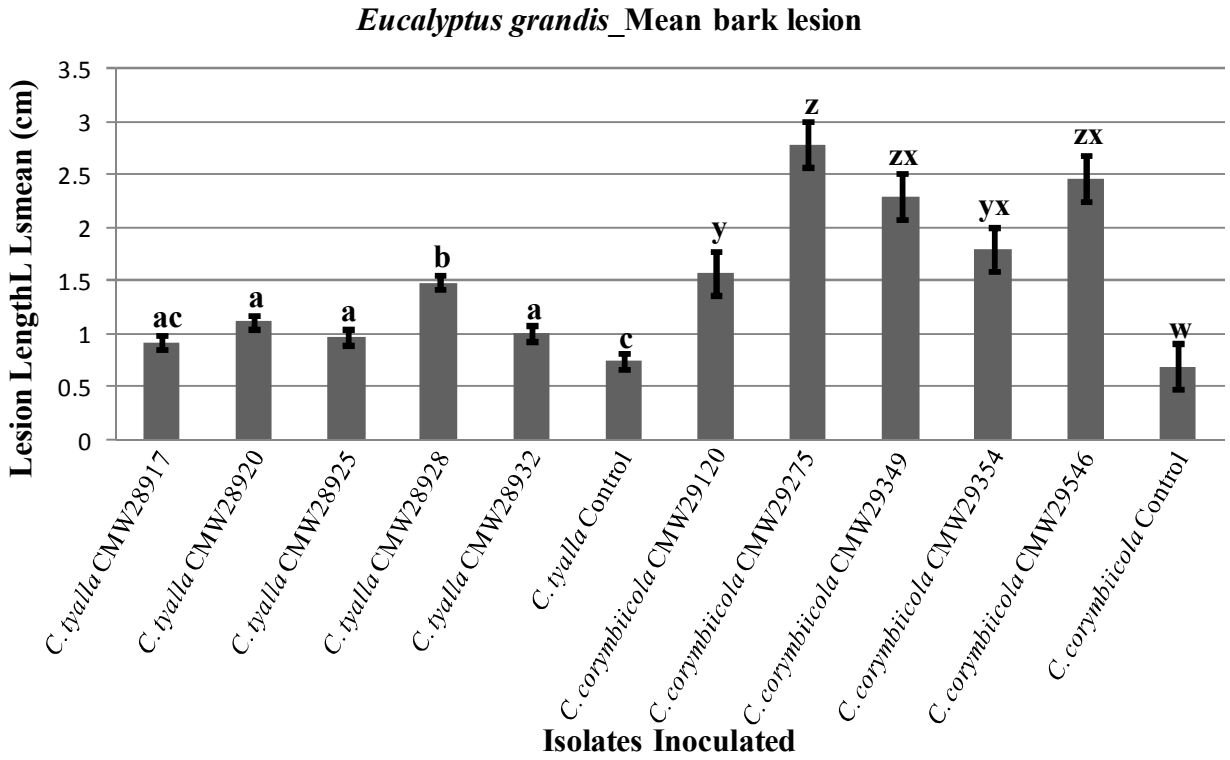

Fig. 7 Vertical bar chart showing results of inoculation trial (bark lesion) with *C. tyalla* isolates (CMW28917, 28920, 28925, 28928, 28932) on *E. grandis* trees. Lsmean = 1.05, R = 0.54, CV = 21.2, P<0.0001, Significance level = 0.05. Average lesion lengths (0.93-1.49) cm; and with *C. corymbiicola* isolates (CMW29120, 29275, 29349, 29354, 29546) on *E. grandis* trees. Lsmean = 1.94, R = 0.54, CV = 34.45, P<0.0001, Significance level = 0.05. Average lesion lengths (1.58-2.79) cm. Lsmeans with similar letters are not statistically significant while those with different letters are statistically significant

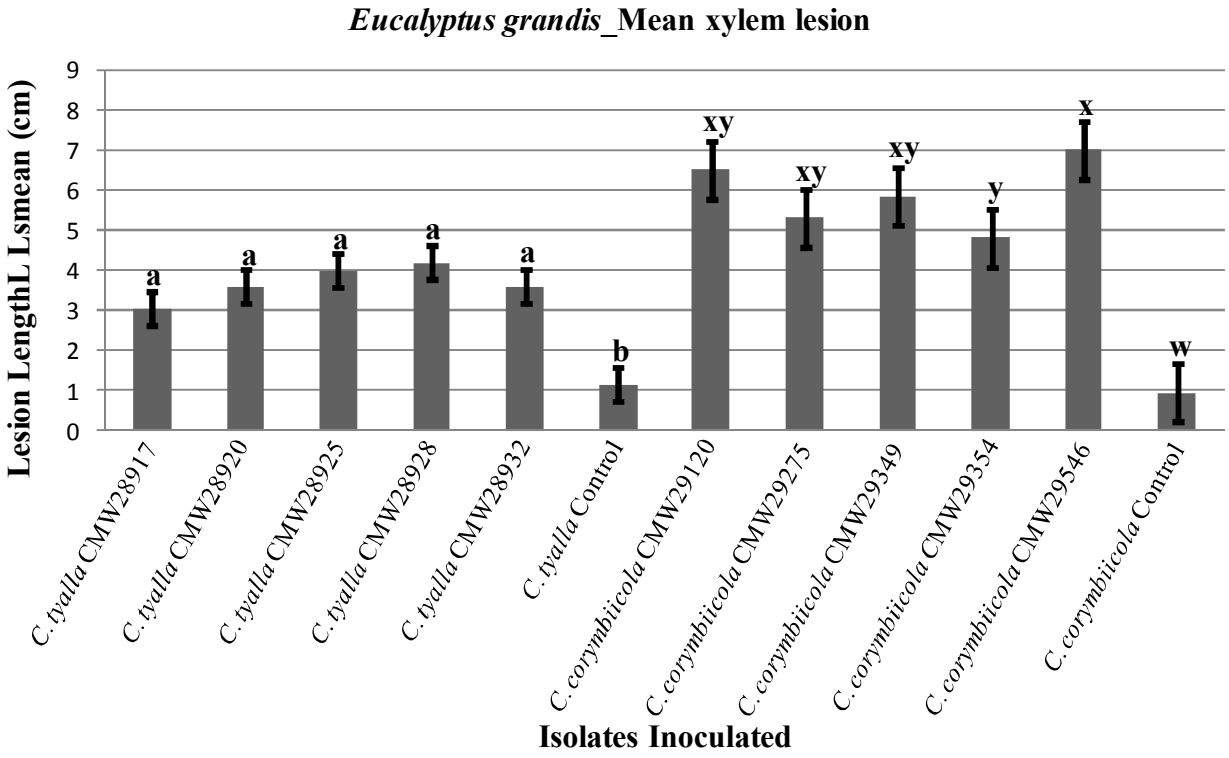

Fig. 8 Vertical bar chart showing results of inoculation trial (xylem lesion) with *C. tyalla* (CMW28917, 28920, 28925, 28928, 28932) on *E. grandis* trees. Lsmean = 3.27, R = 0.39, CV = 40.97, P<0.0001, Significance level = 0.05. Average lesion lengths (3.05-4.2) cm; and with *C. corymbiicola* (CMW29120, 29275, 29349, 29354, 29546) on *E. grandis* trees. Lsmean = 5.06, R = 0.46, CV = 44.76, P<0.0001, Significance level = 0.05. Average lesion lengths (4.8-7) cm. Lsmeans with similar letters are not statistically significant while those with different letters are statistically significant.