



CHAPTER 3

Calonectria species associated with cutting rot of *Eucalyptus*

ABSTRACT

Decline in the productivity of *Eucalyptus* hybrid cutting production in the GuangDong Province of China is linked to cutting rot associated with several *Calonectria* spp. The aim of this study was to identify these fungi using morphological and DNA sequence comparisons. Two previously undescribed *Calonectria* spp., *Ca. pseudoreteaudii* sp. nov. and *Ca. cerciana* sp. nov. were identified as well as *Ca. pauciramosa*. *Calonectria pseudoreteaudii* resides in the *Ca. reteaudii* complex and *Ca. cerciana* is closely related to *Ca. morgani*. Connected to the discovery of *Ca. pseudoreteaudii*, species in the *Ca. reteaudii* complex were re-considered and the group is shown to accommodate two cryptic species. These were collected in Australia and are described as *Ca. queenslandica* sp. nov. and *Ca. terrae-reginae* sp. nov.

Taxonomic novelties: *Calonectria cerciana* L. Lombard, M.J. Wingf. & Crous sp. nov., *Calonectria pseudoreteaudii* L. Lombard, M.J. Wingf. & Crous sp. nov., *Calonectria queenslandica* L. Lombard, M.J. Wingf. & Crous sp. nov., *Calonectria terrae-reginae* L. Lombard, M.J. Wingf. & Crous sp. nov.

INTRODUCTION

Species of *Calonectria* (*Ca.*), and their *Cylindrocladium* (*Cy.*) anamorphs, are important plant pathogens worldwide (Crous 2002). Past taxonomic studies on these pathogens have focused on morphology and sexual compatibility to delimit new species (Peerally 1991, Crous & Wingfield 1994). More recently, DNA sequence comparisons have resulted in the recognition of several species complexes in *Calonectria* (Schoch *et al.* 1999, Crous *et al.* 2004, 2006a).

One of the newly recognised groups in *Calonectria* is the *Ca. reteaudii* complex (Crous & Kang 2001, Kang *et al.* 2001). The complex encompasses several *Calonectria* spp. with *Cylindrocladium* anamorphs morphologically similar to the *Ca. reteaudii* anamorph state, having clavate vesicles with multiseptate macroconidia. These include *Cy. angustatum*, *Cy. hurae*, *Ca. leguminum* and *Ca. rumohrae* (Crous 2002).

The *Ca. morganii* species complex (Crous *et al.* 1993, Schoch *et al.* 2001) includes *Ca. morganii*, *Ca. insularis* and *Cy. hawksworthii* (Schoch *et al.* 1999, 2001, Crous 2002). Species in this complex are characterised by ellipsoidal to obpyriform to clavate vesicles, 1-septate conidia and orange to red perithecia producing 1-septate ascospores (Peerally 1991, Schoch *et al.* 1999, Crous 2002).

Species in both the *Ca. reteaudii* and the *Ca. morganii* complexes are responsible for a wide variety of disease symptoms on several plant hosts in sub-tropical and tropical regions of the world (Bolland *et al.* 1985, Peerally 1991, Sharma & Mohanan 1991, Booth *et al.* 2000, Crous 2002, Rodas *et al.* 2005). Disease symptoms include leaf blight (Sharma & Mohanan 1991, Booth *et al.* 2000, Rodas *et al.* 2005) and cutting rot (Sharma & Mohanan 1982, Sharma *et al.* 1984, Schoch *et al.* 1999, Crous 2002). Of these, leaf blight is most devastating in the tropical regions of South-East Asia and South America and is particularly serious on *Eucalyptus* spp. (Booth *et al.* 2000, Crous & Kang 2001, Crous 2002, Rodas *et al.* 2005).

Decline in *Eucalyptus* hybrid cutting production due to cutting rot has recently been observed in a commercial forest nursery in the GuangDong Province of China. Initial investigations indicated that the causal agents were unknown *Calonectria* spp. that represented species in the *Ca. reteaudii* and *Ca. morganii* complexes (Zhou *et al.* 2008). The aim of this study was to identify these *Calonectria* spp. using morphological characteristics and phylogenetic

inference. In addition, the taxonomic status and circumscription of species in the *Ca. reteaudii* species complex were re-considered.

MATERIALS AND METHODS

Isolates

Hybrid clonal *Eucalyptus* cuttings showing symptoms of cutting rot were collected from the commercial forestry nursery of the China Eucalypt Research Centre (CERC) in GuangDong Province, China. Diseased cuttings were placed in moist chambers and incubated for 48 h at room temperature to induce sporulation. Direct isolations were made onto malt extract agar (2 % w/v; MEA; Biolab, Midrand, South Africa) and cultures were incubated for 7 d at 25 °C under continuous near-ultraviolet light. For each isolate, single conidial cultures were prepared on MEA and representative strains are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and CERC, ZhanJiang Province, China. Isolates of *Ca. reteaudii* were obtained from the culture collection of CBS, including representative isolates used in the study of Kang *et al.* (2001).

DNA sequence comparisons

Calonectria isolates were grown on MEA for 7 d. Mycelium was then scraped from the surfaces of Petri dishes, freeze-dried, and ground to a powder in liquid nitrogen, using a mortar and pestle. DNA was extracted from the powdered mycelium as described by Lombard *et al.* (2008). Three loci were amplified and sequenced. These included a fragment of the β -tubulin (BT) gene region using primers T1 (O'Donnell & Cigelnik 1997) and CYLTUB1R (Crous *et al.* 2004), a fragment of the histone H3 (HIS3) gene region using primers CYLH3F and CYLH3R (Crous *et al.* 2004) and a fragment of the translation elongation factor-1 α (TEF-1 α) gene region using primers EF1-728F (Carbone & Kohn 1999) and EF2 (O'Donnell *et al.* 1998). The PCR reaction mixture used to amplify the different loci consisted of 2.5 units FastStart *Taq* polymerase (Roche Applied Science, USA), 10 \times PCR buffer, 1–1.5 mM MgCl₂, 0.25 mM of each dNTP, 0.5 μ M of each primer and approximately 30 ng of fungal genomic DNA, made up to a total reaction volume of 25 μ L with sterile distilled water.

Amplified fragments were purified using High Pure PCR Product Purification Kit (Roche, USA) and sequenced in both directions. For this purpose, the BigDye terminator sequencing kit v. 3.1 (Applied Biosystems, USA) and an ABI PRISMTM 3100 DNA sequencer (Applied Biosystems) were used. All PCRs and sequencing reactions were performed on an Eppendorf Mastercycler Personal PCR (Eppendorf AG, Germany) with cycling conditions as described in Crous *et al.* (2004, 2006a) for each locus.

Generated sequences were added to other sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov>) and these were assembled and aligned using Sequence Navigator v. 1.0.1 (Applied Biosystems) and MAFFT v. 5.11 (Katoh *et al.* 2005), respectively. The aligned sequences were then manually corrected where needed.

To determine whether the sequence data sets for the separate loci are congruent, tree topologies of 70 % reciprocal Neighbour-Joining bootstrap with Maximum Likelihood distances were compared visually to identify conflicts between partitions (Mason-Gamer & Kellogg 1996, Gueidan *et al.* 2007). Molecular evolution models for the separate partitions were determined in Modeltest v. 3.7 (Posada & Crandall 1998) and bootstrap analyses were run for 10k replicates.

PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b 10, Swofford 2002) was used to analyse the DNA sequence datasets. Phylogenetic relationships were estimated by heuristic searches with 1 000 random addition sequences and tree bisection-reconnection was used, with the branch swapping option set on 'best trees' only.

All characters were weighted equally and alignment gaps were treated as missing data. Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC). Bootstrap analysis (Hillis & Bull 1993) was based on 1 000 replications. All sequences for the isolates studied were analysed using the Basic Local Alignment Search Tool for Nucleotide sequences (BLASTN, Altschul *et al.* 1990). The phylogenetic analysis was done on two separate sequence data sets.

Datasets were separated based on morphological characteristics to allow combinations of gene regions. The first dataset included 40 partial gene sequences per gene, representing 13 *Calonectria* spp. that form part of the *Ca. reteaudii* species complex and other morphologically similar species (Table 1). These included isolates used by Kang *et al.*

(2001), and are *Calonectria* species with anamorph states producing large, multiseptate macroconidia and stipe extensions terminating in clavate vesicles. The second data set consisted of 20 partial gene sequences per gene, representing 12 *Calonectria* spp. of the *Ca. morganii* species complex, and other morphologically similar species. This group of *Calonectria* species is characterised by smaller, 1–3-septate macroconidia, and stipe extensions terminating in ellipsoidal to obpyriform vesicles. *Ca. colombiensis* (CBS 112221) and *Cy. chinense* (CBS 112744) were used as outgroup taxa in both analyses (Lombard *et al.* 2009). All sequences were deposited in GenBank and the alignments in TreeBASE (<http://www.treebase.org>).

A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v. 3.1.1 (Ronquist & Huelsenbeck 2003) for the combined sequence datasets. Models of nucleotide substitution for each gene were determined using MrModeltest v. 2.3 (Nylander 2004) and included for each gene partition. Three analyses of four MCMC chains were run from random trees for one million generations and sampled every 100 generations. All runs converged on the same likelihood score and tree topology and therefore the first 7600 trees for the *Ca. reteaudii* complex and 2000 trees for the *Ca. morganii* complex were discarded as the burn-in phase of each analysis and posterior probabilities determined from the remaining trees.

Sexual compatibility

A total of 29 single conidial *Ca. reteaudii*-like isolates (Table 1), originating from various geographical regions were crossed in all possible combinations including mating tester isolates CBS 112144 (+) and CBS 112147 (-) (Kang *et al.* 2001). Crosses were made as described in Schoch *et al.* (1999) on carnation leaf agar (CLA; Fisher *et al.* 1982, Crous *et al.* 1993). Control inoculations consisted of isolates crossed with themselves to determine whether they had a heterothallic or homothallic mating system. The plates were stacked in plastic containers and incubated at 22 °C for 6 wk. Crosses were regarded as successful when isolate combinations produced perithecia extruding viable ascospores.

Taxonomy

For morphological identification of *Calonectria* isolates, single conidial cultures were prepared on MEA and synthetic nutrient-poor agar (SNA; Nirenburg 1981, Lombard *et al.* 2009). Inoculated plates were incubated at room temperature and examined after 7 d. Gross

morphological characteristics of the anamorph state were determined by mounting fungal structures in lactic acid and 30 measurements at $\times 1\ 000$ magnification were made for each isolate. The 95 % confidence levels were determined and extremes of conidial measurements are given in parentheses. For other structures, only extremes are presented. Optimal growth conditions for cultures were determined in the dark on MEA for each isolate, at temperatures ranging from 5–35 °C at 5 °C intervals. Colony colours were determined after 7 d on MEA at 25 °C in the dark, using the colour charts of Rayner (1970).

RESULTS

DNA phylogeny

Amplicons of approximately 500 bases (BT and TEF-1 α) and 450 bases (HIS3) were obtained for all isolates. The pooled sequence datasets for all three loci showed conflict in tree topology for the 70 % reciprocal bootstrap trees, with *Ca. spathulata* (CBS 112689) and *Ca. multiseptata* (CBS 112682) grouping within different clusters for all three gene regions considered (results not shown). This conflict was resolved by separating the sequence datasets into two datasets representing morphological and phylogenetically closely related species to *Ca. reteaudii* and *Ca. morgani*, and sequence datasets for BT, HIS3 and TEF-1 α were combined for the two separate datasets. Sequence alignments have been deposited in TreeBASE as SN4542.

The combined sequence dataset for isolates representing *Ca. reteaudii* and other closely related species consisted of 1540 characters, of which 926 were constant, 178 were parsimony uninformative and 436 were parsimony informative. Parsimony analysis of the alignment yielded six most parsimonious trees (TL = 1316 steps; CI = 0.703; RI = 0.782; RC = 0.549), one of which is presented in Fig. 1. For Bayesian analyses, a HKY+G model was selected for BT, GTR+I+G for HIS3 and GTR+G for TEF-1 α , and incorporated in the analyses. The consensus tree obtained from Bayesian analyses confirmed the tree topology obtained with parsimony as well as bootstrap support (Fig. 1).

The isolates obtained from the *Eucalyptus* cuttings grouped in the *Ca. reteaudii* cluster, which formed a monophyletic group with a bootstrap value (BP) of 100 and a Bayesian posterior probability (PP) value of 1.00 (Fig. 1). This cluster segregated into two separate clades. The first of these, with a BP of 100 and PP value of 1.00 included the isolates from the *Eucalyptus* cuttings in a clade (BP = 100, PP = 1.00) separate from *Ca. reteaudii*,

possibly representing a distinct species. The second clade (BP = 100, PP = 0.99) was comprised of two sub-clades (BP = 79, PP = 1.00 and BP = 62, PP = 0.86, respectively) representing isolates from the study of Kang *et al.* (2001), and also suggested the existence of distinct species.

For the isolates that are closely related to *Ca. morganii*, the combined dataset consisted of 1518 characters. Of these characters, 969 were constant, 161 were parsimony uninformative and 388 were parsimony informative. Parsimony analysis of the alignment yielded three most parsimonious trees (TL = 1065 steps; CI = 0.736; RI = 0.783; RC = 0.577), one of which is represented in Fig. 2. For Bayesian analyses, a HKY+G model was selected for BT, GTR+I+G for HIS3, and GTR+G for TEF-1 α , and incorporated in the analyses. The consensus tree obtained from Bayesian analyses confirmed the tree topology obtained with parsimony as well as bootstrap support (Fig. 1).

In the tree (Fig. 2), isolates from the *Eucalyptus* cuttings grouped in the cluster representing species in the *Ca. morganii* complex (BP = 89, PP = 0.95), and some in the cluster representing *Ca. pauciramosa* (BP = 100, PP = 1.00). However, those isolates grouping in the *Ca. morganii* cluster formed a separate clade (BP = 100, PP = 1.00), suggesting that they might represent a distinct species.

Sexual compatibility

Protoperithecia formed within 3 wk and mating tests produced viable perithecia within 6 wk on CLA. Mating isolates CBS 112146, CBS 112151, CBS 112155 and CBS 112634 with isolates of *Ca. reteaudii* (represented in the current phylogenetic study), failed to produce perithecia in any combination tested. Crossing isolates CMW 25284, CMW 25285, CMW 25292 and CMW 25310 with isolates of *Ca. reteaudii* also failed to produce perithecia. However, crossed isolates of *Ca. reteaudii* produced perithecia with viable ascospores as reported by Kang *et al.* (2001). Isolate CBS 582.50 did not cross with any of the other *Ca. reteaudii* isolates, possibly due to loss of fertility.

Taxonomy

Morphological observations and DNA sequence comparisons (Fig. 2) showed that isolates CMW 25311 and CMW 25283 clearly represent the anamorph state of *Ca. pauciramosa*. Isolates CMW 25309, CMW 25290 and CMW 25288 represent an undescribed species closely related to other species in the *Ca. morganii* species complex, and it is consequently

described as new. Isolates CMW 25310, CMW 25292, CMW25285 and CMW 25284 obtained from the *Eucalyptus* cuttings are morphologically very similar to the anamorph state of *Ca. reteaudii*. However, some morphological differences were found and this fungus is also treated as a new species. Based on DNA sequence analysis, mating strategies and morphological observations, isolates CBS 112146 (= CPC 3213) and CBS 112155 (= CPC 3210), which were previously regarded as *Ca. reteaudii* (Kang *et al.* 2001), are shown to represent a distinct species. A similar situation was found for isolates CBS 112151 (= CPC 3202) and CBS 112634 (= CPC 4233), and they are also treated as a new species.

Calonectria cerciana L. Lombard, M.J. Wingf. & Crous, **sp. nov.** MycoBank 513263. Fig. 3

Etymology: Name refers to the China Eucalypt Research Centre (CERC), a research institution that is pioneering the study of *Eucalyptus* diseases in China.

Stipa extensiones septatae, rectae vel flexuosae, 148–222 µm longae, ad septum apicale 5–6 µm latae, vesiculo fusiforme vel obpyriforme 8–13 µm diametro terminantes. *Macroconidia* cylindrica, in extremitatibus ambabus rotundata, recta (37–)41–46(–49) × 5–6 µm, 1-septata.

Teleomorph unknown. *Conidiophores* with a stipe bearing a suit of penicillate, fertile branches, a stipe extension, and a terminal vesicle; stipe septate, hyaline, smooth, 48–90 × 6–10 µm; stipe extensions septate, straight to flexuous, 148–222 µm long, 5–6 µm wide at the apical septum, terminating in fusiform to obpyriform vesicles, 8–13 µm diam. *Conidiogenous apparatus* 70–98 µm long, and 62–113 µm wide; primary branches aseptate or 1-septate, 21–31 × 5–7 µm; secondary branches aseptate, 15–22 × 4–5 µm; tertiary and additional branches (–4), aseptate, 10–20 × 4–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 9–12 × 3 µm; apex with minute periclinal thickening and inconspicuous collarete. *Macroconidia* cylindrical, rounded at both ends, straight, (37–)41–46(–49) × 5–6 µm (av. = 44 × 5 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Mega-* and *microconidia* not seen.

Specimens examined: **China**, GuangDong Province, CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, M.J. Wingfield & X.D. Zhou, Herb. PREM 60241, **holotype** of *Calonectria cerciana*, culture ex-type CMW 25309 = CBS 123693; GuangDong Province, CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, M.J. Wingfield & X.D. Zhou (Herb. PREM 60242), culture CMW 25290 = CBS 123695; CERC

nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, M.J. Wingfield & X.D. Zhou, culture CMW 25288.

Culture characteristics: Colonies fast growing with optimal growth temperature at 25 °C (growth at 15–30 °C) on MEA, reverse sepia-brown (13i) after 7 d; abundant white aerial mycelium and sporulation; chlamydospores abundant throughout the medium, forming microsclerotia.

Substrate: *Eucalyptus urophylla* × *grandis* hybrid cuttings

Distribution: China

Notes: *Calonectria cerciana* can be distinguished from *Cy. hawksworthii* (conidia av. 56 × 4 μm), *Ca. morganii* (conidia av. 45 × 4 μm) and *Ca. insularis* (conidia av. 45 × 4 μm) based on its fusoid to ellipsoidal vesicles. Macroconidia of *Ca. cerciana* are also slightly smaller (av. 44 × 5 μm) than those of the above-mentioned species.

Calonectria pseudoreteauidii L. Lombard, M.J. Wingf. & Crous, **sp. nov.** MycoBank 513264. Fig. 4

Etymology: Name reflects the fact that the species resembles the anamorph state of *Ca. reteauidii*.

Stipa extensiones septatae, rectae vel flexuosae, 193–313 μm longae, ad septum apicale 5–6 μm latae, vesiculo anguste clavato, 3–5 μm diametro terminantes. *Macroconidia* cylindrica, apice rotundata, basi complanata, recta (88–96–112(–119) × 7–9(–10) μm, 5–8-septata. *Microconidia* cylindrica, apice rotundata, basi complanata, (30–)34–54(–68) × 3–5(–6) μm, 1–3-septata, cum muco in glomerulis.

Teleomorph unknown. *Macroconidiophores* with a stipe bearing a suite of penicillate fertile branches, a stipe extension, and a terminal vesicle; stipe septate, hyaline, smooth, 43–111 × 5–9 μm; stipe extensions septate, straight to flexuous, 193–313 μm long, 5–6 μm wide at the apical septum, terminating in a narrowly clavate vesicle, 3–5 μm diam. *Conidiogenous apparatus* 45–103 μm long and 26–82 μm wide; primary branches aseptate or 1-septate, 29–42 × 5–6 μm; secondary branches aseptate, 20–36 × 3–6 μm; tertiary branches aseptate, 15–24 × 4–5 μm, each terminal branch producing 1–3 phialides; phialides cylindrical to allantoid, obpyriform when carried singly, hyaline, aseptate, 16–25 × 3–5 μm; apex with minute periclinal thickening and inconspicuous collarette. *Macroconidia* cylindrical, rounded at the apex, flattened at the base, straight, (88–)96–112(–119) × 7–9(–10) μm (av. = 104 × 8

µm), 5–8-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Microconidiophores* simple with some lateral branching, comprising a stipe and a penicillate or subverticillate arrangement of fertile branches. *Stipe* septate, hyaline, thin-walled, 27–70 × 2–3 µm; primary branches aseptate, subcylindrical, straight to curved, 14–19 × 3–4 µm, terminating in 1–3 phialides that are straight to slightly curved, 11–18 × 3–4 µm; apex with minute periclinal thickening and collarete. *Microconidia* cylindrical, straight, rounded at the apex, flattened at the base, (30–)34–54(–68) × 3–5(–6) µm (av. 44 × 4 µm), 1–3-septate, held in fascicles by colourless slime. *Megaconidia* not seen.

Specimens examined: **China**, GuangDong Province, CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, M.J. Wingfield & X.D. Zhou, Herb. PREM 60290, **holotype** of *Calonectria pseudoreteaudii*, culture ex-type CMW 25310 = CBS 123694; GuangDong Province, CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, M.J. Wingfield & X.D. Zhou (Herb. PREM 60291), culture CMW 25292 = CBS 123696; CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, M.J. Wingfield & X.D. Zhou, culture CMW 25284; CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, M.J. Wingfield & X.D. Zhou, culture CMW 25285.

Culture characteristics: Colonies fast growing with optimal growth temperature at 25 °C (growth at 15–30 °C) on MEA, reverse amber (13k) to sepia-brown (13i) after 7 d; abundant white aerial mycelium with moderate sporulation; chlamydospores abundant throughout the medium, forming microsclerotia.

Substrate: *Eucalyptus urophylla* × *grandis* hybrid cuttings

Distribution: China

Notes: The anamorph state of *Ca. pseudoreteaudii* can be distinguished from that of *Ca. reteaudii* based on its larger macroconidia (av. = 104 × 8 µm vs. 84 × 6.5 µm), as well as larger microconidia (av. = 44 × 4 µm vs. 30 × 3 µm). The microconidiophores of *Ca. pseudoreteaudii* do not produce stipe extensions, a feature which is common in *Ca. reteaudii*.

Calonectria queenslandica L. Lombard, M.J. Wingf. & Crous, **sp. nov.** MycoBank 513265.
Fig. 5

Etymology: Name refers to Queensland, Australia where the fungus was collected.

Stipa extensiones septatae, rectae vel flexuosae, 105–156 μm longae, ad septum apicale 4–5 μm latae, vesiculo anguste clavato 3–4 μm diametro terminantes. *Macroconidia* cylindrica, in extremitatibus ambabus rotundata, recta (61–)65–73(–78) \times (4–)5–6(–7) μm , 4–6-septata.

Teleomorph unknown. *Conidiophores* consisting of a stipe bearing a suit of penicillate fertile branches, a stipe extension, and a terminal vesicle; stipe septate, hyaline, smooth, 38–58 \times 6–7 μm ; stipe extensions septate, straight to flexuous, 105–156 μm long, 4–5 μm wide at the apical septum, terminating in narrowly clavate vesicle, 3–4 μm diam. *Conidiogenous apparatus* 39–64 μm long, and 27–68 μm wide; primary branches aseptate or 1-septate, 14–26 \times 4–6 μm ; secondary branches aseptate, 11–22 \times 3–5 μm ; tertiary branches aseptate, 13–17 \times 3–5 μm , each terminal branch producing 1–3 phialides; cylindrical to allantoid, obpyriform when carried singly, hyaline, aseptate, 10–16 \times 3–5 μm ; apex with minute periclinal thickening and inconspicuous collarete. *Macroconidia* cylindrical, rounded at both ends, straight, (61–)65–73(–78) \times (4–)5–6(–7) μm (av. = 69 \times 6 μm), 4–6-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Mega-* and *microconidia* not seen.

Specimens examined: **Australia**, Queensland, Lannercost, on leaves of *E. urophylla*, 15 Apr. 1991, B. Brown, Herb. PREM 60243, **holotype** of *Calonectria queenslandica*, culture ex-type CMW 30604 = CBS 112146 = CPC 3213 = DFRI00147; Queensland, Lannercost, on leaves of *E. pellita*, 10 Mar. 1999, P.Q. Thu & K.M. Old (Herb. PREM 60244), culture CMW 30603 = CBS 112155 = CPC 3210 = DFRI00172.

Culture characteristics: Colonies fast growing, with optimal growth temperature at 25 °C (growth at 15–30 °C) on MEA; reverse sepia-brown (13i) after 7 d; abundant white aerial mycelium and sporulation; chlamydospores abundant throughout the medium, forming microsclerotia.

Substrate: *Eucalyptus urophylla*, *E. pellita*.

Distribution: Australia

Notes: *Calonectria queenslandica* can be distinguished from *Ca. reteaudii* and *Ca. pseudoreteaudii* based on its smaller macroconidia (av. = 69 \times 6 μm) and shorter stipe extensions of the anamorph state. No microconidiophores were observed in *Ca.*

queenslandica, although *Ca. reteaudii* and *Ca. pseudoreteaudii* readily produce these structures on SNA.

Calonectria terrae-reginae L. Lombard, M.J. Wingf. & Crous, *sp. nov.* MycoBank 513266.

Fig. 6

Etymology: Name refers to Queensland, Australia, from where this fungus was isolated.

Stipa extensiones septatae, rectae vel flexuosae, 127–235 μm longae, ad septum apicale 4–6 μm latae, vesiculo anguste clavato 3–5 μm diametro terminantes. *Macroconidia* cylindrica, in extremitatibus ambabus rotundata, recta, 60–83(–87) \times (4–)5–7(–8) μm , 4–6-septata.

Teleomorph unknown. *Conidiophores* consisting of a stipe bearing a suite of penicillate fertile branches, a stipe extension, and a terminal vesicle; stipe septate, hyaline, smooth, 58–86 \times 4–8 μm ; stipe extensions septate, straight to flexuous, 127–235 μm long, 4–6 μm wide at the apical septum, terminating in narrowly clavate vesicle, 3–5 μm diam. *Conidiogenous apparatus* 35–54 μm long, and 33–48 μm wide; primary branches aseptate, 16–25 \times 4–6 μm ; secondary branches aseptate, 13–18 \times 3–6 μm ; tertiary branches aseptate, 10–14 \times 3–5 μm , each terminal branch producing 1–3 phialides; cylindrical to allantoid, obpyriform when carried singly, hyaline, aseptate, 10–17 \times 2–4 μm ; apex with minute periclinal thickening and inconspicuous collarette. *Macroconidia* cylindrical, rounded at both ends, straight, 60–83(–87) \times (4–)5–7(–8) μm (av. = 76 \times 6) μm , 4–6-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Megaconidia* and *microconidia* not seen.

Specimens examined: **Australia**, Queensland, Cardwell, Meunga, on leaves of *E. urophylla*, 11 Apr. 1997, C. Hanwood, Herb. PREM 60239, **holotype** of *Calonectria terrae-reginae*, culture ex-type CMW 30601 = CBS 112151 = CPC 3202 = DFRI00150; Victoria, on *Xanthorrhoea australis*, T. Baigent (Herb. PREM 60240), culture CMW 30602 = CBS 112634 = CPC 4233 = Lynfield 417.

Culture characteristics: Colonies fast growing with optimal growth temperature at 25 °C (growth at 15–30 °C) on MEA; reverse sepia-brown (13i) after 7 d; abundant white aerial mycelium and sporulation; chlamydospores abundant throughout the media, forming microsclerotia.

Substrate: *Eucalyptus urophylla*, *Xanthorrhoea australis*

Distribution: Australia

Notes: *Calonectria terrae-reginae* is distinct from *Ca. queenslandica* in having larger macroconidia (av.= $76 \times 6 \mu\text{m}$), although they are smaller than those of *Ca. reteaudii* and *Ca. pseudoreteaudii*.

DISCUSSION

This study emerged from the collection of isolates of *Calonectria* spp. from infected *Eucalyptus* cuttings in the GuangDong Province of China. The isolates were shown to represent three species including *Ca. pauciramosa*, and two new species that have been described as *Ca. cerciana* and *Ca. pseudoreteaudii*. The former species is related to taxa in the *Ca. morganii* complex, while the latter species resides in the *Ca. reteaudii* complex.

Taxonomic placement of *Ca. pseudoreteaudii* required a re-evaluation of the *Ca. reteaudii* complex. It was consequently found that the group has been poorly defined, and that it encompasses a number of cryptic species. This resulted in the description of *Ca. pseudoreteaudii*, *Ca. queenslandica* and *Ca. terrae-reginae* as three new sibling species in the *Ca. reteaudii* complex based on phylogenetic inference and morphological comparisons with the ex-type culture of *Ca. reteaudii* (CBS 112144). Species in the *Ca. reteaudii* complex are important pathogens of *Eucalyptus* spp. causing *Cylindrocladium* leaf blight and cutting rot in Australia, South-East Asia and South America (Pikethley 1976, Bolland 1985, Sharma & Mohanan 1991, Sharma & Mohanan 1992, Booth *et al.* 2000, Crous & Kang 2001, Crous 2002, Rodas *et al.* 2005) and their refined taxonomy presented here will contribute to efforts to manage this disease.

Discovery of *Ca. queenslandica* and *Ca. terrae-reginae* was serendipitous as isolates used in the phylogenetic component of the study were largely the same as those used in a previous study by Kang *et al.* (2001). Although the latter study focused on the taxonomic position of *Cy. quinqueseptatum* (= *Cy. reteaudii*) and *Ca. quinqueseptata* (= *Ca. leguminum*), it employed a single gene region, and could thus not adequately define the variation in *Ca. reteaudii*, which was later recognised in multi-gene analyses (Crous *et al.* 2006a). Mating tests undertaken by Kang *et al.* (2001) indicated that 15 of the 20 *Ca. reteaudii* isolates used were capable of producing viable progeny. Mating tests conducted in the present study were unsuccessful in crossing strains of *Ca. queenslandica*, *Ca. terrae-reginae*, *Ca. reteaudii* and

Ca. pseudoreteauidi, and only isolates of *Ca. reteaudii* could be induced to produce perithecia with viable ascospores.

Calonectria pauciramosa (anamorph: *Cy. pauciramosum*) is a well-known pathogen in *Eucalyptus* cutting nurseries (Schoch *et al.* 1999, Crous 2002). This pathogen resides in the *Ca. scoparia* species complex and is regarded as the dominant nursery pathogen of various host plants in countries such as Australia, Italy, South Africa and the U.S.A. (Koike *et al.* 1999, Polizzi & Crous 1999, Schoch *et al.* 1999, 2001, Koike & Crous 2001). The present study represents the first report of this pathogen in China, but pathogenicity tests and disease surveys will be required to determine its relevance in that country.

The description of *Ca. cerciana* from *Eucalyptus* cuttings adds a new species to the *Ca. morganii* species complex. *Calonectria cerciana* can be distinguished from the other species in the complex based on its smaller macroconidia and the formation of fusiform to ellipsoidal vesicles. Crous (2002) and Schoch *et al.* (1999) found that there was a low level of fertility among species in this complex, and Schoch *et al.* (2001) used BT to show that they were closely related.

It is unknown whether *Ca. cerciana* and *Ca. pseudoreteauidi* are pathogens of *Eucalyptus*. Other species in the *Ca. reteaudii* (Sharma & Mohanan 1982, Sharma & Mohanan 1991, Sharma & Mohanan 1992) and *Ca. morganii* (Mohanan & Sharma 1985, Crous 2002) species complexes are known to be *Eucalyptus* pathogens, and this is probably also true for *Ca. cerciana* and *Ca. pseudoreteauidi*. However, the pathogenicity of these two *Calonectria* spp. must be tested and these studies would logically also consider the susceptibility of different *Eucalyptus* clones and hybrids being deployed in plantations.

Although no teleomorph states for the four newly described *Calonectria* spp. could be induced in this study, they have all been placed in *Calonectria*, and not in the anamorph genus *Cylindrocladium* (Lombard *et al.* 2009). The decision to use the oldest generic name for a well-defined clade of fungi (*Calonectria*) is consistent with the approach taken previously by Lombard *et al.* (2009), and has also been followed in other groups of fungi such as *Botryosphaeriaceae* (Crous *et al.* 2006b, 2008, Phillips *et al.* 2008), *Mycosphaerellaceae* and *Teratosphaeriaceae* (Crous *et al.* 2009a, 2009b) to name a few. Although it might be considered a broad interpretation, it is allowed by the International Code of Botanical Nomenclature (Hawksworth 2005, McNeill *et al.* 2005). Based on these



regulations and the fact that the anamorph genus *Cylindrocladium* is linked to the single teleomorph genus *Calonectria* (Rossman *et al.* 1999), all new species have been accommodated in *Calonectria*.

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Table 1 Strains of *Calonectria* and *Cylindrocladium* used in the phylogenetic study.

Species	Isolate number ¹	GenBank accession no.			Host	Origin	Collector
		BT	HIS3	TEF-1 α			
<i>Cy. angustatum</i>	CBS 109169	DQ190593	DQ190695	FJ918552	<i>Tillandsia capitata</i>	U.S.A.	R.M. Leahy
	CBS 109065 ^T	AF207543	DQ190656	FJ918551	<i>T. capitata</i>	U.S.A.	R.M. Leahy
<i>Ca. cerciana</i> sp. nov. (= CBS 123693) ^T	CMW 25309	FJ918510	FJ918528	FJ918559	<i>Eucalyptus urophylla</i>	× China	M.J. Wingfield & X.D. Zhou
	CMW 25290 (= CBS 123695)	FJ918511	FJ918529	FJ918560	<i>E. urophylla grandis</i> cutting	× China	M.J. Wingfield & X.D. Zhou
	CMW 25288	GQ25288	GQ267243	GQ267288	<i>E. urophylla grandis</i> cutting	× China	M.J. Wingfield & X.D. Zhou
<i>Ca. colombiensis</i>	CBS112221	AY725620	AY725663	AY725712	Soil	Colombia	M.J. Wingfield
<i>Cy. chinense</i>	CBS112744	AY725618	AY725660	AY725709	Soil	China	E.C.Y. Liew
<i>Ca. brassicae</i>	CBS 111869 ^T	AF232857	DQ190720	FJ918567	<i>Argyrea</i> sp.	South East Asia	
	CBS 111478	DQ190611	DQ190719	FJ918567	Soil	Brazil	A.C. Alfenas
<i>Cy. hawksworthii</i>	CBS 111870 ^T	AF333407	DQ190649	FJ918558	<i>Nelumbo nucifera</i>	Mauritius	A. Peerally
<i>Cy. hurae</i>	CBS 114551	AF333408	DQ190728	FJ918548	<i>Rumhra adiantiformis</i>	Brazil	A.C. Alfenas



Table 1. (Continued)

Species	Isolate number ¹	GenBank accession no.			Host	Origin	Collector
		BT	HIS3	TEF-1 α			
<i>Ca. insularis</i>	CBS 114558	AF210861	FJ918526	FJ918556	Soil	Madagascar	P.W. Crous
	CBS 114559	AF210862	FJ918525	FJ918555	Soil	Madagascar	C.L. Schoch
<i>Ca. leguminum</i>	CBS 728.68 ^T	AF389837	DQ190654	FJ918547	<i>Annona squamosa</i>	Brazil	M.B. Figueiredo
<i>Cy. leucothoës</i>	CBS 109166 ^T	FJ918508	FJ918523	FJ918553	<i>Leucothoë axillaris</i>	USA	N.E. El-Gholl
<i>Cy. multiseptatum</i>	CBS 112682 ^T	DQ190573	DQ190659	FJ918535	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield
<i>Ca. pauciramosa</i>	CMW 5683	FJ918514	FJ918531	FJ918565	<i>Eucalyptus</i> sp.	Brazil	A.C. Alfenas
	CMW 30823	FJ918515	FJ918532	FJ918566	<i>E. grandis</i>	South Africa	P.W. Crous
	CMW 25311	FJ918516	FJ918533	FJ918569	<i>E. urophylla</i> × <i>grandis</i> cutting	China	M.J. Wingfield & X.D. Zhou
	CMW 25283	FJ918517	FJ918534	FJ918570	<i>E. urophylla</i> × <i>grandis</i> cutting	China	M.J. Wingfield & X.D. Zhou
<i>Ca. pseudoreteaudii</i> sp. nov.	CMW 25310 (= CBS 123694) ^T	FJ918504	FJ918519	FJ918541	<i>E. urophylla</i> × <i>grandis</i> cutting	China	M.J. Wingfield & X.D. Zhou



Table 1. (Continued)

Species	Isolate number ¹	GenBank accession no.			Host	Origin	Collector
		BT	HIS3	TEF-1 α			
<i>Ca. pseudoreteauidii</i> sp. nov.	CMW 25292 (= CBS 123696)	FJ918505	FJ918520	FJ918542	<i>E. urophylla</i> × <i>grandis</i> cutting	China	M.J. Wingfield & X.D. Zhou
	CMW 25284	GQ267205	GQ267244	GQ267289	<i>E. urophylla</i> × <i>grandis</i> cutting	China	M.J. Wingfield & X.D. Zhou
	CMW 25285	GQ267206	GQ267245	GQ267290	<i>E. urophylla</i> × <i>grandis</i> cutting	China	M.J. Wingfield & X.D. Zhou
<i>Ca. pseudospathiphylli</i>	CBS 109165	FJ918513	AF348241	FJ918562	Soil	Ecuador	M.J. Wingfield
<i>Ca. pteridis</i>	CBS 111793	DQ190578	DQ190679	FJ918563	<i>Arachnoides</i> <i>adiantiformis</i>	USA	
	CBS 111871	DQ190579	DQ190680	FJ918564	<i>Pinus</i> sp.	Spain	T.L. Krugner
<i>Ca. queenslandica</i> sp. nov.	CMW 30604 (= CBS 112146 = CPC 3213 = DRFI000147) ^T	AF389835	FJ918521	FJ918543	<i>E. urophylla</i>	Australia	B. Brown
	CMW 30603 (= CBS 112155 = CPC 3210 = DFRI00172)	AF389834	DQ190667	FJ918544	<i>E. pellita</i>	Australia	K.M. Old



Table 1. (Continued)

Species	Isolate number ¹	GenBank accession no.			Host	Origin	Collector
		BT	HIS3	TEF-1 α			
<i>Ca. reteaudii</i>	CBS 582.50	AF389836	DQ190673	FJ918540	<i>Hibiscus sabdariffa</i>	Indonesia	—
	CBS 112143	GQ240642	DQ190660	FJ918536	<i>E. camaldulensis</i>	Vietnam	M.J. Dudzinski
	CBS 112144 ^T	AF389833	DQ190661	FJ918537	<i>E. camaldulensis</i>	Vietnam	M.J. Dudzinski
	CBS 112147	AF389830	DQ190663	FJ918539	<i>E. camaldulensis</i>	Vietnam	M.J. Dudzinski
	CBS 112153	AF389831	FJ918518	FJ918538	<i>E. camaldulensis</i>	Vietnam	M.J. Dudzinski
	CBS 113582	GQ240643	GQ240659	GQ240675	<i>Eucalyptus</i> sp.	Thailand	—
	CBS 113583	GQ240644	GQ240660	GQ240676	<i>Eucalyptus</i> sp.	Madagascar	P.W. Crous
	CMW 18446	GQ240649	GQ240665	GQ240681	<i>E. urophylla</i>	Indonesia	M.J. Wingfield
	CMW 18448	GQ240650	GQ240666	GQ240682	<i>E. urophylla</i>	Indonesia	M.J. Wingfield
	CMW 18450	GQ240651	GQ240667	GQ240683	<i>E. grandis</i>	Thailand	M.J. Wingfield
	CMW 18458	GQ240654	GQ240670	GQ240686	<i>E. urophylla</i>	Indonesia	M.J. Wingfield
	CMW 18462	GQ240653	GQ240669	GQ240685	<i>E. urophylla</i>	Indonesia	M.J. Wingfield
	CMW 18463	GQ240646	GQ240662	GQ240678	<i>E. urophylla</i>	Indonesia	M.J. Wingfield
	CMW 20597	GQ240645	GQ240661	GQ240677	<i>E. grandis</i>	Thailand	M.J. Wingfield
	CMW 31177	GQ240657	GQ240673	GQ240689	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield
	CMW 31178	GQ240656	GQ240672	GQ240688	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield
	CMW 31179	GQ240655	GQ240671	GQ240687	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield



Table 1. (Continued)

Species	Isolate number ¹	GenBank accession no.			Host	Origin	Collector
		BT	HIS3	TEF-1 α			
<i>Ca. reteaudii</i>	CMW 31188	GQ240658	GQ240674	GQ240690	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield
	CMW 31189	GQ240647	GQ240663	GQ240679	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield
	CMW 31190	GQ240648	GQ240664	GQ240680	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield
	CMW 31191	GQ240652	GQ240668	GQ240684	<i>Eucalyptus</i> sp.	Thailand	M.J. Wingfield
<i>Ca. rumohrae</i>	CBS 111431 ^T	AF232871	DQ190675	FJ918549	<i>Rumohra adiantiformis</i>	Panama	J.W. Miller
	CBS 109062	AF232873	DQ190676	FJ918550	<i>Adiantum</i> sp.	The Netherlands	R. Pieters
<i>Ca. morganii</i>	CBS 110666	FJ918509	FJ918527	FJ918557	<i>Rosa</i> sp.	USA	N.E. Ell-Gholl
<i>Ca. spathiphylli</i>	CBS 116168 ^T	FJ918512	FJ918530	FJ918561	<i>Spathiphyllum</i> sp.	USA	C.L. Schoulties
<i>Ca. spathulata</i>	CBS 112689	AF308463	FJ918524	FJ918554	<i>E. viminalis</i>	Brazil	N.E. Ell-Gholl
	CMW 30601						
<i>Ca. terrae-reginae</i> sp. nov.	(= CBS 112151 = CPC 3202 = DFRI00150 = Lynfield 417) ^T	FJ918506	FJ918522	FJ918545	<i>E. urophylla</i>	Australia	C. Hanwood
	CMW 30602 (= CBS 112634)	FJ918507	DQ190668	FJ918546	<i>Xanthorrhoea australis</i>	Australia	T. Baigent

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW: culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria Pretoria, South Africa; ^T All ex-type cultures.

Fig. 1 One of six most parsimonious trees obtained from a heuristic search with 1 000 random addition of the combined sequences of β -tubulin, histone H3 and translation elongation factor-1 alpha sequence alignments of the *Ca. reteaudii* complex and other closely related species. Scale bar shows 10 changes and bootstrap support values from 1 000 replicates are shown above the nodes in bold. Bayesian posterior probability values are indicated below the nodes. Red lines indicate bootstrap support values of 100 and posterior probability values of 1.00. Thickened lines indicate branches in the strict consensus tree and the consensus tree of the Bayesian analyses. The tree was rooted to *Ca. colombiensis* (CBS 112221) and *Cy. chinense* (CBS 112744).

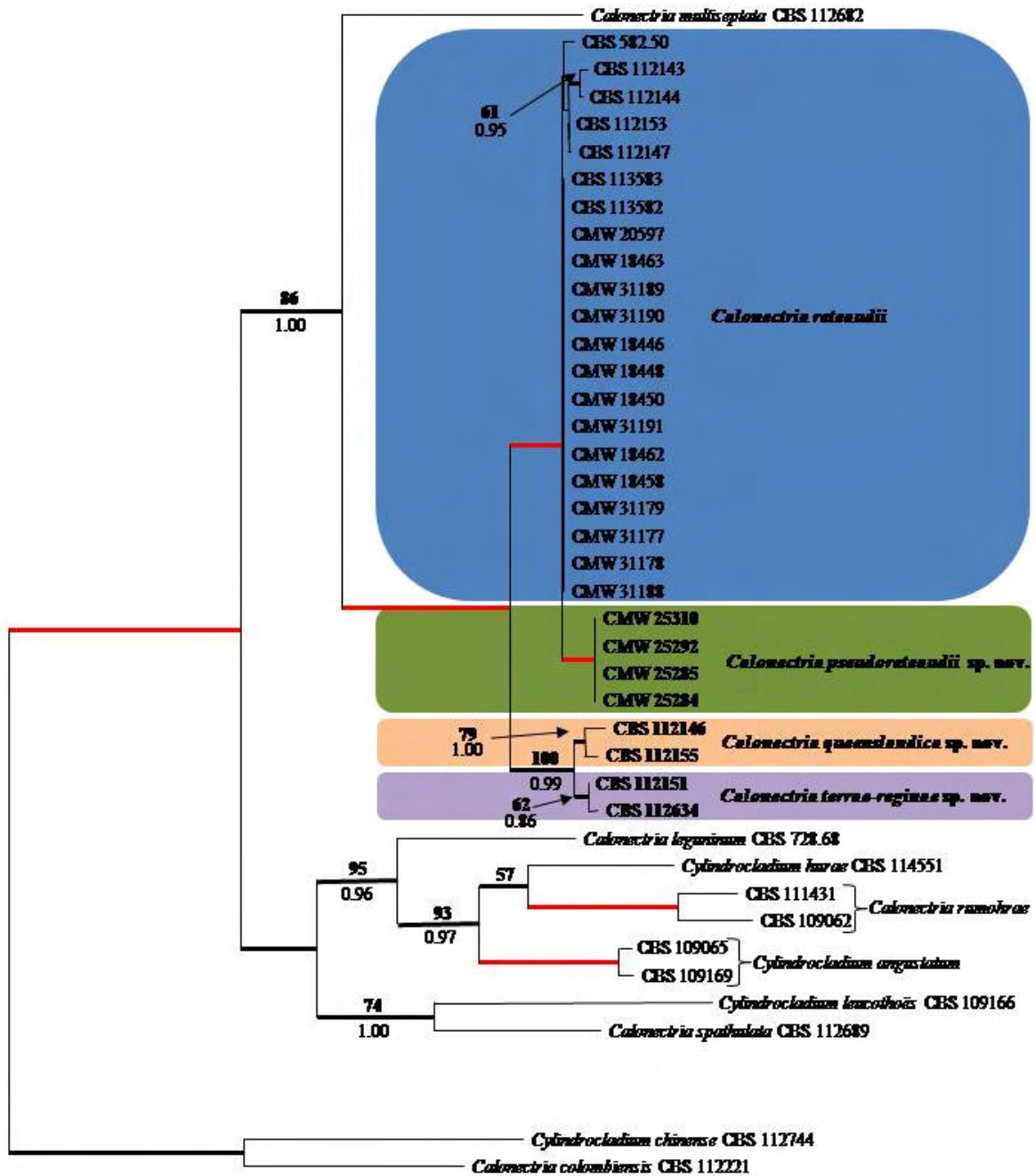


Fig. 2 One of three most parsimonious trees obtained from a heuristic search with 1 000 random addition sequences of the combined sequences of β -tubulin, histone H3 and translation elongation factor-1 alpha sequence alignments of the *Ca. morgani* complex and other closely related species. Scale bar shows 10 changes and bootstrap support values from 1 000 replicates are above the nodes in bold. Bayesian posterior probability values are indicated below the nodes. Red lines indicate bootstrap support values of 100 and posterior probability values of 1.00. Thickened lines indicate branches in the strict consensus tree and the consensus tree of the Bayesian analyses. The tree was rooted to *Ca. colombiensis* (CBS 112221) and *Cy. chinense* (CBS 112744).

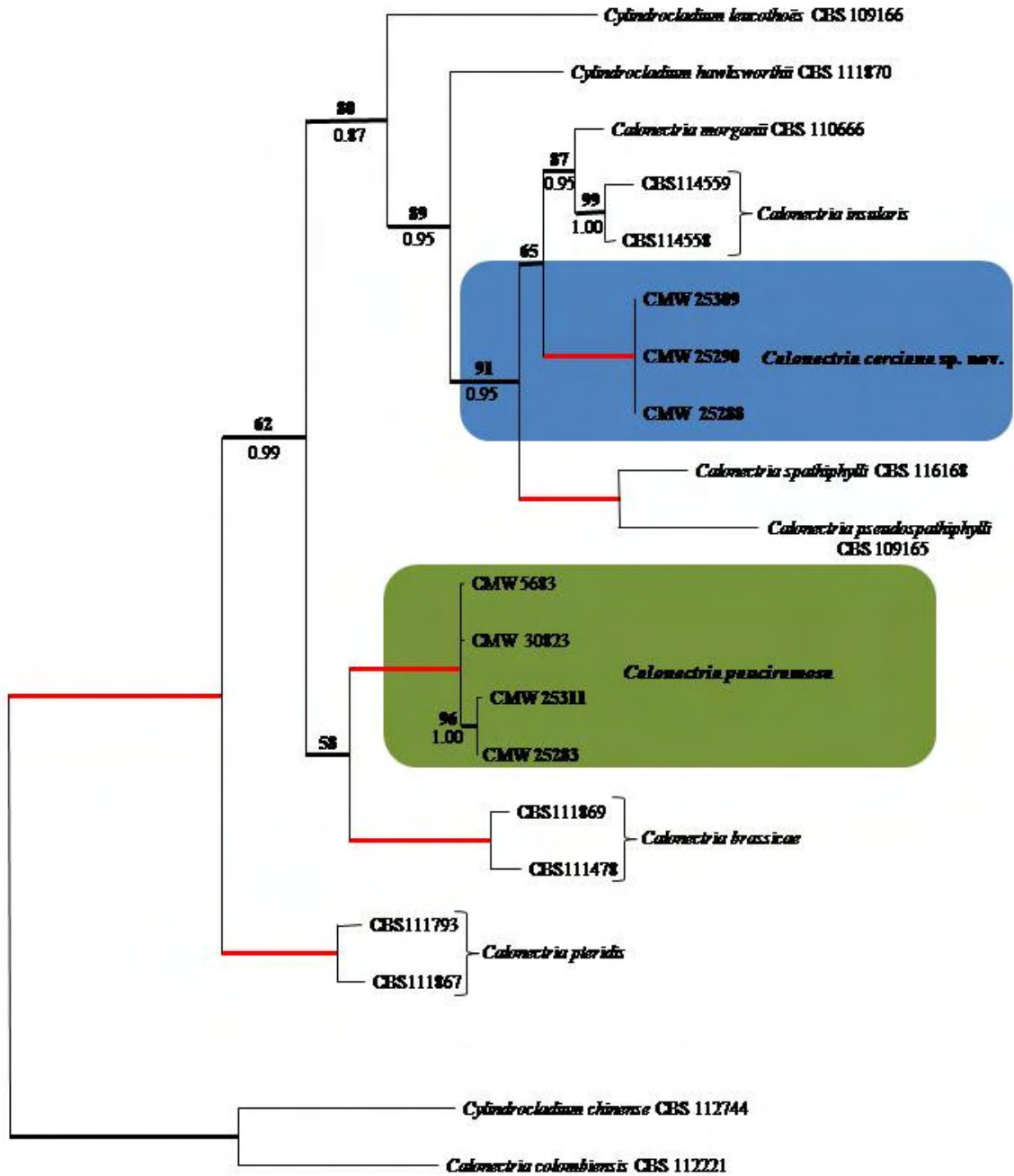




Fig. 3 *Calonectria cerciana*. A. Macroconidiophore. B, C. fusiform to ellipsoidal vesicles. D, E. Fertile branches with reniform to doliiform phialides. F, G. Macroconidia. Scale bars A = 20 μm , B–G = 10 μm .

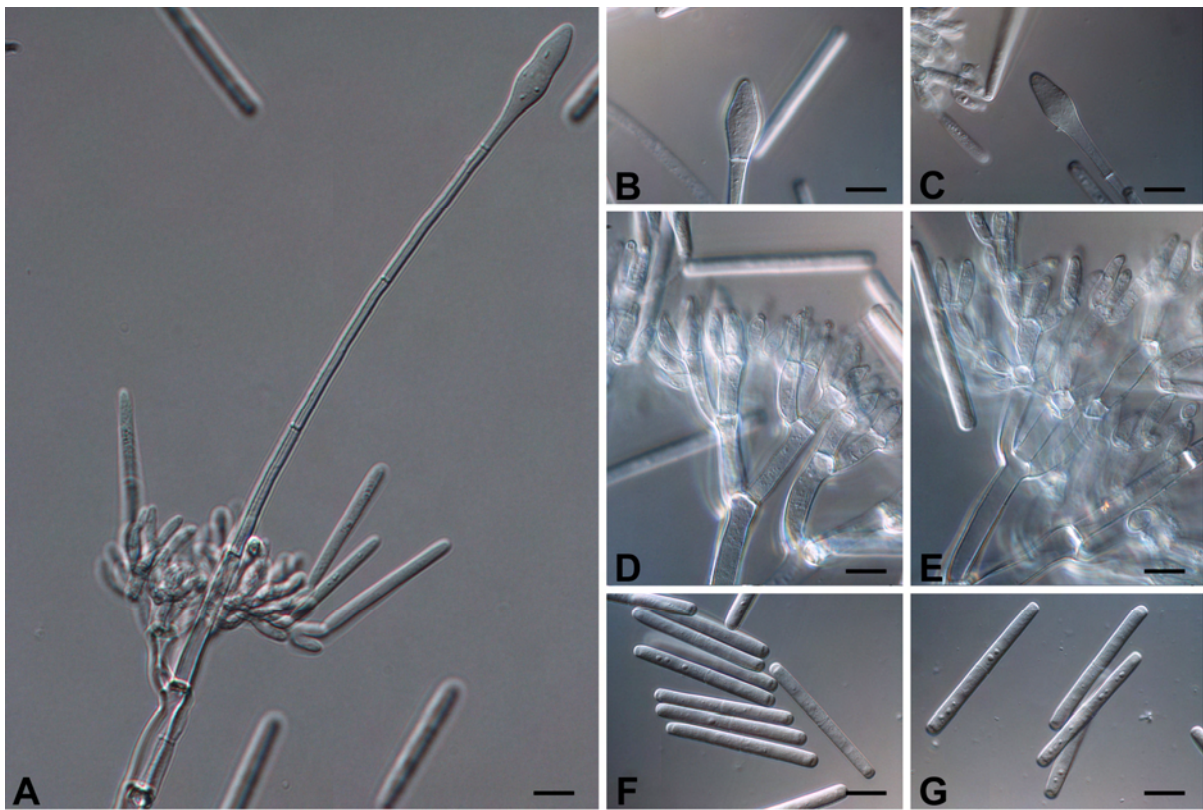




Fig. 4 *Calonectria pseudoreteauidii*. A. Macroconidiophore. B, C. Clavate vesicle, D, E. Macroconidia, F, G. Fertile branches with cylindrical to allantoid phialides. H, I. Microconidiophores. J. Microconidia. K. Comparison of macroconidia and microconidia. Scale bars A, H, I, K = 20 μm , B–G, J = 10 μm .

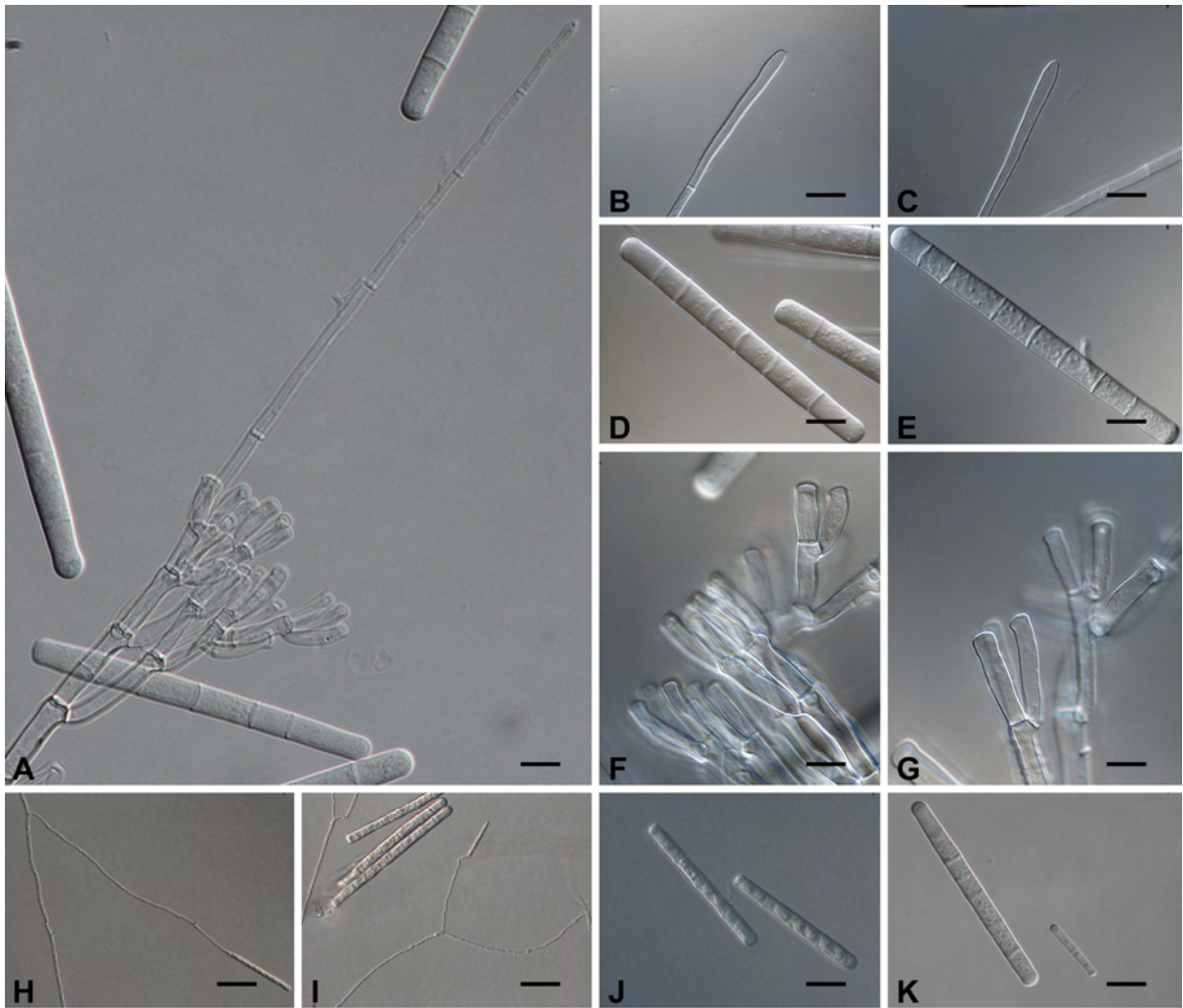




Fig. 5 *Calonectria queenslandica*. A. Macroconidiophore. B, C. Clavate vesicles. D, E. Fertile branches with cylindrical to allantoid phialides. F, G. Macroconidia. Scale bars A = 20 μm , B–G = 10 μm .

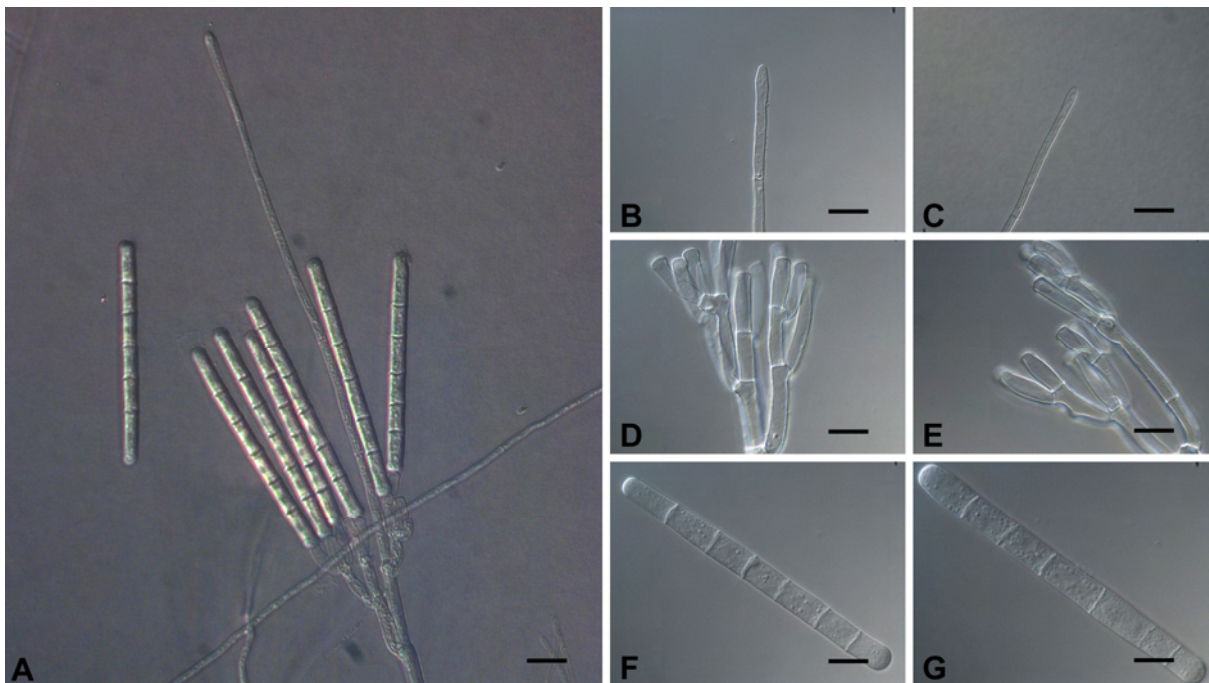




Fig. 6 *Calonectria terrae-reginae*. A. Macroconidiophore. B, C. Clavate vesicles. D, E. Fertile branches with cylindrical to allantoid phialides. F, G. Macroconidia. Scale bars A = 20 μm , B–G = 10 μm .

