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Calonectria species associated with cutting rot of *Eucalyptus*

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Key words

Australia
Calonectria
China
Cylindrocladium
Eucalyptus
systematics

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 together with *Ca. pauciramosa*. *Calonectria pseudoreteaudii* resides in the *Ca. reteaudii* complex and *Ca. cerciana* is closely related to *Ca. morganii*. 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 originate from Australia and are described as *Ca. queenslandica* sp. nov. and *Ca. terrae-reginae* sp. nov.

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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. insularis*, *Ca. morganii* and *Cy. hawksworthii* (Schoch et al. 1999, 2001, Crous 2002). Species in this complex are characterised by ellipsoid 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 subtropical 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 in the 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 the China Eucalypt Research Centre (CERC), ZhanJiang, 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

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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
		β-tubulin	Histone H3	Translation elongation factor -1α			
Cy. angustatum	CBS 109169	DQ190593	DQ190695	FJ918552	*Tillandsia capitata*	USA	R.M. Leahy
	CBS 109065^{T}	AF207543	DQ190656	FJ918551	*T. capitata*	USA	R.M. Leahy
Ca. cerciana sp. nov.	CMW 25309 (= CBS 123693)^{T}	FJ918510	FJ918528	FJ918559	*Eucalyptus urophylla* × *E. grandis* cutting	China	M.J. Wingfield & X.D.Zhou
	CMW 25290 (= CBS 123695)	FJ918511	FJ918529	FJ918560	*E. urophylla* × *E. grandis* cutting	China	M.J. Wingfield & X.D.Zhou
	CMW 25288	GQ25288	GQ267243	GQ267288	*E. urophylla* × *E. grandis* cutting	China	M.J. Wingfield & X.D.Zhou
Ca. colombiensis	CBS112221	AY725620	AY725663	AY725712	Soil	Colombia	M.J. Wingfield
Cy. chinense	CBS112744	AY725618	AY725660	AY725709	Soil	China	E.C.Y. Liew
Ca. brassicae	CBS 111869^{T}	AF232857	DQ190720	FJ918567	*Argyreia* sp.	South East Asia	
	CBS 111478	DQ190611	DQ190719	FJ918567	Soil	Brazil	A.C. Alfenas
Cy. hawksworthii	CBS 111870^{T}	AF333407	DQ190649	FJ918558	*Nelumbo nucifera*	Mauritius	A. Peerally
Cy. hurae	CBS 114551	AF333408	DQ190728	FJ918548	*Rumohra adiantiformis*	Brazil	A.C. Alfenas
Ca. insularis (= *Cy. insulare*)	CBS 114558	AF210861	FJ918526	FJ918556	Soil	Madagascar	P.W. Crous
	CBS 114559	AF210862	FJ918525	FJ918555	Soil	Madagascar	C. L. Schoch
Ca. leguminum (= *Cy. leguminum*)	CBS 728.68^{T}	AF389837	DQ190654	FJ918547	*Annona squamosa*	Brazil	M.B. Figueiredo
Cy. leucothoës	CBS 109166^{T}	FJ918508	FJ918523	FJ918553	*Leucothoë axillaris*	USA	N.E. El-Gholl
Cy. multiseptatum	CBS 112682^{T}	DQ190573	DQ190659	FJ918535	*Eucalyptus* sp.	Indonesia	M.J. Wingfield
Ca. pauciramosa (= Cy. *pauciramosum*)	CMW 5683	FJ918514	FJ918531	FJ918565	*Eucalyptus* sp.	Brazil	A.C. Alfenas
	CMW 30823	FJ918515	FJ918532	FJ918566	*E. grandis*	South Africa	P.W. Crous
	CMW 25311	FJ918516	FJ918533	FJ918569	*E. urophylla* × *E. grandis* cutting	China	M.J. Wingfield & X.D. Zhou
	CMW 25283	FJ918517	FJ918534	FJ918570	*E. urophylla* × *E. grandis* cutting	China	M.J. Wingfield & X.D. Zhou
Ca. pseudoreteaudii sp. nov.	CMW 25310 (= CBS 123694)^{T}	FJ918504	FJ918519	FJ918541	*E. urophylla* × *E. grandis* cutting	China	M.J. Wingfield & X.D. Zhou
	CMW 25292 (= CBS 123696)	FJ918505	FJ918520	FJ918542	*E. urophylla* × *E. grandis* cutting	China	M.J. Wingfield & X.D. Zhou
	CMW 25284	GQ267205	GQ267244	GQ267289	*E. urophylla* × *E. grandis* cutting	China	M.J. Wingfield & X.D. Zhou
	CMW 25285	GQ267206	GQ267245	GQ267290	*E. urophylla* × *E. grandis* cutting	China	M.J. Wingfield & X.D. Zhou
Ca. pseudospathiphylli	CBS 109165	FJ918513	AF348241	FJ918562	Soil	Ecuador	M.J. Wingfield
Ca. pteridis	CBS 111793	DQ190578	DQ190679	FJ918563	*Arachnoides adiantiformis*	USA	
	CBS 111871	DQ190579	DQ190680	FJ918564	*Pinus* sp.	Spain	T.L. Krugner
Ca. queenslandica sp. nov.	CMW 30604 (= CBS 112146 = CPC 3213 = DRFI000147)^{T}	AF389835	FJ918521	FJ918543	*E. urophylla*	Australia	B. Brown
	CMW 30603 (= CBS 112155 = CPC 3210 = DFRI00172)	AF389834	DQ190667	FJ918544	*E. pellita*	Australia	K.M. Old
Ca. reteaudii	CBS 582.50	AF389836	DQ190673	FJ918540	*Hibiscus sabdariffa*	Indonesia	—
	CBS 112143	GQ240642	DQ190660	FJ918536	*E. camaldulensis*	Vietnam	M.J. Dudzinski
	CBS 112144^{T}	AF389833	DQ190661	FJ918537	*E. camaldulensis*	Vietnam	M.J. Dudzinski
	CBS 112147	AF389830	DQ190663	FJ918539	*E. camaldulensis*	Vietnam	M.J. Dudzinski
	CBS 112153	AF389831	FJ918518	FJ918538	*E. camaldulensis*	Vietnam	M.J. Dudzinski
	CBS 113582	GQ240643	GQ240659	GQ240675	*Eucalyptus* sp.	Thailand	—
	CBS 113583	GQ240644	GQ240660	GQ240676	*Eucalyptus* sp.	Madagascar	P.W. Crous
	CMW 18446	GQ240649	GQ240665	GQ240681	*E. urophylla*	Indonesia	M.J. Wingfield

	CMW 18448	GQ240650	GQ240666	GQ240682	*E. urophylla*	Indonesia	M.J. Wingfield
	CMW 18450	GQ240651	GQ240667	GQ240683	*E. grandis*	Thailand	M.J. Wingfield
	CMW 18458	GQ240654	GQ240670	GQ240686	*E. urophylla*	Indonesia	M.J. Wingfield
	CMW 18462	GQ240653	GQ240669	GQ240685	*E. urophylla*	Indonesia	M.J. Wingfield
	CMW 18463	GQ240646	GQ240662	GQ240678	*E. urophylla*	Indonesia	M.J. Wingfield
	CMW 20597	GQ240645	GQ240661	GQ240677	*E. grandis*	Thailand	M.J. Wingfield
	CMW 31177	GQ240657	GQ240673	GQ240689	*Eucalyptus* sp.	Indonesia	M.J. Wingfield
	CMW 31178	GQ240656	GQ240672	GQ240688	*Eucalyptus* sp.	Indonesia	M.J. Wingfield
	CMW 31179	GQ240655	GQ240671	GQ240687	*Eucalyptus* sp.	Indonesia	M.J. Wingfield
	CMW 31188	GQ240658	GQ240674	GQ240690	*Eucalyptus* sp.	Indonesia	M.J. Wingfield
	CMW 31189	GQ240647	GQ240663	GQ240679	*Eucalyptus* sp.	Indonesia	M.J. Wingfield
	CMW 31190	GQ240648	GQ240664	GQ240680	*Eucalyptus* sp.	Indonesia	M.J. Wingfield
	CMW 31191	GQ240652	GQ240668	GQ240684	*Eucalyptus* sp.	Thailand	M.J. Wingfield
Ca. rumohrae	CBS 111431[T]	AF232871	DQ190675	FJ918549	*Rumohra adiantiformis*	Panama	J.W. Miller
	CBS 109062	AF232873	DQ190676	FJ918550	*Adiantum* sp.	The Netherlands	R. Pieters
Ca. morganii	CBS 110666		FJ918509	FJ918527	FJ918557	*Rosa* sp.	USA	N.E. Ell-Gholl
Ca. spathiphylli	CBS 116168[T]		FJ918512	FJ918530	FJ918561	*Spathiphyllum* sp.	USA	C.L. Schoulties
Ca. spathulata	CBS 112689		AF308463	FJ918524	FJ918554	*E. viminalis*	Brazil	N.E. Ell-Gholl
Ca. terrae-reginae sp. nov.	CMW 30601 (= CBS 112151 = CPC 3202 = DFR100150 = Lynfield 417)[T]		FJ918506	FJ918522	FJ918545	*E. urophylla*	Australia	C. Hanwood
	CMW 30602 (= CBS 112634)		FJ918507	DQ190668	FJ918546	*Xanthorrhoea australis*	Australia	T. Baigent

[1] 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.

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-1α (TEF-1α) 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× PCR buffer, 1–1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 µm of each primer and ± 30 ng of fungal genomic DNA, made up to a total reaction volume of 25 µL 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 (v3.1, Applied Biosystems, USA) and an ABI PRISM™ 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 (v1.0.1, Applied Biosystems) and MAFFT (v5.11, Katoh et al. 2005), respectively. The aligned sequences were then manually corrected where needed.

To determine whether the sequence datasets 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 v3.7 (Posada & Crandall 1998) and bootstrap analyses were run for 10k replicates.

PAUP (Phylogenetic Analysis Using Parsimony, v4.0b10, Swofford 2002) was used to analyse the DNA sequence datasets. Phylogenetic relationships were estimated by a 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 datasets.

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 dataset 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 macrocondia, and stipe extensions terminating in ellipsoidal to obpyriform vesicles. *Calonectria*

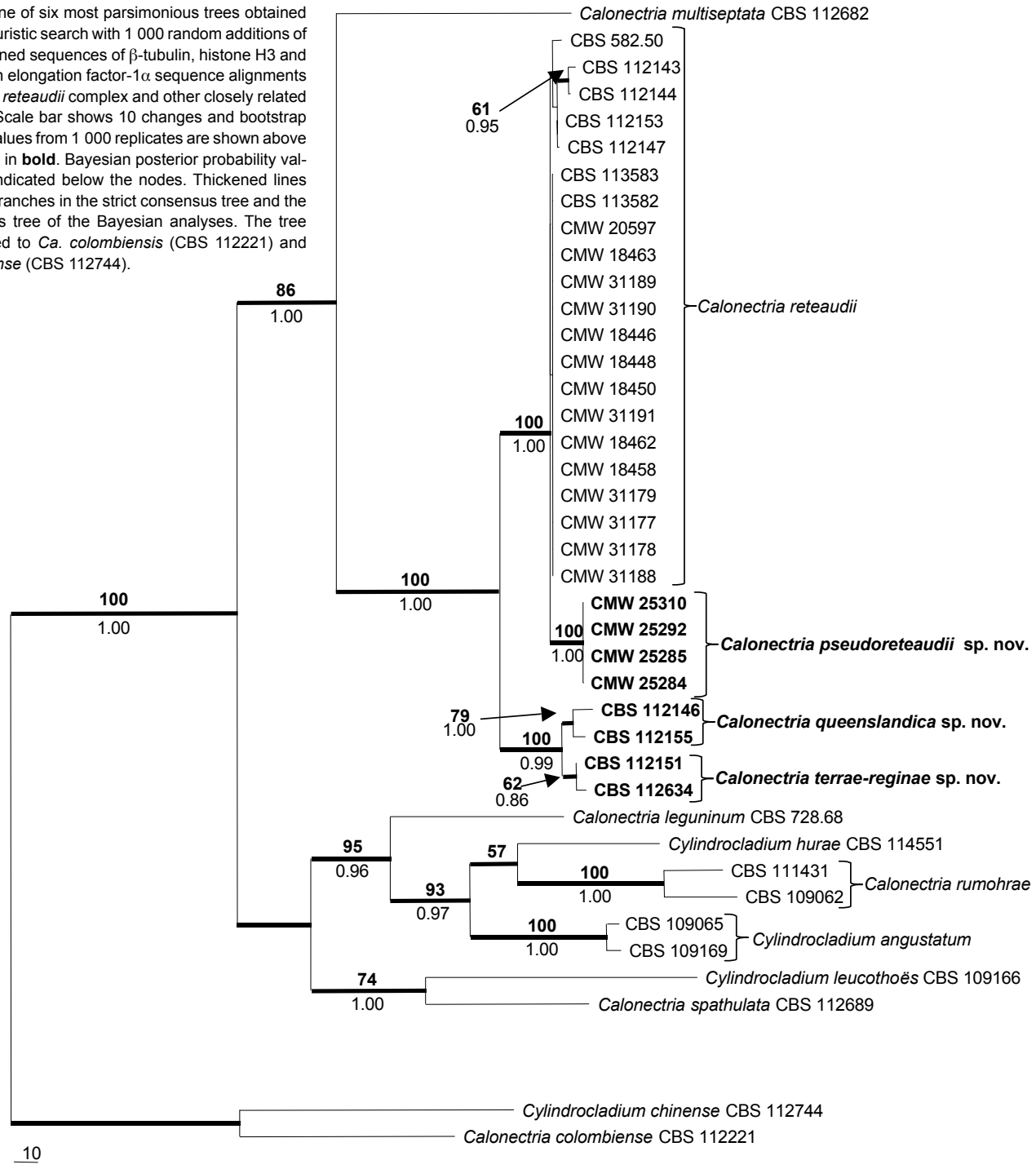

Fig. 1 One of six most parsimonious trees obtained from a heuristic search with 1 000 random additions of the combined sequences of β-tubulin, histone H3 and translation elongation factor-1α sequence alignments of the *Ca. reteaudii* complex and other closely related species. Scale bar shows 10 changes and bootstrap support values from 1 000 replicates are shown above the nodes in **bold**. Bayesian posterior probability values are indicated below the nodes. 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).

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 v3.1.1 (Ronquist & Huelsenbeck 2003) for the combined sequence datasets. Models of nucleotide substitution for each gene were determined using MrModeltest v2.3 (Nylander 2004) and included for each gene partition. Three analyses of four MCMC chains were run from random trees for 1 000 000 generations and sampled every 100 generations. All runs converged on the same likelihood score and tree topology and therefore, the first 7 600 trees for the *Ca. reteaudii* complex and 2 000 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 a 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.

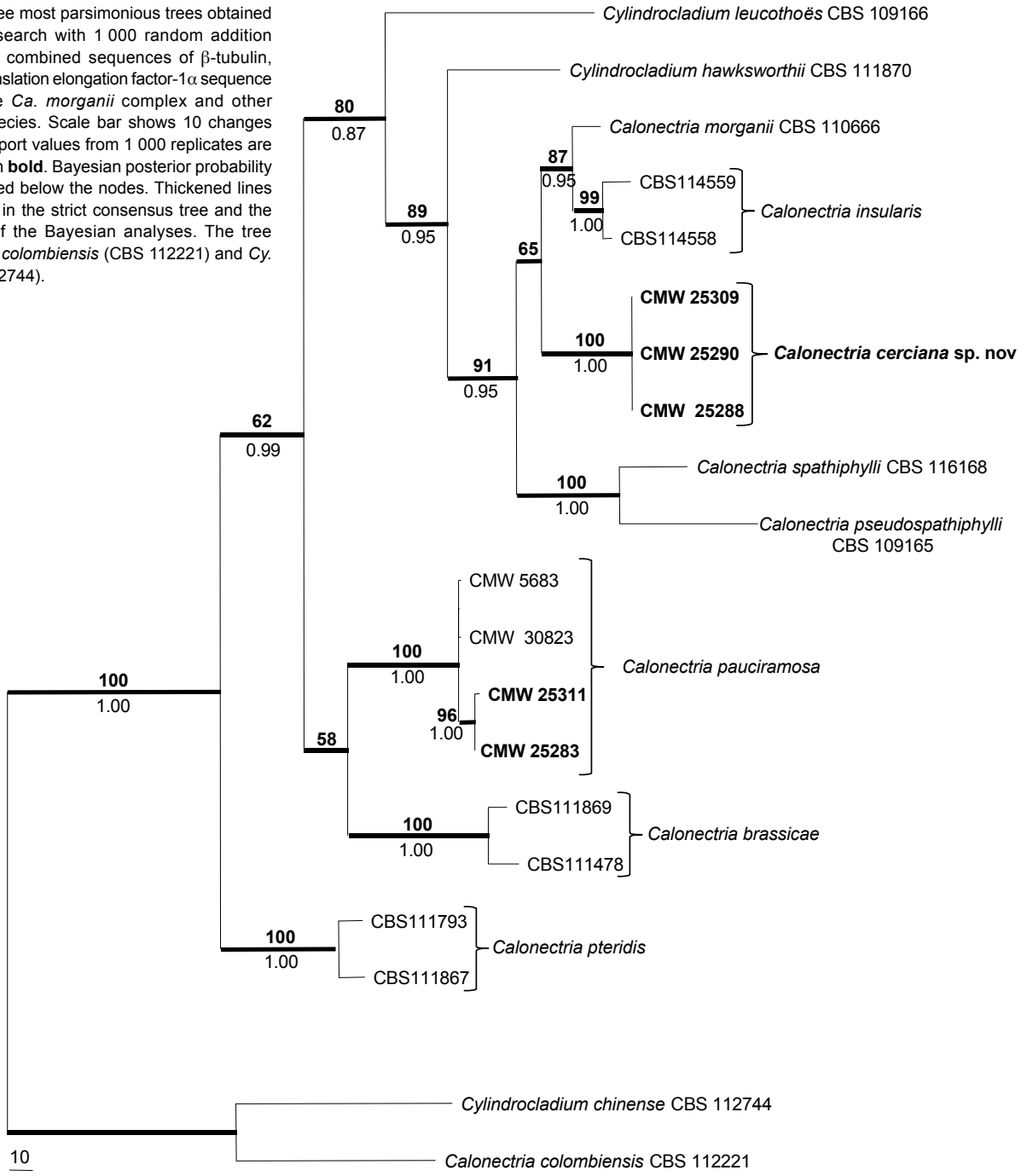

Fig. 2 One of three most parsimonious trees obtained from a heuristic search with 1 000 random addition sequences of the combined sequences of β-tubulin, histone H3 and translation elongation factor-1α sequence alignments of the *Ca. morganii* complex and other closely related species. Scale bar shows 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. 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).

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 × 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 ± 500 bases (BT and TEF-1α) 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 *Cy. multiseptatum* (CBS 112682) grouping within different clusters for all three gene regions considered (results not shown). This conflict was resolved by separating the sequence datasets for those representing morphological and phylogenetically closely related species to *Ca. reteaudii* and *Ca. morganii*. Sequence datasets for BT, HIS3 and TEF-1α were then combined for the two separate datasets. Sequence alignments were deposited in TreeBASE as SN4542.

The combined sequence dataset for isolates representing *Ca. reteaudii* and other closely related species consisted of 1 540 characters, of which 926 were constant, 178 were parsi-

mony uninformative and 436 were parsimony informative. Parsimony analysis of the alignment yielded six most parsimonious trees (TL = 1 316 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-1α, 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 subclades (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 1 518 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 = 1 065 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-1α, 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. 2).

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. Crossing isolates CBS 112146, CBS 112151, CBS 112155 and CBS 112634 with isolates of *Ca. reteaudii* (represented in this phylogenetic study), failed to produce perithecia in any combination tested. Likewise, when isolates CMW 25284, CMW 25285, CMW 25292 and CMW 25310 were crossed with the isolates of *Ca. reteaudii* in this study, perithecia were also not found. However, the crossed isolates of *Ca. reteaudii* in this study, previously shown to represent different mating types by Kang et al. (2001), produced perithecia with viable ascospores. Isolate CBS 582.50, representing *Ca. reteaudii*, 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, CMW 25285 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 treated as a new species. Based on DNA sequence analysis, mating strategy and morphological observations, isolates CBS 112146 (= CPC 3213) and CBS 112155

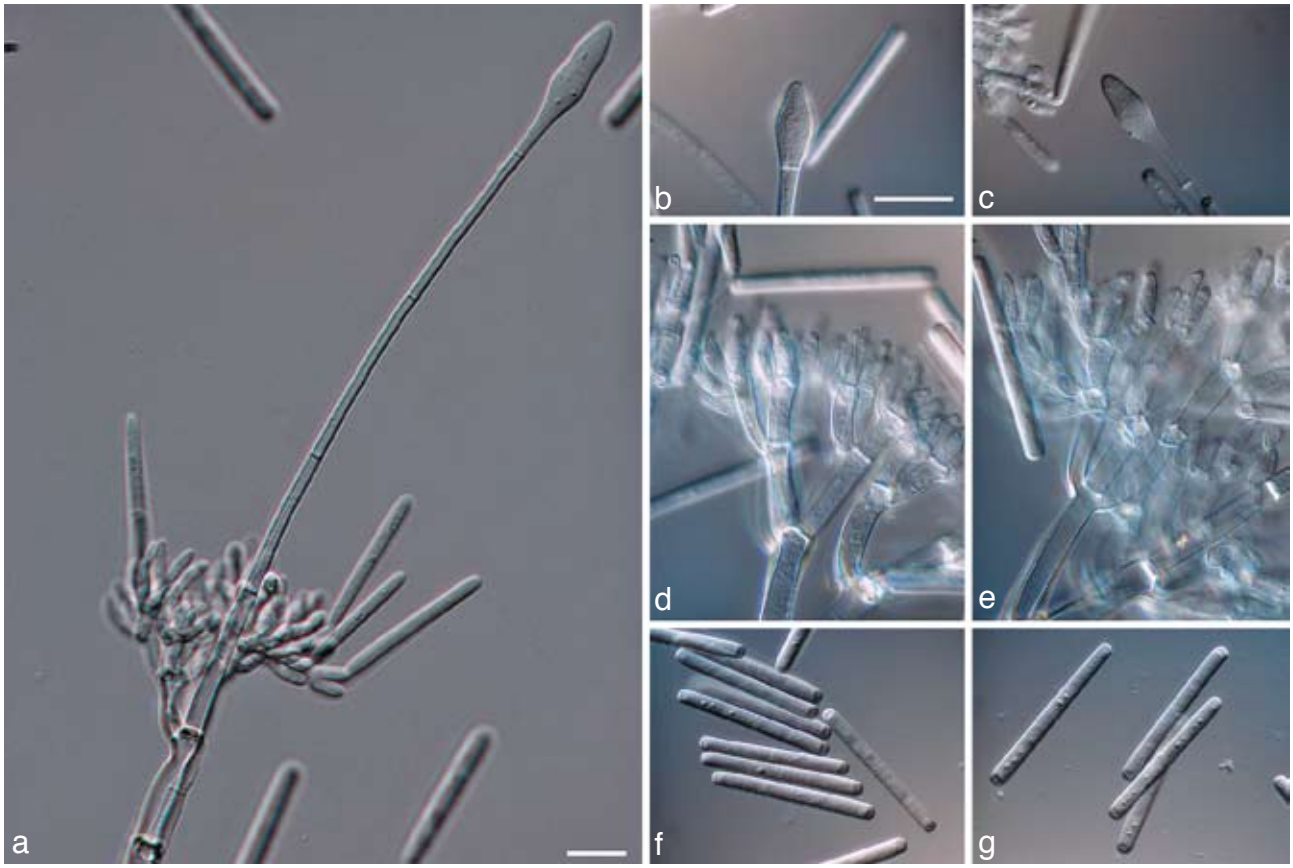

Fig. 3 *Calonectria cerciana*. a. Macroconidiophore; b, c. fusiform to ellipsoidal vesicles; d, e. fertile branches with reniiform to doliiform phialides; f, g. macroconidia. — Scale bars = 10 µm.

(= 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) also previously believed to represent *Ca. reteaudii*, and they are treated as a new species.

Calonectria cerciana L. Lombard, M.J. Wingf. & Crous, *sp. nov*. — MycoBank MB513263; Fig. 3

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.

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

Teleomorph unknown. *Conidiophores* with a stipe bearing a suite 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 collarette. *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. *Megaconidia* and *microconidia* not seen.

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

Specimens examined. China, Guangdong Province, CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, *M.J. Wingfield & X.D. Zhou*, holotype PREM 60241, culture ex-type CMW 25309 = CBS 123693; CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, *M.J. Wingfield & X.D. Zhou*, 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.

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.

Fig. 4 *Calonectria pseudoreteaudii*. a. Macroconidiophore; b, c. clavate vesicle; d, e. macrocondia; f, g. fertile branches with cylindrical to allantoid phialides; h, i. microconidiophores; j. microconidia; k. comparison of macroconidia and microconidia. — Scale bars = 10 µm.

Calonectria pseudoreteaudii L. Lombard, M.J. Wingf. & Crous, *sp. nov*. — MycoBank MB513264; Fig. 4

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.

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

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 periclinical thickening and collarette. *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.

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

Specimens examined. China, Guangdong Province, CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, *M.J. Wingfield & X.D. Zhou*, holotype PREM 60290, culture ex-type CMW 25310 = CBS 123694; CERC nursery, on stems of *E. urophylla* × *grandis* hybrid cutting, Nov. 2007, *M.J. Wingfield & X.D. Zhou*, 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*, cultures CMW 25284 and CMW 25285.

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 MB513265; Fig. 5

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) × (4–)5–6(–7) µm, 4–6-septata.

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

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, 38–58 × 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 × 4–6 µm; secondary branches aseptate, 11–22 × 3–5 µm; tertiary branches aseptate, 13–17 × 3–5

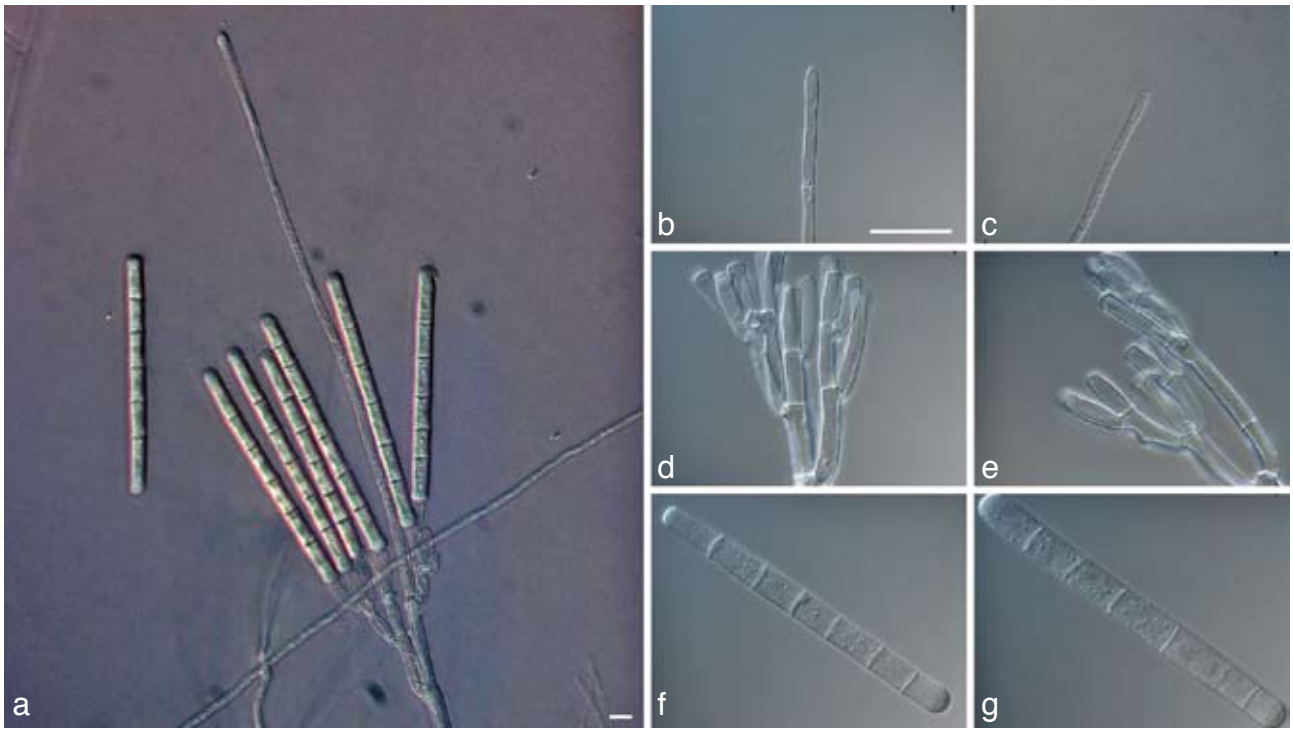

Fig. 5 *Calonectria queenslandica*. a. Macroconidiophore; b, c. clavate vesicles; d, e. fertile branches with cylindrical to allantoid phialides; f, g. macroconidia — Scale bars = 10 µm.

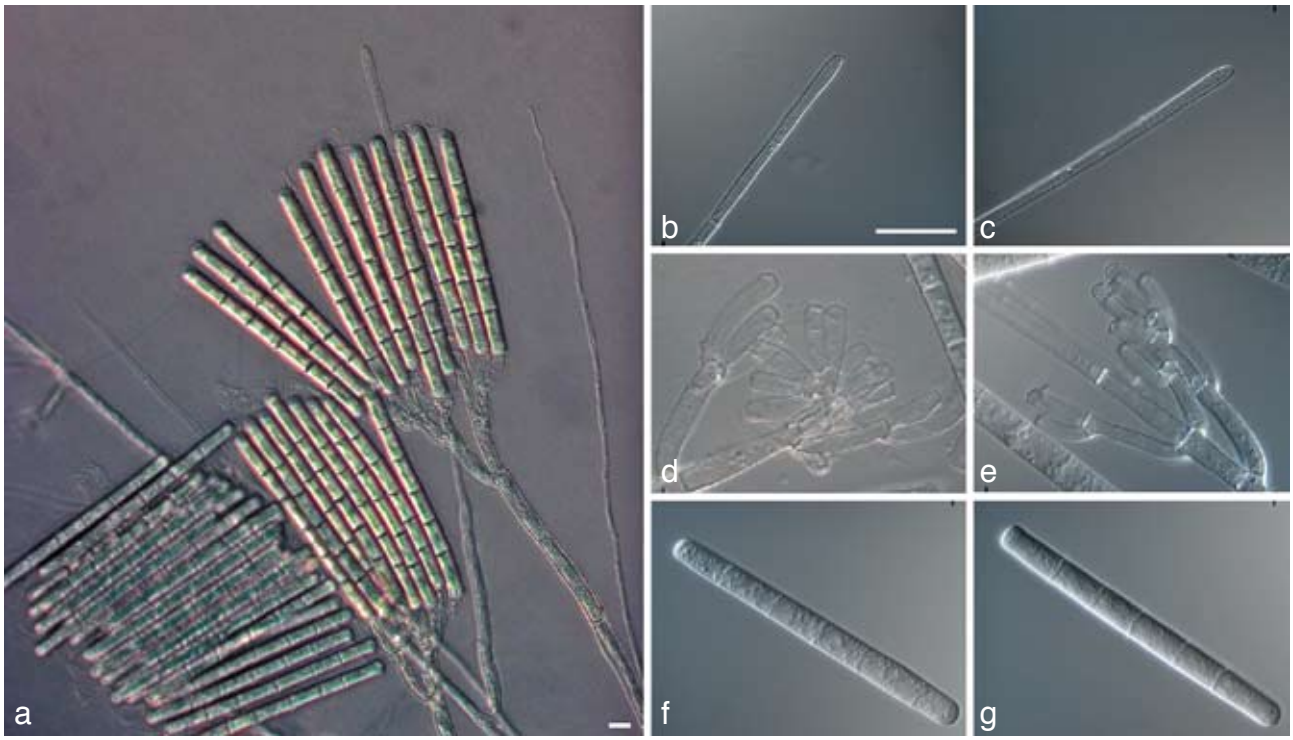

Fig. 6 *Calonectria terrae-reginae*. a. Macroconidiophore; b, c. clavate vesicles; d, e. fertile branches with cylindrical to allantoid phialides; f, g. macroconidia. — Scale bars = 10 µm.

µm, each terminal branch producing 1–3 phialides; cylindrical to allantoid, obpyriform when carried singly, hyaline, aseptate, 10–16 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. *Macroconidia* cylindrical, rounded at both ends, straight, (61–)65–73(–78) × (4–)5–6(–7) µm (av. = 69 × 6 µm), 4–6-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Megaconidia* and *microconidia* not seen.

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

Specimens examined. Australia, Queensland, Lannercost, on leaves of *E. urophylla*, 15 Apr. 1991, *B. Brown*, holotype PREM 60243, 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*, PREM 60244, culture CMW 30603 = CBS 112155 = CPC 3210 = DFRI00172.

Notes — *Calonectria queenslandica* can be distinguished from *Ca. reteaudii* and *Ca. pseudoreteaudii* based on its smaller macrocondia (av. = 69 × 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

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) × (4–)5–7(–8) µm, 4–6-septata.

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

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 × 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 × 4–6 µm; secondary branches aseptate, 13–18 × 3–6 µm; tertiary branches aseptate, 10–14 × 3–5 µm, each terminal branch producing 1–3 phialides; cylindrical to allantiod, obpyriform when carried singly, hyaline, aseptate, 10–17 × 2–4 µm; apex with minute periclinal thickening and inconspicuous collarette. *Macroconidia* cylindrical, rounded at both ends, straight, 60–83(–87) × (4–)5–7(–8) µm (av. = 76 × 6 µm), 4–6-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Megaconidia* and *microconidia* not seen.

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

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

Notes — *Calonectria terrae-reginae* is distinct from *Ca. queenslandica* by having larger macroconidia (av. = 76 × 6 µm), 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*, three new sibling species in the *Ca. reteaudii* complex and distinguished 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 et al. 1985, Sharma & Mohanan 1991, 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 diseases caused by them.

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. In the present study, it was not possible to successfully cross strains of *Ca. queenslandica*, *Ca. terrae-reginae*, and *Ca. pseudoreteaudii*, 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 plants in countries such as Australia, Italy, South Africa and the USA (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 diseases 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 a 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. pseudoreteaudii* are pathogens of *Eucalyptus*. Other species in the *Ca. reteaudii* (Sharma & Mohanan 1982, 1991, 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. pseudoreteaudii*. 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*. 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, b), to name but 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* (1892) is linked to the single teleomorph genus *Calonectria* (1867) (Rossman et al. 1999), all new species have been accommodated in *Calonectria*.

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