Chapter 4

Multigene phylogeny and mating tests reveal three cryptic species related to *Calonectria pauciramosa*

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ABSTRACT

*Calonectria pauciramosa* is a pathogen of numerous plant hosts worldwide. Recent studies have suggested that it accommodates cryptic species and the aim of this study was to identify these taxa. Isolates from various geographical origins were collected and compared based on morphology, DNA sequence data of the β-tubulin, histone H3 and translation elongation factor-1α regions and mating compatibility. Comparisons of the DNA sequence data and mating compatibility revealed three new species. These included *Ca. colombiana* sp. nov. from Colombia, *Ca. zuluensis* sp. nov. from South Africa and *Ca. polizzii* sp. nov. from Italy, all of which had distinguishing morphological features. Based on DNA sequence data, *Ca. brasiliensis* is also elevated to species level.

**Taxonomic novelties:** *Calonectria brasiliensis* (Bat. & Cif.) L. Lombard, M.J. Wingf. & Crous comb. nov., *Calonectria colombiana* L. Lombard, M.J. Wingf. & Crous sp. nov., *Calonectria polizzii* L. Lombard, M.J. Wingf. & Crous sp. nov., *Calonectria zuluensis* L. Lombard, M.J. Wingf. & Crous sp. nov.
INTRODUCTION

Several past studies have focused on the taxonomy of Calonectria spp. with small, 1-septate macroconidia and ellipsoidal to obpyriform vesicles (Crous et al. 1993, Overmeyer et al. 1996, Schoch et al. 1999, 2000). These Calonectria spp. were initially regarded as either Ca. morganii (= Cy. scoparium) or Ca. scoparia (= Cy. candelabrum) based on their morphological similarities. However, the anamorph state of Ca. morganii was circumscribed as having ellipsoidal to pyriform vesicles and Ca. scoparia having ellipsoidal to obpyriform vesicles by Crous et al. (1993). Later studies, incorporating DNA sequence data, have shown that Ca. morganii is restricted to the Northern Hemisphere and Brazil (Crous et al. 1993, Overmeyer et al. 1996, Schoch et al. 2000). In contrast, Ca. scoparia is found worldwide and forms part of a species complex consisting of four mating groups, each representing a different Calonectria species that includes Ca. pauciramosa (anamorph: Cy. pauciramosum), Ca. scoparia, Ca. mexicana (anamorph: Cy. mexicanum) and Ca. insularis (anamorph: Cy. insulare) (Schoch et al. 1999).

Calonectria pauciramosa has been reported worldwide on numerous plant hosts (Schoch et al. 1999, Koike et al. 1999, Koike & Crous 2001, Polizzi & Crous 1999, Polizzi 2000, Polizzi & Catara 2001, Polizzi & Vitale 2001, Crous 2002, Polizzi et al. 2006, 2007), where it causes diseases such as cutting rot, damping-off, root rot and leaf blight. In South Africa and Australia, Ca. pauciramosa is regarded as the dominant pathogen in commercial forest nurseries (Crous 2002) and it is also found on various horticultural crops in commercial nurseries in Italy and the U.S.A. (Schoch et al. 2001, Crous 2002).

Schoch et al. (2001) considered female fertility in populations of Ca. pauciramosa from various geographical regions to determine the ratio of mating types present, and based on these data suggested that Ca. pauciramosa could be endemic to South America. The latter study also indicated that Ca. pauciramosa isolates from California were represented by only one mating type, supporting the view that this represented an introduced pathogen. Isolates from Italy showed higher ratios of hermaphrodites, also indicative of a recent introduction, although some variation was observed in the β-tubulin sequences. In contrast, South African isolates had close to a 1:1 mating type ratio and showed variation in β-tubulin sequence data (Schoch et al. 1999, 2001), suggesting that this was either a native pathogen or that there had been multiple introductions into the country.
Initial investigations using DNA sequence comparisons and mating studies on *Ca. pauciramosa* isolates from South Africa and Colombia showed some variation amongst isolates. These findings and those of Schoch *et al.* (2001) suggested that *Ca. pauciramosa* might accommodate a number of cryptic species. The aim of this study was, therefore, to consider the phylogenetic relationships, morphological characters and mating compatibility of available isolates of *Ca. pauciramosa* and to determine whether this species represented an assemblage of cryptic taxa.

**MATERIALS AND METHODS**

**Isolates**

Isolates of *Ca. pauciramosa* were obtained from culture collections (Table 1) or were isolated from infected plant material and soil samples following the methods of Crous (2002). For each isolate, single conidial cultures were prepared on 2 % (w/v) malt extract agar (MEA, Biolab, Midrand, South Africa). Representative strains are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

**Sexual compatibility**

A total of 57 single conidial *Ca. pauciramosa*-like isolates (Table 1), originating from various geographic regions and hosts were crossed in all possible combinations. Mating-tester strains CMW 30823 (= STE-U 416) and CMW 5683 (= STE-U 971) for *Ca. pauciramosa* defined by Schoch *et al.* (2001) were also crossed with these isolates. Matings were done as described in Schoch *et al.* (1999) on carnation leaf agar (CLA; Fisher *et al.* 1982, Crous *et al.* 1993) and on minimal salt agar (MSA; Guerber & Correll 2001, Halleen *et al.* 2006) with sterile toothpicks placed on the surface of the agar. Control tests, where isolates were crossed with themselves, were undertaken to determine whether strains had a heterothallic or homothallic mating system. The plates were stacked in plastic containers and incubated at 22 °C for 6 wk. Matings were regarded as successful when isolate combinations produced perithecia extruding viable ascospores.

**DNA sequence comparisons**

*Calonectria pauciramosa*-like isolates were grown on MEA for 7 d. Mycelium was then scraped from the surface of the cultures, 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 including fragments of the β-tubulin (BT), histone H3 (HIS3) and translation elongation factor-1 alpha (TEF-1α) gene regions were sequenced. Primers used to sequence these regions were T1 (O’Donnell & Cigelnik 1997) and CYLTUB1R (Crous et al. 2004b) for the BT region, CYLH3F and CYLH3R (Crous et al. 2004b) for the HIS3 region and EF1-728F (Carbone & Kohn 1999) and EF2 (O’Donnell et al. 1998) for the TEF-1α region. 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 MgCl2, 0.25 mM of each dNTP, 0.5 µm of each primer and approximately 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 v. 3.1 (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. (2006) for BT and HIS3. The same cycling conditions for HIS3 were used for TEF-1α amplifications.

The generated sequences were added to other sequences of closely related Calonectria spp. 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. Single nucleotide polymorphisms (SNP’S) were determined for each gene region analysed using DnaSP v. 5.00.07 (Librado & Rozas 2009).

To determine whether the DNA sequence datasets for the three gene regions were congruent, a 70 % reciprocal bootstrap method using Neighbour-Joining with Maximum Likelihood distance was employed (Mason-Gamer & Kellogg 1996, Gueidan et al. 2007). Models of evolution were estimated in Modeltest v. 3.7 (Posada & Crandall 1998) using the Akaike Information Criterion for each separate gene region. The bootstrap analyses were run in PAUP (Phylogenetic Analysis Using Parsimony v. 4.0b10, Swofford 2002) for 10 000 replicates. Resulting tree topologies were compared visually for conflicts between the
separate gene regions. Phylogenetic relationships were estimated in PAUP, by heuristic searches based on 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 included 73 partial gene sequences per gene, representing 11 Calonectria and Cylindrocladium species (Table 1). Calonectria colombiensis (CBS 112221) and Cy. chinense (CBS 112744) were used as outgroup taxa (Lombard et al. 2009). Novel sequences were deposited in GenBank and all alignments in TreeBASE (http://treebase.org) as SN4773.

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). Models of nucleotide substitution for each gene were determined using Mrmodeltest (Nylander 2004) and included for each gene partition. Two analyses of four MCMC chains were run from random trees for 1 000 000 generations and sampled every 100 generations. Both runs converged on the same likelihood score and tree topology. Therefore, the first 1 000 trees were discarded as the burn-in phase of each analysis and posterior probabilities were determined from the remaining trees.

**Taxonomy**

For morphological identification of the anamorphs, single conidial cultures were prepared on synthetic nutrient-poor agar (SNA; Nirenburg 1981, Lombard et al. 2009). Inoculated plates were incubated at room temperature and examined after 7d. Gross morphological characteristics were determined by mounting fungal structures in lactic acid and 30 measurements at ×1 000 magnification were made for each isolate. Teleomorph morphology was determined by mounting perithecia obtained from the sexual compatibility tests in Leica mountant (Setpoint Premier, Johannesburg, South Africa) and hand-sectioned with a Leica CM1100 cryostat (Setpoint Technologies) at –20 °C. The 10 μm sections were mounted in lactophenol or 3 % KOH. Gross morphological characteristics were observed as above. The
95% confidence levels were calculated and extreme measurements of conidia are given in parentheses. For other structures, only the extremes are indicated. Optimal growth temperatures were determined for each isolate on MEA at 5–35 °C in 5 °C intervals in the dark. Colony colours were determined after 7 d on MEA at 25 °C in the dark, using the colour charts of Rayner (1970) for comparison. Descriptions, nomenclature, and illustrations were deposited in MycoBank (Crous et al. 2004a).

RESULTS

Sexual compatibility
Protoperithecia formed within 3 wk and successful matings produced perithecia with viable ascospores within 6 wk on both CLA and MSA. A total of 1 649 crosses were made using the 57 putative Ca. pauciramosa isolates and mating tester strains for Ca. pauciramosa s. str. This resulted in 642 tests where perithecia produced viable ascospores. Self-self crosses indicated that 11 of the 57 isolates were self-fertile (homothallic). These included the Colombian isolates CBS 111041, CBS 111136, CBS 115127, CBS 115638, CBS 115694 and CMW 9058, and South African isolates CMW 9115, CMW 9188, CMW 9208, CMW 9215 and CMW 9896. Sixteen of the 57 putative Ca. pauciramosa did not cross with the mating tester strains for that species or with any other isolate included in this study. These included isolates CMW 7578 from Argentina; CBS 114257, CBS 116078, CBS 116076, CBS 116081, CMW 31505, CMW 31507 and CMW 31508, from Brazil; CMW 7804, CMW 10151 and CBS 123402 from Italy, CMW 30814 and CMW 30815 from Kenya; CMW 30817 from New Zealand; CMW 1786 and CMW 30815 from South Africa. The remaining 30 isolates produced perithecia containing viable ascospores when crossed with the Ca. pauciramosa mating tester strains and between them. This resulted in 203 successful heterothallic matings (Table 2).

DNA sequence comparisons
Amplicons of approx. 500 bp were generated for the BT and TEF-1α gene regions and those for the HIS3 region were approx. 450 bp. Comparing the tree topologies of the 70% reciprocal bootstrap trees indicated no conflicts. Subsequently, the datasets were combined and this resulted in a data set consisting of 1 529 characters including gaps. Of these characters, 1 151 were constant and parsimony uninformative. The 378 parsimony informative characters included in the parsimony analyses yielded eight most parsimonious
trees (TL = 993, CI = 0.732, RI = 0.903, RC = 0.661), one of which is presented (Fig. 1). For Bayesian analyses, a HKY+I model was selected for BT, GTR+I+G model for HIS3 and a GTR+G model for TEF-1α and incorporated into the analyses. The consensus tree obtained for the Bayesian analyses confirmed the tree topology obtained with parsimony as well as bootstrap support (Fig. 1).

The majority of the Ca. pauciramosa isolates grouped together to form a monophyletic cluster with a bootstrap (BP) value of 100 and a Bayesian posterior probability (PP) value of 1.00. Within this cluster, two separate clades could be distinguished. The first (BP = 66, PP = 0.92) represented isolates obtained from South Africa (Table 1) and analyses of the SNP’s (Table 3) showed one fixed allele for BT, two for HIS3 and one indel for TEF-1α. The second clade (BP = 97, PP = 1.00) represented isolates from Italy (Table 1) that were closely related to Ca. pauciramosa and have a number of shared fixed polymorphisms; five BT and two HIS3 (Table 3). Isolates from Colombia (Table 1) grouped together (BP = 100, PP = 1.00), separate from the Ca. pauciramosa cluster and SNP analyses show that six BT, 13 HIS3 and nine TEF-1α shared fixed alleles including three indels are characteristic for this group (Table 3). These isolates were closely related to Ca. spathulata. Isolates from Brazil grouped together with isolate CBS 230.51 (ex-type of Cy. brasiliensis; BP = 100, PP = 1.00), closely related to Ca. morganii and Ca. insularis, but separate from both of these species. Analyses of the SNP’s for the isolates from Brazil compared to Ca. morganii and Ca. insulare also show several fixed alleles for these isolates, which include the ex-type culture of Cy. brasiliensis (CBS 230.51) (Table 4). The DNA sequence data for the three gene regions used in the present study showed 16 fixed alleles between Ca. brasiliensis, Ca. insularis and Ca. morganii (Table 4). An additional 10 fixed alleles were shared between Cy. brasiliensis and Ca. insularis and distinguished both species from Ca. morganii.

**Taxonomy**

Isolates CMW 9115, CMW 9188, CMW 9208, CMW 9215 and CMW 9896 represent a distinct species closely related to Ca. pauciramosa, based on phylogenetic inference. Mating studies also showed that these isolates have a homothallic mating system, distinguishing them from Ca. pauciramosa s. str. A similar situation was found for the isolates CBS 111136, CBS 115127, CBS 115638 and CBS 115694 from Colombia and they are also treated as a new species based on their homothallic mating system and phylogenetic inference. Furthermore, isolates CBS 123402, CMW 7804 and CMW 10151 from Italy are closely related to Ca.
pauciramosa and failed to cross with the mating tester strains of that species. However, morphological observations and DNA sequence data indicate that these isolates represent an undescribed taxon.

Species of Cylindrocladium (1892) represent anamorph states of Calonectria (1867) (Rossman et al. 1999). In this study, these fungi are described as new species of Calonectria, which represents the older generic name for these holomorphs. This is irrespective whether the teleomorph states of these fungi have been found or not. This is consistent with the new regulations on fungal nomenclature as proposed by Hawksworth (2005) stating that for all newly described pleomorphic fungal species, the teleomorph name takes precedence over the anamorph name when both types belong to the same holomorph taxon.

**Calonectria brasiliensis** (Bat. & Cif.) L. Lombard, M.J. Wingf. & Crous, **comb. nov.** MycoBank MB 515110. Fig. 2.

*Basionym:* Cylindrocladium brasiliensis (Bat. & Cif.) Peerally, (as braziliensis) CMI Descriptions of Pathogenic Fungi and Bacteria 427. 1974.

≡ Cylindrocladium scoparium var. brasiliensis Bat. & Cif., (as brasiilense) Boletim de SA.I.C. Pernambuco 18: 188–191. 1951.

*Teleomorph* unknown. *Conidiophores* with a stipe bearing a penicillate suite of fertile branches, stipe extensions, and terminal vesicles. *Stipe* septate, hyaline, smooth, 63–103 × 7–14 µm; stipe extensions septate, straight to flexuous, 204–266 µm long, 6–7 µm wide at the apical septum, terminating in a broadly clavate to ellipsoidal to fusiform vesicle, 7–11 µm diam. *Conidiogenous apparatus* 58–90 µm long, and 81–103 µm wide; primary branches aseptate or 1-septate, 25–34 × 5–8 µm; secondary branches aseptate, 14–25 × 4–7 µm; tertiary branches aseptate, 8–20 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 8–12 × 2–4 µm; apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (35–)36–40(–41) × 3–5 µm (av. = 38 × 3.5 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Megaconidia* and *microconidia* not seen.

*Specimens examined:* Brazil, Ceara State, *Eucalyptus* sp., Sep. 1948, T.R. Ciferri, ex-type culture CBS 230.51 = IMI 299576 = CMW 23671. Aracruz, *Eucalyptus* sp., June 1998, A.C.
Alfenas, CBS 114257 = CMW 32949. Rio de Janeiro, *E. citriodora*, A.O. Carvalho, CBS 116078 = CMW 32950. Champion nursery, *Eucalyptus* sp., P.W. Crous, CPC 602 = CMW 31507. Aracruz, *Eucalyptus* sp., P.W. Crous, CPC 1943 = CMW 31508.

**Culture characteristics:** Colonies fast growing with optimal growth temperature at 25 ºC (growth at 10–30 ºC) on MEA, reverse amber (13k) to sepiia-brown (13i) after 7 d; sparse white aerial mycelium with sparse sporulation; chlamydospores moderate throughout the medium, forming microsclerotia.

**Substrates:** *Eucalyptus* spp.

**Distribution:** Brazil.

**Notes:** Based on morphological observations, Crous & Wingfield (1994) reduced *Ca. brasiliensis* to synonymy with *Ca. morganii*. However, phylogenetic inference in this study has shown that the ex-type culture of *Ca. brasiliensis* (CBS 230.51) is distinct from *Ca. morganii* (CBS 110666). Morphological observations in this study also indicated that conidia of *Ca. brasiliensis* (av. 38 × 3.5 µm) are smaller than those of *Ca. morganii* (av. 45 × 4 µm). *Calonectria brasiliensis* only produces up to three branches per conidiophore, where as *Ca. morganii* can have up to six branches per conidiophore.

**Calonectria colombiana** L. Lombard, Crous & M.J. Wingf., sp. nov. MycoBank MB515065, Fig. 3.

**Etymology:** Name refers to Colombia, the country this fungus was isolated from.

Telomorpha *Calonectriae pauciramosa* similis, sed ascosporis brevioribus, (28–)31–36(–40) × 3–5 µm (in medio 34 × 4 µm). Culturae homothallicae. Anamorpha *Cylindrocladio pauciramoso* simile, sed vesiculis obpyriforme vel fusiforme (8–12 µm diam.) et conidiis maioribus (33–)35–39(–40) × 3–4 µm, in medio 37 × 3 µm.

**Perithecia** solitary or in groups, orange to red, becoming red-brown with age; in section, apex and body yellow to orange, base red-brown, sub-globose to ovoid, 270–410 µm high, 175–285 µm diam, body turning dark red, and base dark red-brown (KOH+). Perithecial walls rough, consisting of 2 thick-walled layers: outside layer of *textura globulosa*, 24–90 µm wide; becoming more compressed towards inner layer of *textura angularis*, 18–22 µm wide; becoming thin-walled and hyaline towards the center, outer cells, 38–55 × 16–40 µm; inner cells, 3–12 × 3–7 µm: perithecial base up to 114 µm wide; consisting of dark red, angular
cells; merging with an erumpent stroma, cells of the outer wall layer continuing into the pseudoparenchymatous cells of the erumpent stroma. *Asci* 8-spored, clavate, 87–162 × 12–18 µm, tapering to a long thin stalk. *Ascospores* aggregated in the upper third of the ascus, hyaline, glutulate, fusoid with rounded ends, straight to slightly curved, 1-septate, not or slightly constricted at the septum, (28–)31–36(–40) × 3–5 µm (av. = 34 × 4 µm). Cultures homothallic. *Conidiophores* with a stipe bearing a penicillate suite of fertile branches, stipe extensions, and terminal vesicles. *Stipe* septate, hyaline, smooth, 45–126 × 6–9 µm; stipe extensions septate, straight to flexuous, 143–173 µm long, 5–7 µm wide at the apical septum, terminating in an obpyriform to fusiform vesicle, 8–12 µm diam. *Conidiogenous apparatus* 38–115 µm long, and 35–91 µm wide; primary branches aseptate or 1-septate, 19–37 × 5–8 µm; secondary branches aseptate, 9–17 × 4–5 µm; tertiary and additional branches (–4), aseptate, 8–13 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 9–12 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (33–)35–39(–40) × 3–4 µm (av. = 37 × 3 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Megaconidia* and *microconidia* not seen.

**Specimen examined:** Colombia, La Selva, from soil, June 1995, M.J. Wingfield, Herb. PREM 60295, **holotype** of *Calonectria colombiana*, cultures ex-type CBS 115127 = CMW 30871 = CPC 1160; La Selva, June 1995, M.J. Wingfield, CBS 111041 = CMW 30767 = CPC 1163; La Selva, June 1995, M.J. Wingfield, CBS 111136 = CMW 30812 = CPC 1151; CBS 115638 = CMW 30766 = CPC 1161 (Herb. PREM 60296); La Selva, June 1995, M.J. Wingfield, CBS 115694 = CMW 30813 = CPC 1162; La Selva, June 1995, M.J. Wingfield, CMW 9058.

**Culture characteristics:** Colonies fast growing with optimal growth temperature at 25 ºC (growth at 10–30 ºC) on MEA, reverse amber (13k) to sephia-brown (13i) after 7 d; abundant white aerial mycelium with sparse sporulation; chlamydospores extensive throughout the medium, forming microsclerotia.

**Substrate:** Soil.

**Distribution:** Colombia.
Notes: Isolates of *Ca. colombiana* were previously regarded as either *Ca. pauciramosa* or *Ca. scoparia* (Crous 2002) based on the morphological similarity of the anamorph states of these species. Based on macroconidial dimensions, *Ca. colombiana* (av. 37 × 3 µm) can be distinguished from *Ca. pauciramosa* (av. 50 × 4.5 µm) and *Ca. scoparia* (av. 60 × 4.5 µm) in having smaller, 1-septate macroconidia. Both *Ca. pauciramosa* and *Ca. scoparia* have a biallelic, heterothallic mating system (Schoch et al. 1999, 2001), whereas *Ca. colombiana* is homothallic.

*Calonectria polizzii* L. Lombard, Crous & M.J. Wingf., sp. nov. MycoBank MB515066, Fig. 4.

*Etymology:* The name honours Prof. dr. Giancarlo Polizzi, who isolated the fungus in Italy.

Teleomorth ignota. *Cylindrocladio pauciramoso* simile, sed vesiculis clavato vel obpyriforme (6–9 µm diam.) et conidiis maioribus (31–)32–42(–49) × 3–5 µm, in medio 37 × 4 µm.

*Teleomorph* unknown. *Conidiophores* with a stipe bearing a penicillate suite of fertile branches, stipe extensions, and terminal vesicles. *Stipe* septate, hyaline, smooth, 58–108 × 5–7 µm; stipe extensions septate, straight to flexuous, 111–167 µm long, 5–6 µm wide at the apical septum, terminating in a broadly clavate to pyriform vesicle, 6–9 µm diam. *Conidiogenous apparatus* 27–57 µm long, and 28–51 µm wide; primary branches aseptate or 1-septate, 15–35 × 4–6 µm; secondary branches aseptate, 12–26 × 3–5 µm; tertiary branches aseptate, 10–15 × 4–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 8–13 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (31–)32–42(–49) × 3–5 µm (av. = 37 × 4 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Megaconidia* and *microconidia* not seen.

*Specimen examined:* Italy, Sicily, Carrubba, on *Arbutus unedo*, 1997, G. Polizzi, Herb. PREM 60297, *holotype* of *Calonectria polizzii*, cultures ex-type CBS 123402 = CMW 30872; *Callistemon citrinus*, 1997, G. Polizzi, CMW 7804 = CPC 2681 = CBS 125270; *Callistemon citrinus*, 1997, G. Polizzi CMW 10151 = CPC 2771 = CBS 125271 (Herb. PREM 60298).

*Culture characteristics:* Colonies fast growing with optimal growth temperature at 25 ºC (growth at 10–30 ºC) on MEA, reverse amber (13k) to sepia-brown (13i) after 7 d; abundant
white aerial mycelium with sparse sporulation; chlamydospores extensive throughout the medium, forming microsclerotia.

**Substrates:** *Arbutus unedo, Callistemon citrinus.*

**Distribution:** Italy.

**Notes:** *Calonectria polizzii* is morphologically similar to *Ca. pauciramosa* and *Ca. zuluensis.* The macroconidia of *Ca. polizzii* (av. 37 × 4 µm) are smaller to those of *Ca. pauciramosa* (av. 50 × 4.5 µm). Mating tests also showed that *Ca. polizzii* does not mate with either of the tester strains of *Ca. pauciramosa* (Schoch *et al.* 2001) used in this study. However, the isolates of *Ca. polizzii* tested might represent a single mating type, or might have lost their ability to mate, and further studies incorporating more isolates will be required to confirm this.

*Calonectria zuluensis* L. Lombard, Crous & M.J. Wingf., **sp. nov.** MycoBank MB515067, Fig. 5.

**Etymology:** Name refers to KwaZulu-Natal, South Africa, the province where the fungus was isolated.

Telomorpha *Calonectria pauciramosa* similis, sed ascosporis brevioribus, (26–)29–34(–38) × 4–5 µm (in medio 32 × 4 µm). Culturae homothallicae. Anamorpha *Cylindrocladion pauciramoso* simile, sed vesiculis clavato vel obpyriforme (6–10 µm diam) et conidiis maioribus (31–)34–38(–40) × 3–5 µm, in medio 36 × 4 µm.

**Perithecia** solitary or in groups, orange to red, becoming red-brown with age; in section apex and body yellow to orange, base red-brown, sub-globose to ovoid, 292–394 µm high, 170–285 µm diam, body turning dark red, and base dark red-brown (KOH+). Perithecial walls rough, consisting of 2 thick-walled layers: outside layer of *textura globulosa*, 30–80 µm wide; becoming more compressed towards inner layer of *textura angularis*, 20–22 µm wide; becoming thin-walled and hyaline towards the center, outer cells, 40–50 × 18–40 µm; inner cells, 4–12 × 3–5 µm: perithecial base up to 116 µm wide; consisting of dark red, angular cells; merging with an erumpent stroma, cells of the outer wall layer continuing into the pseudoparenchymatous cells of the erumpent stroma. *Asci* 8-spored, clavate, 92–140 × 10–16 µm, tapering to a long thin stalk. *Ascospores* aggregate in the upper third of the ascus, hyaline, gluttulate, fusoid with rounded ends, straight to slightly curved, 1-septate, not or slightly constricted at the septum, (26–)29–34(–38) × 4–5 µm (av. = 32 × 4 µm). Cultures
homothallic. **Conidiophores** with a stipe bearing penicillate clusters of fertile branches, stipe extensions, and terminal vesicles. **Stipe** septate, hyaline, smooth, 57–84 × 6–9 µm; stipe extensions septate, straight to flexuous, 110–171 µm long, 5–8 µm wide at the apical septum, terminating in a broadly clavate to obpyriform vesicle, 6–10 µm diam. **Conidiogenous apparatus** 35–67 µm long, and 37–70 µm wide; primary branches aseptate or 1-septate, 16–28 × 4–6 µm; secondary branches aseptate, 11–20 × 3–5 µm; tertiary branches aseptate, 8–13 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides doliform to reniform, hyaline, aseptate, 10–13 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. **Conidia** cylindrical, rounded at both ends, straight, (31–)34–38(–40) × 3–5 µm (av. = 36 × 4 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. **Megaconidia** and **microconidia** not seen.

**Specimen examined:** **South Africa**, KwaZulu-Natal, Kwambonambi, from *Eucalyptus grandis* clonal cutting, Feb. 2001, L. Lombard, Herb. PREM 60292, **holotype** of *Calonectria zuluensis*, cultures ex-type CBS 125268 = CMW 9188; KwaZulu-Natal, Kwambonambi, *E. grandis × urophylla* hybrid cutting, Feb. 2001, L. Lombard, CMW 9115, CMW 9208 (Herb. PREM 60293), CMW 9215, Pietermarizburg, *E. grandis × urophylla* hybrid cutting, Mar. 2001, L. Lombard, CMW 9896 = CBS 125272.

**Culture characteristics:** Colonies fast growing with optimal growth temperature at 25 ºC (growth at 10–30 ºC) on MEA, reverse amber (13k) to sepia-brown (13i) after 7 d; abundant white aerial mycelium with sparse sporulation; chlamydospores extensive throughout the medium, forming microsclerotia.

**Substrate:** *Eucalyptus grandis* rooted cuttings, *E. grandis × urophylla* rooted cuttings

**Distribution:** South Africa.

**Notes:** *Calonectria zuluensis* can be distinguished from *Ca. pauciramosa* and *Ca. scoparia* based on its homothallic mating system. Macroconidia of *Ca. zuluensis* (av. 36 × 4 µm) are also smaller than those of *Ca. pauciramosa* (av. 50 × 4.5 µm) and *Ca. scoparia* (av. 60 × 4.5 µm). This species is morphologically very similar to *Ca. colombiana*. However, *Ca. zuluensis* can be distinguished from *Ca. colombiana* based on the fact that it has a broadly clavate to obpyriform vesicle as compared with a obpyriform to fusiform vesicle in *Ca.
colombiana. Furthermore, Ca. zuluensis can easily be distinguished based on phylogenetic inference.

DISCUSSION

Considerable variation observed amongst isolates of “Ca. pauciramosa” from different geographical localities was illustrated in this study. Morphological characteristics, phylogenetic inference and mating studies revealed the presence of three cryptic species accommodated in cultures that have collectively been treated as Ca. pauciramosa. This is consistent with the results of previous studies (Schoch et al. 1999, 2001), which noted variation within Ca. pauciramosa, although at that time the sample size was inordinately small to consider the matter further. Schoch et al. (2001) also noted a high level of variation among isolates from South America, but concluded that this most likely reflected diversity consistent with an endemic population.

Crous (2002) suggested that mating isolates with recognised mating tester strains represented an important step in identifying isolates of Ca. pauciramosa. Various studies (Crous et al. 1993, Crous & Wingfield 1994, Crous et al. 1998, Schoch et al. 1999, 2001, Crous 2002) have used CLA as standardised medium to study sexual compatibility amongst isolates of Cylindrocladium. However, CLA has its limitations in that carnation leaf pieces are not always available and the present study used both CLA and MSA amended with sterile tooth picks, which proved to be very successful. Effective application of the latter technique to induce teleomorphs in culture has also been achieved for various other plant pathogenic genera, including Glomerella (Geurber & Correll 2001) and Neonectria (Halleen et al. 2006).

The descriptions of Ca. colombiana, Ca. zuluensis and Ca. polizzii add three new species to the Ca. scoparia species complex. This complex is characterised by species having ellipsoidal to obpyriform vesicles and producing 1-septate macroconidia (Schoch et al. 1999, Crous 2002). The complex was previously regarded as having a biallelic, heterothallic mating system (Schoch et al. 1999, 2001). However, both the newly described Ca. colombiana and Ca. zuluensis are homothallic. The occurrence of both heterothallic and homothallic Calonectria species in a single complex is not unique, having previously been found in the Ca. kyotensis species complex (Crous et al. 2004b).
Schoch *et al.* (2001) considered female fertility of *Ca. pauciramosa*, and found variation in BT sequence data for isolates from Italy. This variation has most likely been captured in the description of *Ca. polizzii* in the present study. This new species has thus been shown as unique based on morphological, phylogenetic inference and biological characteristics, separating it from *Ca. pauciramosa*. Morphologically, *Ca. polizzii* can be distinguished from *Ca. pauciramosa* by its smaller 1-septate macroconidia. Isolates of *Ca. polizzii* were also not capable of mating with the *Ca. pauciramosa* mating-tester strains or other *Ca. pauciramosa* isolates from different geographic regions.

Schoch *et al.* (2001), noted variation amongst isolates of *Ca. pauciramosa* from South America, and suggested that the fungus could be native to that continent. Results of the present study, including isolates from Colombia, led to the description of *Ca. colombiana*. This fungus is distinct from *Ca. pauciramosa* in having a homothallic mating system, smaller macroconidia and quaternary branches on the conidiophores. Although *Ca. insularis* also forms conidiophores with quaternary branches (Schoch *et al.* 1999), *Ca. colombiana* can easily be distinguished from it based on DNA sequence comparisons and its homothallic mating system.

Various species of *Calonectria* have been recorded from South Africa (Crous *et al.* 1991, Crous *et al.* 1993, Schoch *et al.* 1999, Crous 2002) and the description of *Ca. zuluensis* adds another species to those already reported from the country. *Calonectria zuluensis* has a homothallic mating system, which is different from *Ca. pauciramosa* with a biallelic, heterothallic mating system (Schoch *et al.* 2001). The two species can also easily be distinguished from each other based on DNA sequence comparisons.

In the analyses of the SNP’s for the three gene regions used in this study, several fixed and shared SNP alleles were found for *Ca. colombiana*, *Ca. polizzii* and *Ca. zuluensis*. The majority of the fixed SNPs are shared between *Ca. polizzii* and *Ca. zuluensis*, indicating that these are sibling species, and that genetic isolation between them occurred recently (Taylor *et al.* 2000). For *Ca. colombiana*, fewer of the fixed SNPs are shared with *Ca. polizzii* and *Ca. zuluensis*, indicating that speciation occurred less recently than that of *Ca. polizzii* and *Ca. zuluensis*. These three species do not share the same alleles with *Ca. pauciramosa*, clearly distinguishing it from them.
*Calonectria brasiliensis* has been elevated to species level based on phylogenetic inference. Although Peerally (1974) indicated that the macroconidia of *Ca. brasiliensis* (24–38 × 2–3 µm) are smaller than those of *Ca. morganii* (av. 45 × 4 µm), Crous & Wingfield (1994) reduced *Ca. brasiliensis* to *Ca. morganii*, based on similar conidial dimensions and vesicles morphology observed in culture. It is possible, however, that the original ex-type strain of *Ca. brasiliensis* was in fact morphologically degenerated, appearing atypical for the species. Several isolates from Brazil, previously identified as *Ca. pauciramosa*, grouped with the ex-type strain of *Ca. brasiliensis* (CBS 230.51). Previous DNA sequence comparisons and mating studies with *Ca. morganii* (Crous et al. 1993, Overmeyer et al. 1996, Schoch et al. 2000, 2001) failed to include the ex-type strain CBS 230.51 of *Ca. brasiliensis*, as this species was seen as a synonym of *Ca. morganii* (Crous 2002).

This study has shown the importance of combining morphological, biological and phylogenetic data to identify cryptic species of *Calonectria*. Although the biological species concept is regarded as insufficient for this purpose and needs to be clearly defined in *Calonectria* (Crous 2002), this study has shown that it has some use in identifying cryptic species within *Ca. pauciramosa*. However, morphology in combination with phylogenetic inference provides the most useful approach to identify cryptic species in *Calonectria* (Lombard et al. 2009). The present study has also shown the importance of the multi-gene approach in studying the phylogenetic relationships of phenotypic closely related *Calonectria* spp.
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Table 1. Isolates of *Calonectria pauciramosa* and other *Calonectria* species studied.

| Species           | Isolate     | Mating system | GenBank Assession nr. | Host         | Origin | Collector       |
|-------------------|-------------|---------------|-----------------------|--------------|--------|-----------------|
|                   |             | β-tubulin     | Histone H3 | TEF-1α   |           |                 |
| *Ca. brasiliensis*| CBS 230.51T | GQ267241      | GQ267259 | GQ267328 | Eucalyptus sp. | Brazil | T.R. Ciferri    |
|                   | (= IMI 299576) |             |           |         |           |       |                 |
|                   | CBS 114257  | GQ267242      | GQ267260 | GQ267329 | Leaf litter | Brazil | A.C. Alfenas    |
|                   | CBS 116078  | GQ421772      | GQ421780 | GQ421788 | *E. citriodora* | Brazil | A.O. Carvalo    |
|                   | (= UFO 202) |             |           |         |           |       |                 |
|                   | CMW 31505   | GQ421775      | GQ421783 | GQ421791 | Prunus sp.   | South Africa | C. Linde       |
|                   | (= CPC 2581) |             |           |         |           |       |                 |
|                   | CMW 31507   | GQ421773      | GQ421781 | GQ421789 | Eucalyptus sp. | Brazil | P.W. Crous      |
|                   | (= CPC 602) |             |           |         |           |       |                 |
|                   | CMW 31508   | GQ421774      | GQ421782 | GQ421790 | Leaf litter | Brazil | A.C. Alfenas    |
|                   | (= CPC 1943) |             |           |         |           |       |                 |
| *Ca. colombiana* sp. nov. | CBS 111136 | homothallic   | FJ972424 | FJ972443 | Soil | Colombia | M.J. Wingfield |
|                   | CBS 115127T | homothallic   | FJ972423 | FJ972442 | Soil | Colombia | M.J. Wingfield |
|                   | CBS 115638  | homothallic   | FJ972422 | FJ972441 | Soil | Colombia | M.J. Wingfield |
Table 1. (Continued)

| Species          | Isolate | Mating Type | GenBank Assession nr. | Host            | Origin   | Collector  |
|------------------|---------|-------------|-----------------------|-----------------|----------|------------|
|                  |         |             | β-tubulin | Histone H3 | TEF-1α |             |            |
|                  | CBS 115694 | homothallic | FJ972425 | FJ972444 | FJ972494 | Soil | Colombia | M.J. Wingfield |
| Ca. colombiensis | CMW 9058 | homothallic | FJ972420 | FJ972439 | FJ972489 | Soil | Colombia | M.J. Wingfield |
| Ca. insularis    | CBS 112221 |                  | AY725620 | AY725663 | AY725712 | Soil | Colombia | M.J. Wingfield |
| Ca. mexicana     | CBS 114558 |                  | AF210861 | FJ918526 | FJ918556 | Soil | Madagascar | P.W. Crous |
|                  | CBS 114559 |                  | AF210862 | FJ918525 | FJ918555 | Soil | Madagascar | C. L. Schoch |
| Ca. morganii     | CBS 110918T |                  | AF210863 | FJ972460 | FJ972526 | Soil | Mexico | M.J. Wingfield |
|                  | CBS 110666 |                  | FJ918509 | FJ918527 | FJ918557 | Ilex vomitoria | USA | N.E. El-Gholl |
|                  | CBS 119669 |                  | DQ521599 | DQ521601 | GQ421796 | Pistacia lentiscus | Italy | G. Polizzi |
|                  | CBS 119670 |                  | DQ521600 | DQ521602 | GQ421797 | Pistacia lentiscus | Italy | G. Polizzi |
|                  | CMW 31506 (= P94-4359) |                  | AF210875 | GQ421787 | GQ421795 | Dodenaea vicosa | USA | N.E. El-Gholl |
| Ca. pauciramosa  | CMW 1786 | Unknown       | FJ972378 | FJ972445 | FJ972495 | Eucalyptus smithii | South Africa | M.J. Wingfield |
|                  | CMW 2151 | Mat1-2        | FJ972400 | FJ972468 | FJ972517 | E. nitens | South Africa | M.J. Wingfield |
|                  | CMW 5683T | Mat1-2        | FJ918514 | FJ918531 | FJ918565 | E. grandis | South Africa | P.W. Crous |
Table 1. (Continued)

| Species          | Isolate | Mating Type | GenBank Assesstnr. | Host     | Origin | Collector     |
|------------------|---------|-------------|--------------------|----------|--------|---------------|
| *Ca. pauciramosa* | CMW 7592| Mat1-1      | FJ972380 FJ972447 FJ972497 | *E. grandis* | Uruguay | M.J. Wingfield |
|                  | CMW 7597| Mat1-1      | FJ972406 FJ972474 FJ972523 | *E. grandis* | Uruguay | M.J. Wingfield |
|                  | CMW 7600| Mat1-1      | FJ972405 FJ972473 FJ972522 | *E. grandis* | Uruguay | M.J. Wingfield |
|                  | CMW 7826| Mat1-2      | FJ972392 FJ972459 FJ972509 | Soil     | Australia | P.W. Crous     |
|                  | CMW 7827| Mat1-2      | FJ972385 FJ972452 FJ972502 | Soil     | Australia | P.W. Crous     |
|                  | CMW 7828| Mat1-2      | FJ972391 FJ972458 FJ972508 | Soil     | Australia | P.W. Crous     |
|                  | CMW 7849| Mat1-2      | FJ972383 FJ972450 FJ972500 | *Erica sp.* | USA     | S.T. Koike     |
|                  | CMW 7851| Mat1-2      | FJ972382 FJ972449 FJ972499 | *Mytrus communis* | USA     | S.T. Koike     |
|                  | CMW 7852| Mat1-2      | FJ972381 FJ972448 FJ972498 | *M. communis* | USA     | S.T. Koike     |
|                  | CMW 8061| Mat1-2      | FJ972386 FJ972453 FJ972503 | Soil     | Australia | P.W. Crous     |
|                  | CMW 9151| Mat1-2      | FJ972384 FJ972451 FJ972501 | *Acacia mearnsii* | South Africa | L. Lombard |
|                  | CMW 9172| Mat1-2      | FJ972379 FJ972446 FJ972496 | *A. mearnsii* | South Africa | L. Lombard |
|                  | CMW 10148| Mat1-2      | FJ972387 FJ972454 FJ972504 | *Erica sp.* | USA     | S.T. Koike     |
| Species        | Isolate | Mating Type | GenBank Assession nr. | Host      | Origin   | Collector |
|---------------|---------|-------------|-----------------------|-----------|----------|-----------|
| *Ca. pauciramosa* | CBS 102296 | Mat1-2      | FJ972404 FJ972472 FJ972521 | Vriessea sp. | New Zealand | H.M. Dance |
|               | CBS 110945 | Mat1-1      | FJ972389 FJ972456 FJ972506 | *Podocarpus* sp. | South Africa | P.W. Crous |
|               | CBS 111873 | Mat1-1      | FJ972399 FJ972467 FJ972516 | *Prunus* sp. | South Africa | C. Linde  |
|               | CBS 114861 | Mat1-1      | FJ972403 FJ972471 FJ972520 | *Eucalyptus* sp. | South Africa | P.W. Crous |
|               | CBS 115670 | Mat1-1      | FJ972393 FJ972461 FJ972510 | *Pinus* sp. | South Africa | P.W. Crous |
|               | CBS 115893 | Unknown     | FJ972411 FJ972430 FJ972480 |           |          |           |
|               | CMW 30819  | Mat1-2      | FJ972402 FJ972470 FJ972519 | *E. grandis* | South Africa | P.W. Crous |
|               | CMW 30875  | Mat1-1      | FJ972390 FJ972457 FJ972507 | *Eucalyptus* sp. | South Africa | P.W. Crous |
|               | CMW 30823  | Mat1-1      | FJ918515 FJ918532 FJ918566 | *E. grandis* | South Africa | P.W. Crous |
|               | CMW 30814  | Unknown     | FJ972408 FJ972427 FJ972477 | *Eucalyptus* sp. | Kenya       | J. Roux   |
|               | CMW 30822  | Unknown     | FJ972409 FJ972428 FJ972478 | *Eucalyptus* sp. | Kenya       | J. Roux   |
|               | CMW30873   | Mat1-2      | FJ972388 FJ972455 FJ972505 | *Eucalyptus* sp. | South Africa | L. Lombard |
|               | CMW 27203  | Mat1-2      | FJ972398 FJ972466 FJ972515 | *Eucalyptus* sp. | China       | S. Chen   |
Table 1. (Continued)

| Species                     | Isolate | Mating Type | GenBank Assession nr. | Host              | Origin     | Collector      |
|-----------------------------|---------|-------------|-----------------------|-------------------|------------|----------------|
| *Ca. pauciramosa*           | CMW 27206 | Mat1-2      | FJ972396 FJ972464 FJ972513 | *Eucalyptus* sp. | China      | S. Chen        |
|                             | CMW 27283 | Mat1-2      | FJ972397 FJ972465 FJ972514 | *Eucalyptus* sp. | China      | S. Chen        |
|                             | CMW 30878 | Mat1-1      | FJ972401 FJ972469 FJ972518 | *Prunus* sp.     | South Africa | C. Linde       |
|                             | CMW 30818 | Mat1-2      | FJ972395 FJ972463 FJ972512 | *Limonium* sp.   | New Zealand | I. Brice       |
|                             | CMW 30817 | Unknown     | FJ972394 FJ972462 FJ972511 | *Rhododendron* sp.| New Zealand | R.A.J. White   |
|                             | CMW 30879 | Mat1-2      | FJ972407 FJ972475 FJ972524 | *Azalea* sp.     | Germany     | G. Hagedorn    |
|                             | CMW 30815 | Unknown     | FJ972410 FJ972429 FJ972479 | *Eucalyptus* sp. | South Africa | P.W. Crous     |
| *Ca. polizii* sp. nov.     | CBS 123402T |            | FJ972419 FJ972438 FJ972488 | *Arbutus unedo*  | Italy       | G. Polizzi     |
|                             | CMW 7804  |             | FJ972417 FJ972436 FJ972486 | *Callistemon citrinus* | Italy     | G. Polizzi     |
|                             | CMW 10151 |             | FJ972418 FJ972437 FJ972487 | *A. unedo*       | Italy       | G. Polizzi     |
| *Ca. scoparia*              | CMW 31000 |             | FJ972426 FJ972476 FJ97252 | *Eucalyptus* sp. | Brazil      | A.C. Alfenas   |
|                             | CMW 31001 |             | GQ421779 GQ267246 GQ267246 | *Eucalyptus* sp. | Brazil      | A.C. Alfenas   |
|                             | CBS 116076|             | GQ421776 GQ421784 GQ421792 | *Eucalyptus* sp. | Brazil      | P.W. Crous     |
| Species | Isolate | Mating Type | GenBank Assession nr. | Host          | Origin  | Collector       |
|---------|---------|-------------|----------------------|---------------|---------|-----------------|
| Ca. scoparia | CBS 116081 |  | GQ421777 GQ42178 GQ421793 | Soil         | Brazil | M.J. Wingfield |
| Ca. scoparia | CMW 7578 |  | GQ421778 GQ421786 GQ421794 | E. grandis   | Argentina | L. Lombard |
| Ca. spathulata | CBS 112689 |  | AF308463 FJ918524 FJ918554 | E. viminalis  | Brazil | N.E. El-Gholl |
| Ca. spathulata | CBS555.92T | homothallic | GQ267215 GQ267261 GQ267331 | Araucaria angustifolia | Brazil | C. Hodges |
| Ca. zuluensis sp. nov. | CMW 9115 | homothallic | FJ972413 FJ972432 FJ972482 | Eucalyptus sp. | South Africa | L. Lombard |
| Ca. zuluensis sp. nov. | CMW 9188T | homothallic | FJ972414 FJ972433 FJ972483 | Eucalyptus sp. | South Africa | L. Lombard |
| Ca. zuluensis sp. nov. | CMW 9208 | homothallic | FJ972412 FJ972431 FJ972481 | Eucalyptus sp. | South Africa | L. Lombard |
| Ca. zuluensis sp. nov. | CMW 9215 | homothallic | FJ972416 FJ972435 FJ972485 | Eucalyptus sp. | South Africa | L. Lombard |
| Ca. zuluensis sp. nov. | CMW 9896 | homothallic | FJ972415 FJ972434 FJ972484 | Eucalyptus sp. | South Africa | L. Lombard |
| Cy chinense | CBS 112744 |  | AY725618 AY725660 AY725709 | Soil         | China   | M.J. Wingfield |
| Cy. hawksworthii | CBS 111870T |  | AF333407 DQ190649 FJ918558 | Nelumbo nucifera | Mauritius | A. Peerally |
| Cy. leucothoës | CBS 109166T |  | FJ918508 FJ918523 FJ918553 | Leucothoë axillaris | USA | N.E. El-Gholl |

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 Ex-type cultures.
Table 2. Results of mating studies between isolates of *Calonectria pauciramosa* from various geographic regions.

| CBS 102296 | CBS 110945 | CBS 110953 | CBS 111873 | CBS 114861 | CBS 115670 | CMW 2151 | CMW 5683 | CMW 7592 | CMW 7597 | CMW 7600 | CMW 7826 | CMW 7827 | CMW 7828 | CMW 7849 | CMW 7851 | CMW 7852 | CMW 8061 | CMW 9151 | CMW 9172 | CMW 27203 | CMW 27206 | CMW 27283 | CMW 30817 | CMW 30818 | CMW 30819 | CMW 30823 | CMW 30873 | CMW 30875 | CMW 30876 | CMW 30877 | CMW 30878 | CMW 30879 | CMW 30880 |
|------------|------------|------------|------------|------------|------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| -          | -          | +          | -          | -          | +          | -        | -        | -        | +        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        |
| +          | -          | -          | +          | -          | -          | -        | +        | +        | +        | +        | +        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        |
|            |            |            |            |            |            |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| Isolates in bold indicate *Ca. pauciramosa* mating tester strains. + indicates formation of perithecia with viable ascospores; - indicates no perithecial formation.
Table 3. Single nucleotide polymorphisms from the β-tubulin, histone H3 and translation elongation factor-1α sequence data of *Calonectria* isolates from Colombia, Italy and South Africa.

| Species          | Isolate no.   | β-tubulin | Histone H3 | Translation elongation factor-1α |
|------------------|---------------|-----------|------------|--------------------------------|
| *Ca. pauciramosa*| CMW 5685      | A A C G A | C C G C C  | T T G C G C A T G C A C T T C C T T G C A A T T T - A C C T |
|                  | CMW 9825      | A A C G A | C C G C C  | T T G C G C A T G C A C T T C C T T G C A A T T T - A C C T |
| *Ca. colombiana* | CBS 111135    | G C A G A | C C A A C  | T T A T G C A T G A T T T C C T T C T C A A G G T T T |
|                  | CBS 115127    | G C A G A | C C A A C  | T T A T G C A T G A T T T C C T T C T C A A G G T T T |
|                  | CBS 115638    | G C A G A | C C A A C  | T T A T G C A T G A T T T C C T T C T C A A G G T T T |
|                  | CBS 115694    | G C A G A | C C A A C  | T T A T G C A T G A T T T C C T T C T C A A G G T T T |
|                  | CMW 9058      | G C A G A | C C A A C  | T T A T G C A T G A T T T C C T T C T C A A G G T T T |
|                  | CMW 9115      | G A T C G | A G T G T G G C T C C G A A T C T C C T C C C T T G C A A A T T A C C C |
|                  | CMW 9180      | G A T C G | A G T G T G G C T C C G A A T C T C C T C C C T T G C A A A T T A C C C |
|                  | CMW 9208      | G A T C G | A G T G T G G C T C C G A A T C T C C T C C C T T G C A A A T T A C C C |
|                  | CMW 9215      | G A T C G | A G T G T G G C T C C G A A T C T C C T C C C T T G C A A A T T A C C C |
|                  | CMW 9896      | G A T C G | A G T G T G G C T C C G A A T C T C C T C C C T T G C A A A T T A C C C |

Yellow = unique SNP’s; Green = shared SNP’s
Table 4. Single nucleotide polymorphisms from the sequence data of β-tubulin, histone H3 and translation elongation factor-1α of Ca. brasiiliensis, Ca. insulare and Ca. morganii used in this study.

| Species     | Isolate no. | β-tubulin | Histone H3 | Translation elongation factor-1α |
|-------------|-------------|-----------|------------|-----------------------------------|
| Ca. brasiliensis | CBS 230.51  | C C A T   | T G - - -  | G T G T C T A T G T A C G - - - T T T F C C T G T |
|             | CBS 114257  | C C A T   | T G - - -  | G T G T C T A T G T A C G - - - T T T F C C T G T |
|             | CBS 116078  | C C A T   | T G - - -  | G T G T C T A T G T A C G - - - T T T F C C T G T |
|             | CMW 31508   | C C A T   | T G - - -  | G T G T C T A T G T A C G - - - T T T F C C T G T |
| Ca. insulare | CBS114558   | C C T G C | T G T T C A | G T G T C C C A C G T A C G T A C C C G A T C A A C C |
|             | CBS 114559  | C C T G C | T G T T C A | G T G T C C C A C G T A C G T A C C C G A T C A A C C |
| Ca. morganii | CBS114354   | A A T A T C C | A C T C T C C C C G T A G G A A - - - C - T C G A T C A C |
|             | CBS 115068  | A A T A T C C | A C T C T C C C C G T A G G A A - - - C - T C G A T C A C |
|             | CBS 119069  | A A T A T C C | A C T C T C C C C G T A G G A A - - - C - T C G A T C A C |
|             | CMW 31506   | A A T A T C C | A C T C T C C C C G T A G G A A - - - C - T C G A T C A C |
|             | CMW 31505   | A A T A T C C | A C T C T C C C C G T A G G A A - - - C - T C G A T C A C |

Yellow = unique SNP’s; Green = shared SNP’s
Fig. 1. One of eight most parsimonious trees obtained from a heuristic search with 1 000 random addition of the combined BT, HIS3 and TEF-1α sequence alignments. 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 and Bayesian consensus tree. The tree was rooted to Calonectria colombiensis (CBS 112221) and Cylindrocladium chinense (CBS 112744). Mating tester strains of Ca. pauciramosa used in this study are indicated in bold.
**Fig. 2.** *Calonectria brasiliensis.* A–E. Anamorph state of *Ca. brasiliensis.* A. Macroconidiophore. B–C. Vesicles. D. Fertile branches of the conidiophore with doliiform to reniform phialides. E. Macroconidia. Scale bars in A = 20 µm, B–E = 10 µm.
**Fig. 3.** *Calonectria colombiana.* A–F. Teleomorph state of *Ca. colombiana.* G–L. Anamorph state of *Ca. colombiana.* A. Perithecium on toothpick. B. A 10 µm thick vertical section through perithecium. C. Section through perithecial wall. D. Ostiolar region of perithecium. E. Asci and ascospores. F. Ascospore. G–H. Macroconidiophores of *Ca. colombiana.* I–J. Vesicles. K. Fertile branches of the conidiophore with doliiform to reniform phialides. L. Macroconidia. Scale bars: A = 50 µm, B, D = 20 µm, C, E–H = 10 µm.
**Fig. 4.** *Calonectria polizzi*. A–E. Anamorph state of *Ca. polizzi*. A. Macroconidiophore. B–C. Vesicles. D. Fertile branches of the conidiophore with doliiform to reniform phialides. E. Macroconidia. Scale bars in A = 20 µm, B–E = 10 µm.
**Fig. 5.** *Calonectria zuluensis.* A–F. Teleomorph state of *Ca. zuluensis.* G–L. Anamorph state of *Ca. zuluensis.* A. Perithecium on toothpick. B. A 10 µm thick vertical section through perithecium. C. Section through perithecial wall. D. Ostiolar region of perithecium. E. Asci and ascospores. F. Ascospore. G–H. Macroconidiophores of *Ca. zuluensis.* I–J. Vesicles. K. Fertile branches of the conidiophore with doliiform to reniform phialides. L. Macroconidia. Scale bars: A = 50 µm, B, D = 20 µm, C, E–H = 10 µm.
