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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 indicated that it included cryptic species, some of which are identified in this study. 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. polizzii* sp. nov. from Italy and *Ca. zuluensis* sp. nov. from South Africa, all of which had distinguishing morphological features. Based on DNA sequence data, *Ca. brasiliensis* is also elevated to species level.

Key words: *Calonectria*, plant pathogens, sexual compatibility, systematics.
Taxonomic novelties: *Calonectria brasiliensis* (Bat. & Cif.) L. Lombard, M.J. Wingf. & Crous, comb. nov., *Calonectria colombiana* L. Lombard, Crous & M.J. Wingf., sp. nov., *Calonectria polizzii* L. Lombard, Crous & M.J. Wingf., sp. nov., *Calonectria zuluensis* L. Lombard, Crous & M.J. Wingf., 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* (= *Cylindrocladium 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, 2009, Vitale *et al.* 2009), 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, Polizzi *et al.* 2006, 2007, 2009, Vitale *et al.* 2009).

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* was endemic to South America given that the ratio of both mating types approached 1:1. Furthermore, the 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 and 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), indicating 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 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

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Table 1. Isolates of *Calonectria pauciramosa* and other *Calonectria* species studied.

Species	Isolate	Mating type	GenBank accession no.			Host	Country	Collector
			β-tubulin	Histone H3	Translation elongation factor-1α			
Ca. brasiliensis	CBS 230.51[T] (= IMI 299576)		GQ267241	GQ267259	GQ267328	*Eucalyptus* sp.	Brazil	T.R. Ciferri
	CBS 114257		GQ267242	GQ267260	GQ267329	Leaf litter	Brazil	A.C. Alfenas
	CBS 116078 (= UFO 202)		GQ421772	GQ421780	GQ421788	*E. citriodora*	Brazil	A.O. Carvalo
	CMW 31505 (= CPC 2581)		GQ421775	GQ421783	GQ421791	*Prunus* sp.	South Africa	C. Linde
	CMW 31507 (= CPC 602)		GQ421773	GQ421781	GQ421789	*Eucalyptus* sp.	Brazil	P.W. Crous
	CMW 31508 (= CPC 1943)		GQ421774	GQ421782	GQ421790	Leaf litter	Brazil	A.C. Alfenas
Ca. colombiana sp. nov.	CBS 111136	Homothallic	FJ972424	FJ972443	FJ972493	Soil	Colombia	M.J. Wingfield
	CBS 115127[T]	Homothallic	FJ972423	FJ972442	FJ972492	Soil	Colombia	M.J. Wingfield
	CBS 115638	Homothallic	FJ972422	FJ972441	FJ972491	Soil	Colombia	M.J. Wingfield
	CBS 115694	Homothallic	FJ972425	FJ972444	FJ972494	Soil	Colombia	M.J. Wingfield
	CMW 9058	Homothallic	FJ972420	FJ972439	FJ972489	Soil	Colombia	M.J. Wingfield
Ca. colombiensis	CBS 112221		AY725620	AY725663	AY725712	Soil	Colombia	M.J. Wingfield
Ca. insularis	CBS 114558		AF210861	FJ918526	FJ918556	Soil	Madagascar	P.W. Crous
	CBS 114559		AF210862	FJ918525	FJ918555	Soil	Madagascar	C.L. Schoch
Ca. mexicana	CBS 110918[T]		AF210863	FJ972460	FJ972526	Soil	Mexico	M.J. Wingfield
Ca. morganii	CBS 110666		FJ918509	FJ918527	FJ918557	*Ilex vomitoria*	U.S.A.	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 (= CPC1722 = P94-4359)		AF210875	GQ421787	GQ421795	*Dodenaea vicosa*	U.S.A.	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 5683[T]	Mat1-2	FJ918514	FJ918531	FJ918565	*E. grandis*	South Africa	P.W. Crous
	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.	U.S.A.	S.T. Koike
	CMW 7851	Mat1-2	FJ972382	FJ972449	FJ972499	*Mytrus communis*	U.S.A.	S.T. Koike
	CMW 7852	Mat1-2	FJ972381	FJ972448	FJ972498	*M. communis*	U.S.A.	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.	U.S.A.	S.T. Koike
	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

Table 1. (Continued).

Species	Isolate	Mating type	GenBank accession no. β-tubulin	Histone H3	Translation elongation factor-1α	Host	Country	Collector
	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
	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. polizzii sp. nov.	CBS 123402[T]		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
	CBS 116081		GQ421777	GQ421785	GQ421793	Soil	Brazil	M.J. Wingfield
	CMW 7578		GQ421778	GQ421786	GQ421794	*E. grandis*	Argentina	L. Lombard
Ca. spathulata	CBS 112689		AF308463	FJ918524	FJ918554	*E. viminalis*	Brazil	N.E. El-Gholl
	CBS555.92[T]		GQ267215	GQ267261	GQ267331	*Araucaria angustifolia*	Brazil	C. Hodges
Ca. zuluensis sp. nov.	CMW 9115	Homothallic	FJ972413	FJ972432	FJ972482	*Eucalyptus* sp.	South Africa	L. Lombard
	CMW 9188[T]	Homothallic	FJ972414	FJ972433	FJ972483	*Eucalyptus* sp.	South Africa	L. Lombard
	CMW 9208	Homothallic	FJ972412	FJ972431	FJ972481	*Eucalyptus* sp.	South Africa	L. Lombard
	CMW 9215	Homothallic	FJ972416	FJ972435	FJ972485	*Eucalyptus* sp.	South Africa	L. Lombard
	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 111870[T]		AF333407	DQ190649	FJ918558	*Nelumbo nucifera*	Mauritius	A. Peerally
Cy. leucothoës	CBS 109166[T]		FJ918508	FJ918523	FJ918553	*Leucothoë axillaris*	U.S.A.	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.

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, U.S.A.), 1× PCR buffer, 1–1.5 mM $MgCl_2$, 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, U.S.A.) and sequenced in both directions. For this purpose, the BigDye terminator sequencing kit v. 3.1 (Applied Biosystems, U.S.A.) 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://www.treebase.org).

A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v. 3.1.1 (Ronquist & Huelsenbeck 2003). 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, 2010). 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*. 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. insularis* 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 *Cy. 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*. 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. 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. This is irrespective whether the teleomorph states of these fungi have been found or not and follows the approach of Lombard *et al.* (2009, 2010).

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 *brasiliense*) 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 an ellipsoidal to obpyriform 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. *Macroconidia* 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., Sept. 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, *Corymbia citriodora* sub. sp. *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 (30–45 mm diam after 7 d) with optimal growth temperature at 25 ºC (growth at 10–30 ºC) on MEA, reverse amber to sepia-brown after 7 d; sparse white aerial mycelium with sparse sporulation; chlamydospores moderate throughout the medium, forming microsclerotia.

Substrate: *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

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

	CBS 102296	CBS 110945	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 10148	CMW 27203	CMW 27206	CMW 27283	CMW 30817	CMW 30818	CMW 30819	**CMW 30823**	CMW 30873	CMW 30875	CMW 30878	CMW 30879
CBS 102296	-																														
CBS 110945	+	-																													
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 10148	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-											
CMW 27203	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-										
CMW 27206	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-									
CMW 27283	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-								
CMW 30817	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-							
CMW 30818	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
CMW 30819	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
CMW 30823	+	-	-	-	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-				
CMW 30873	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-			
CMW 30875	+	-	-	-	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-		
CMW 30878	+	-	-	-	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	
CMW 30879	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-

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 (SNP's)[1] 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											
		87	187	200	201	208	336	375	382	385	401	406	496	514	36	39	76	209	251	257	266	274	276	281	282	292	329	350	53	86	94	95	103	126	127	128	269	423	426	515
Ca. pauciramosa	CMW 5683	A	A	C	G	A	C	C	C	G	C	C	T	T	G	C	G	C	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	T
	CMW 30823	A	A	C	G	A	C	C	C	G	C	C	T	T	G	C	G	C	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	T
Ca. colombiana	CBS 111136	G	C	C	A	G	C	C	C	A	C	A	C	T	C	T	A	T	G	C	A	T	G	T	A	T	T	C	C	T	-	-	G	A	A	G	T	T	T	-
	CBS 115127	G	C	C	A	G	C	C	C	A	C	A	C	T	C	T	A	T	G	C	A	T	G	T	A	T	T	C	C	T	-	-	G	A	A	G	T	T	T	-
	CBS 115638	G	C	C	A	G	C	C	C	A	C	A	C	T	C	T	A	T	G	C	A	T	G	T	A	T	T	C	C	T	-	-	G	A	A	G	T	T	T	-
	CBS 115694	G	C	C	A	G	C	C	C	A	C	A	C	T	C	T	A	T	G	C	A	T	G	T	A	T	T	C	C	T	-	-	G	A	A	G	T	T	T	-
	CMW 9058	G	C	C	A	G	C	C	C	A	C	A	C	T	C	T	A	T	G	C	A	T	G	T	A	T	T	C	C	T	-	-	G	A	A	G	T	T	T	-
Ca. polizzii	CBS 123402	G	A	C	G	A	T	G	T	G	G	C	T	C	C	C	G	G	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	-
	CMW 7804	G	A	C	G	A	T	G	T	G	G	C	T	C	C	C	G	G	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	-
	CMW 10151	G	A	C	G	A	T	G	T	G	G	C	T	C	C	C	G	G	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	-
Ca. zuluensis	CMW 9115	G	A	T	G	A	C	C	C	G	C	C	T	T	C	C	G	G	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	-
	CMW 9188	G	A	T	G	A	C	C	C	G	C	C	T	T	C	C	G	G	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	-
	CMW 9208	G	A	T	G	A	C	C	C	G	C	C	T	T	C	C	G	G	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	-
	CMW 9215	G	A	T	G	A	C	C	C	G	C	C	T	T	C	C	G	G	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	-
	CMW 9896	G	A	T	G	A	C	C	C	G	C	C	T	T	C	C	G	G	A	T	G	C	A	C	T	C	C	T	T	G	C	A	A	T	T	-	A	C	C	-

[1]Polymorphisms are highlighted; Yellow = unique fixed alleles, Blue = shared fixed alleles.

Table 4. Single nucleotide polymorphisms (SNP's)[1] from the sequence data of β-tubulin, histone H3 and translation elongation factor-1α of *Ca. brasiliensis*, *Ca. insulare* and *Ca. morganii* used in this study.

Species	Isolate no.	β-tubulin					Histone H3														Transation elongation factor-1α																			
		53	61	117	360	472	9	10	68	69	70	94	204	252	257	359	389	416	451	460	9	10	20	47	48	102	110	112	113	114	115	142	280	378	406	407	408	414	437	479
Ca. brasiliensis	CBS 230.51	C	C	C	A	T	T	G	-	-	-	G	T	G	T	C	T	T	A	T	A	G	T	A	C	G	C	-	-	-	-	T	T	T	T	C	C	T	G	T
	CBS 114257	C	C	C	A	T	T	G	-	-	-	G	T	G	T	C	T	T	A	T	A	G	T	A	C	G	C	-	-	-	-	T	T	T	T	C	C	T	G	T
	CBS 116078	C	C	C	A	T	T	G	-	-	-	G	T	G	T	C	T	T	A	T	A	G	T	A	C	G	C	-	-	-	-	T	T	T	T	C	C	T	G	T
	CPC1943	C	C	C	A	T	T	G	-	-	-	G	T	G	T	C	T	T	A	T	A	G	T	A	C	G	C	-	-	-	-	T	T	T	T	C	C	T	G	T
	CPC602	C	C	C	A	T	T	G	-	-	-	G	T	G	T	C	T	T	A	T	A	G	T	A	C	G	C	-	-	-	-	T	T	T	T	C	C	T	G	T
	CPC2581	C	C	C	A	T	T	G	T	T	C	A	T	G	T	C	T	T	A	T	A	G	T	A	C	G	C	-	-	-	-	T	T	T	T	C	C	T	G	T
Ca. insulare	CBS114558	C	C	T	G	C	T	G	T	T	C	A	T	G	T	C	C	C	A	C	G	T	G	C	C	A	A	C	A	C	C	-	C	C	G	A	T	C	A	C
	CBS 114559	C	C	T	G	C	T	G	T	T	C	A	C	G	T	C	C	C	A	C	G	T	G	C	C	A	A	C	A	C	C	-	C	C	G	A	T	C	A	C
Ca. morganii	CBS 110666	A	A	T	A	T	C	T	-	-	-	A	C	T	C	T	C	C	C	C	G	T	T	A	G	G	A	-	-	-	C	-	T	C	G	A	T	C	A	C
	CBS 119669	A	A	T	A	T	C	T	-	-	-	A	C	T	C	T	C	C	C	C	G	T	T	A	G	G	A	-	-	-	C	-	T	C	G	A	T	C	A	C
	CBS 119670	A	A	T	A	T	C	T	-	-	-	A	C	T	C	T	C	C	C	C	G	T	T	A	G	G	A	-	-	-	C	-	T	C	G	A	T	C	A	C
	CPC 1722	A	A	T	A	T	C	T	-	-	-	A	C	T	C	T	C	C	C	C	G	T	T	A	G	G	A	-	-	-	C	-	T	C	G	A	T	C	A	C

[1]Polymorphisms are highlighted; Yellow = unique fixed alleles, Blue = shared fixed alleles.l

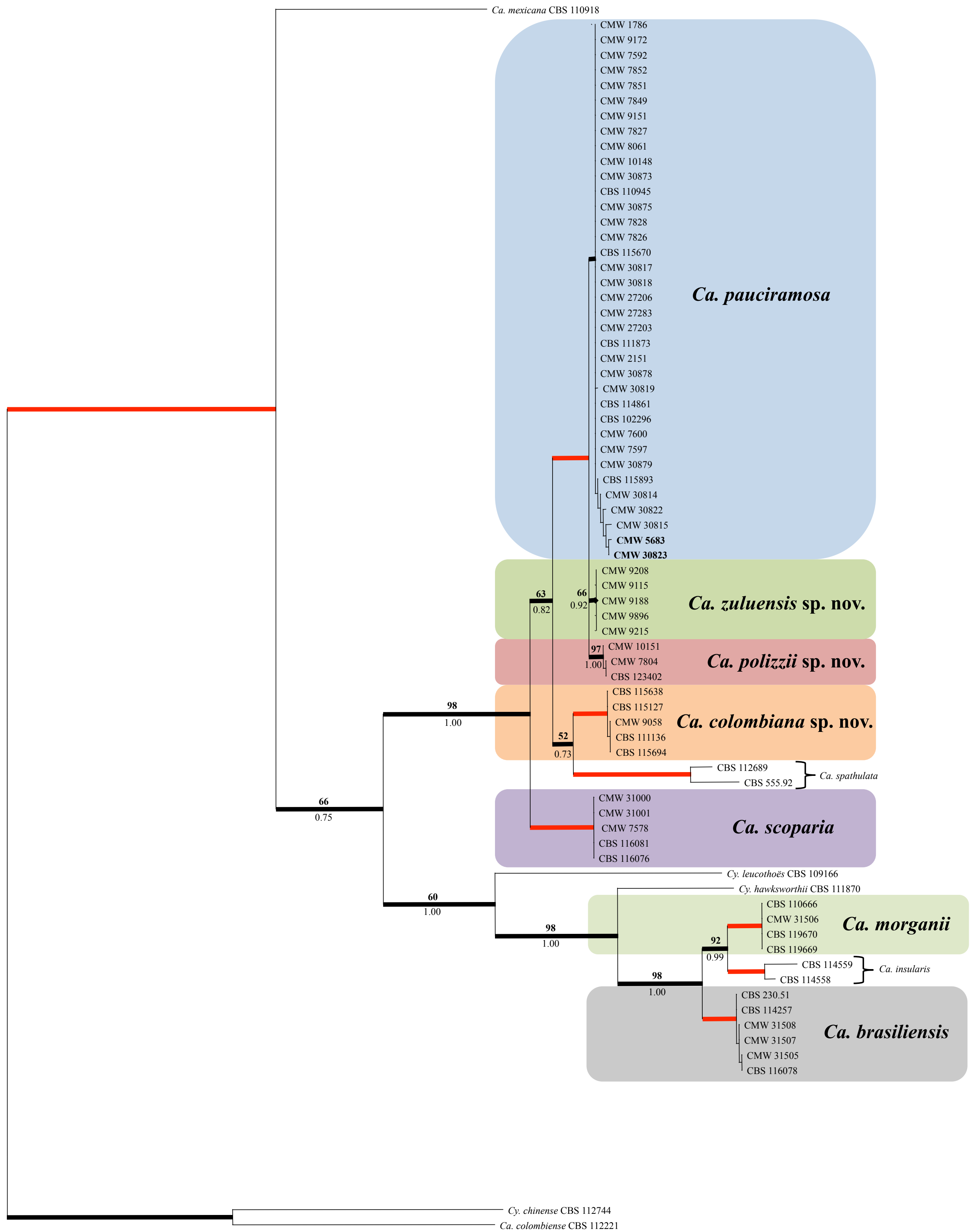

Fig. 1. One of eight most parsimonious trees obtained from a heuristic search with 1 000 random addition of the combined BT, HIS3 and TEF-1α sequence alignments. Scale bar shows 10 changes and bootstrap support values from 1 000 replicates are shown above the nodes in bold. Bayesian posterior probability 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 *Ca. colombiensis* (CBS 112221) and *Ca. chinensis* (CBS 112744). Mating tester strains of *Ca. pauciramosa* used in this study are indicated in bold.

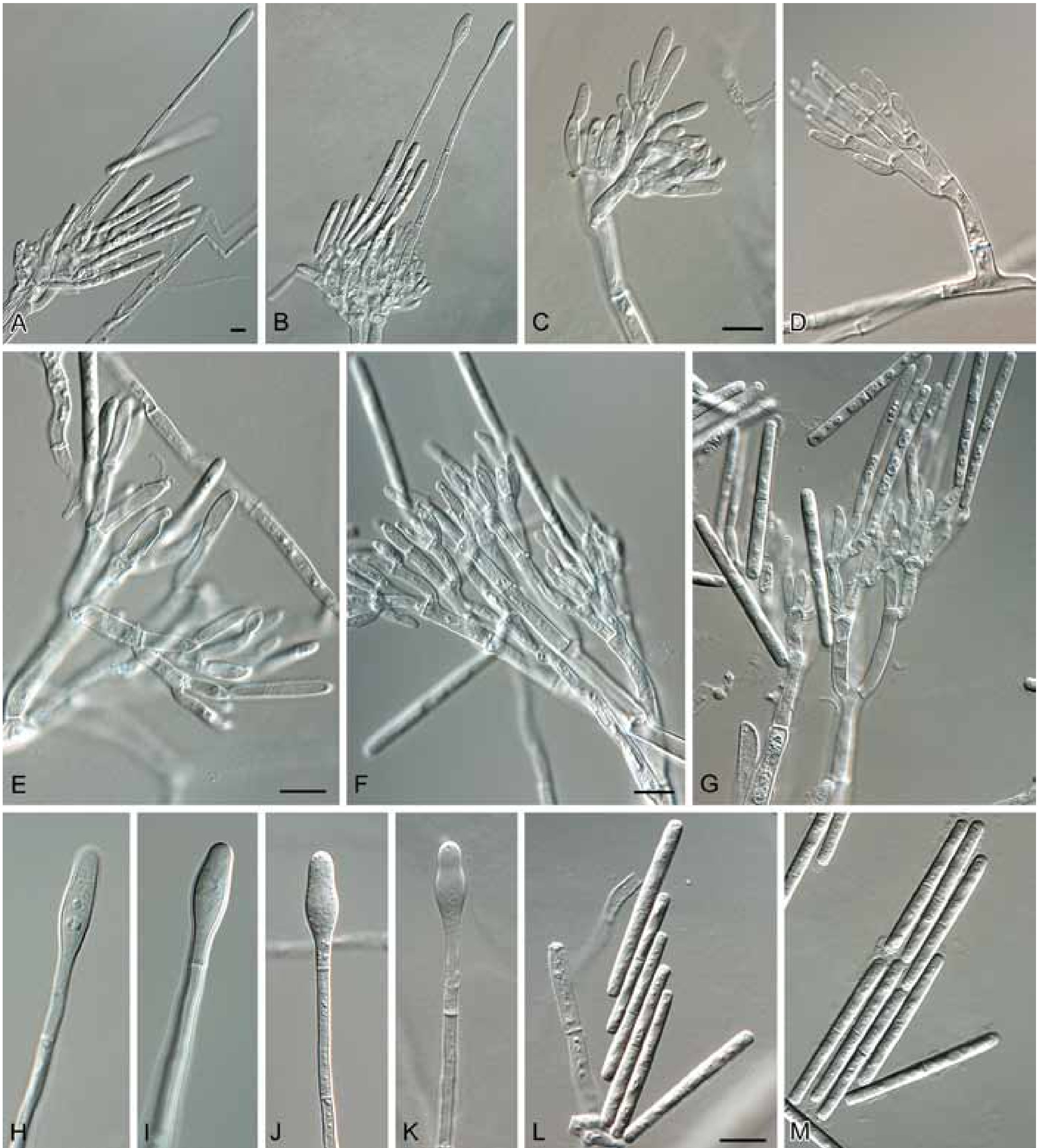

Fig. 2. *Calonectria brasiliensis*. A–B. Macroconidiophores. C–G. Conidiogenous apparatus with conidiophore branches and doliiform to reniform phialides. H–K. Ellipsoidal to obpyriform vesicles. L–M. One-septate macroconidia. Scale bars = 10 µm.

Ca. morganii (CBS 110666, CBS 119669, CBS 119670 and CMW 31506). 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, whereas *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.

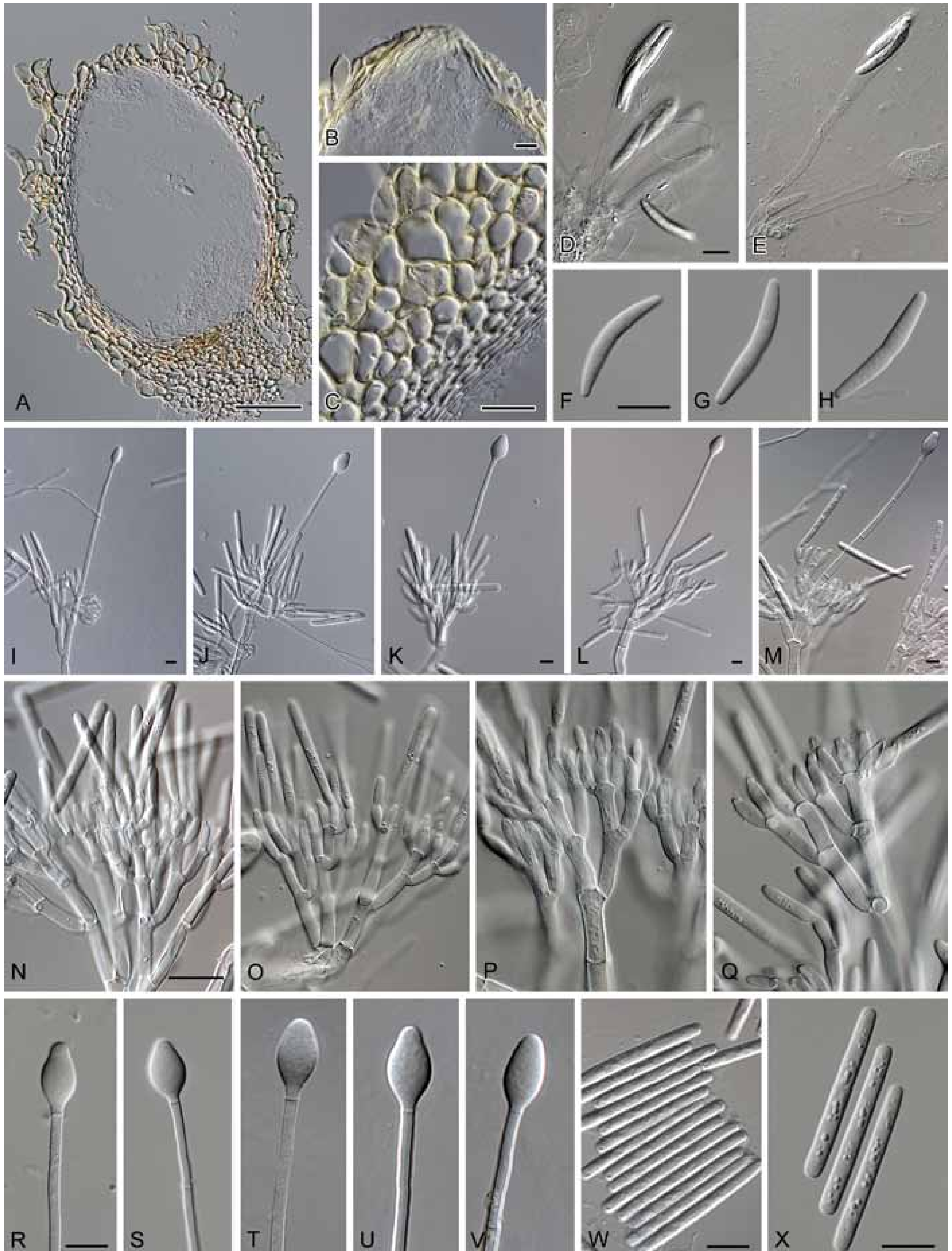

Fig. 3. *Calonectria colombiana*. A. Perithecium. B. Ostiolar region of perithecium. C. Vertical section through perithecium, showing wall structure. D–E. Asci. F–H. Ascospores. I–M. Macroconidiophores. N–Q. Conidiogenous apparatus with conidiophore branches and doliiform to reniform phialides. R–V. Obpyriform to ellipsoid vesicles. W–X. One-septate macroconidia. Scale bars: A = 70 µm, B–C = 30 µm, other scale bars = 10 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, gluttulate, 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 ellipsoid 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. *Macroconidia* 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.

Specimens 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; La Selva, June 1995, M.J. Wingfield, CBS 115638 = CMW 30766 = CPC 1161 (Herb. PREM 60296); La Selva, June 1995, M.J. Wingfield, CBS 115694 = CMW 30813 = CPC 1162, CMW 9058.

Culture characteristics: Colonies fast growing (35–55 mm diam after 7 d) with optimal growth temperature at 25 ºC (growth at 10–30 ºC) on MEA, reverse amber to sepia-brown 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.

Teleomorpha 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 an obpyriform to ellipsoid 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. *Macroconidia* 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.

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

Culture characteristics: Colonies fast growing (35–40 mm diam after 7 d) with optimal growth temperature at 25 ºC (growth at 10–30 ºC) on MEA, reverse amber to sepia-brown 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 were the fungus was isolated.

Telomorpha *Calonectriae pauciramosa* similis, sed ascosporis brevioribus, (26–)29–34(–38) × 4–5 µm (in medio 32 × 4 µm). Culturae homothallicae. Anamorpha *Cylindrocladio 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.

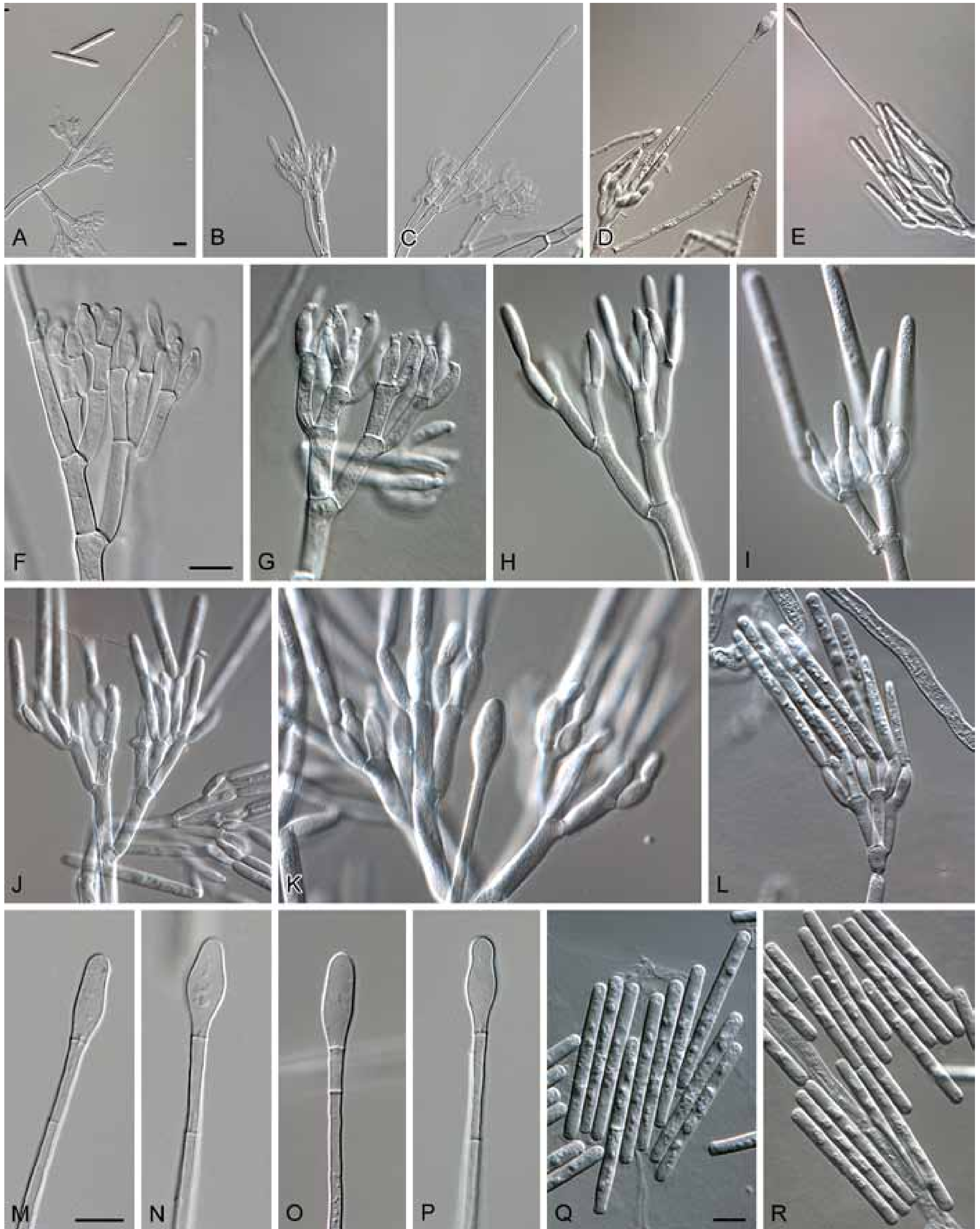

Fig. 4. *Calonectria polizzii*. A–E. Macroconidiophores. F–L. Conidiogenous apparatus with conidiophore branches and doliiform to reniform phialides. M–P. Obpyriform to ellipsoid vesicles. Q–R. One-septate macroconidia. Scale bars = 10 µm.

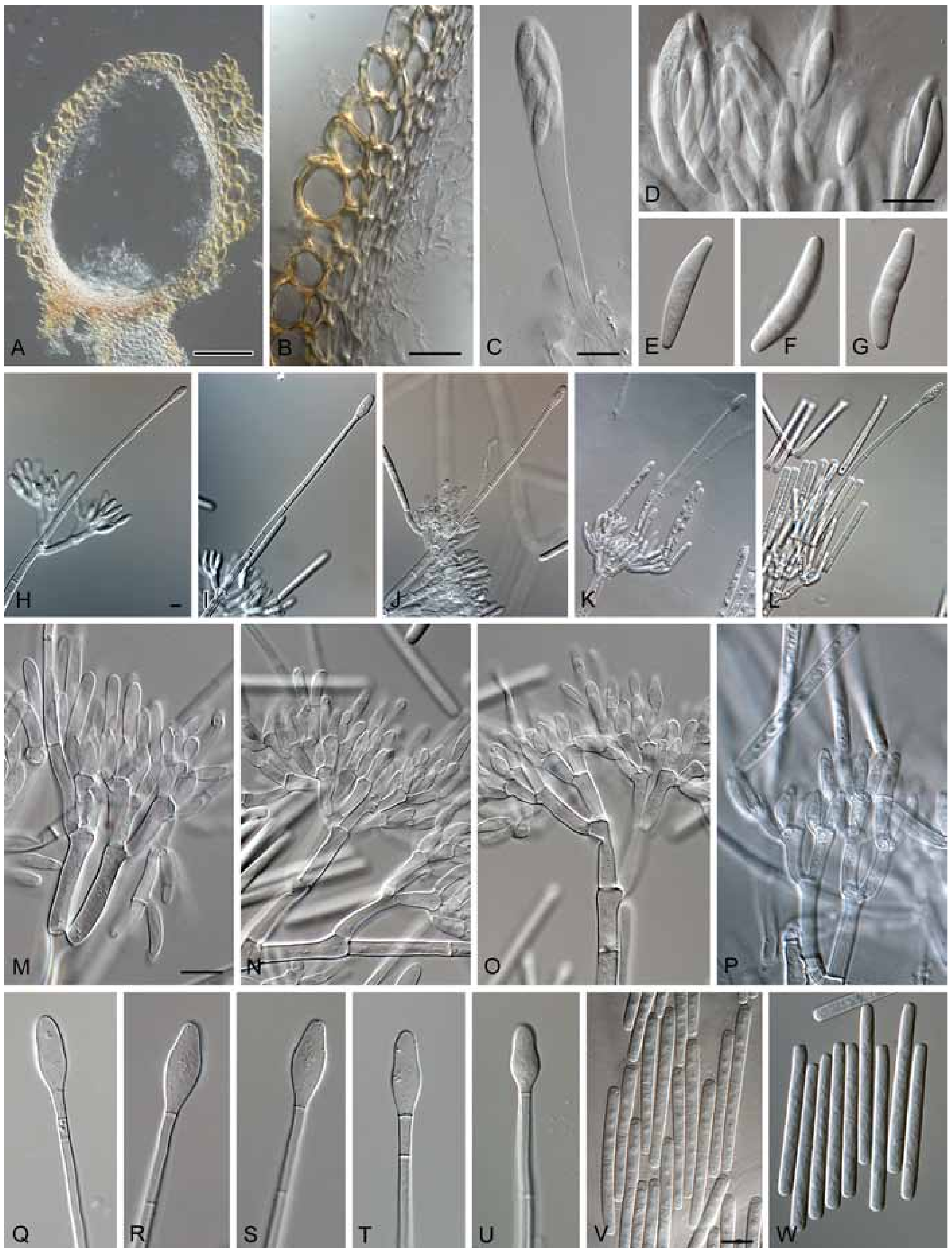

Fig. 5. *Calonectria zuluensis*. A. Perithecium. B. A vertical section through a perithecium, showing the wall layers. C–D. Asci. E–G. Ascospores. H–L. Macroconidiophores. M–P. Conidiogenous apparatus with conidiophore branches and doliiform to reniform phialides. Q–U. Ellipsoid to obpyriform vesicles. V–W. One-septate macroconidia. Scale bars: A = 70 µm, B = 30 µm, other scale bars = 10 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 ellipsoid to obpyriform vesicles, 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 doliiform to reniform, hyaline, aseptate, 10–13 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. *Macroconidia* 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.

Specimens 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 (35–40 mm diam after 7 d) with optimal growth temperature at 25 ºC (growth at 10–30 ºC) on MEA, reverse amber to sepia-brown after 7 d; abundant white aerial mycelium with sparse sporulation; chlamydospores extensive throughout the medium, forming microsclerotia.

Substrate: *Eucalyptus grandis* and *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 broadly clavate to obpyriform vesicles as compared with the obpyriform to fusiform vesicles 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.

More than eight 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 synonymy under *Ca. morganii*, based on similar conidial dimensions and vesicle 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*. The presence of homothallic and heterothallic mating strategies in closely related fungi is interesting and could well provide another opportunity to analyse the genetics of mating systems in ascomycetes. This study has shown, however, that 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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