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Calonectria (*Cylindrocladium*) species associated with dying *Pinus* cuttings

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Key words

β-tubulin
Calonectria
Cylindrocladium
histone
Pinus
root disease

Abstract *Calonectria* (*Ca.*) species and their *Cylindrocladium* (*Cy.*) anamorphs are well-known pathogens of forest nursery plants in subtropical and tropical areas of the world. An investigation of the mortality of rooted *Pinus* cuttings in a commercial forest nursery in Colombia led to the isolation of two *Cylindrocladium* anamorphs of *Calonectria* species. The aim of this study was to identify these species using DNA sequence data and morphological comparisons. Two species were identified, namely one undescribed species, and *Cy. gracile*, which is allocated to *Calonectria* as *Ca. brassicae*. The new species, *Ca. brachiatica*, resides in the *Ca. brassicae* species complex. Pathogenicity tests with *Ca. brachiatica* and *Ca. brassicae* showed that both are able to cause disease on *Pinus maximinoi* and *P. tecunumanii*. An emended key is provided to distinguish between *Calonectria* species with clavate vesicles and 1-septate macroconidia.

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INTRODUCTION

Species of *Calonectria* (anamorph *Cylindrocladium*) are plant pathogens associated with a large number of agronomic and forestry crops in temperate, subtropical and tropical climates, worldwide (Crous & Wingfield 1994, Crous 2002). Infection by these fungi gives rise to symptoms including cutting rot (Crous et al. 1991), damping-off (Sharma et al. 1984, Ferreira et al. 1995), leaf spot (Sharma et al. 1984, Ferreira et al. 1995, Crous et al. 1998), shoot blight (Crous et al. 1991, Crous et al. 1998), stem cankers (Sharma et al. 1984, Crous et al. 1991) and root disease (Mohanan & Sharma 1985, Crous et al. 1991) on various forest trees species.

The first report of *Ca. morganii* (as *Cy. scoparium*) infecting *Pinus* spp. was by Graves (1915), but he failed to re-induce disease symptoms and assumed that it was a saprobe. There have subsequently been several reports of *Cylindrocladium* spp. infecting *Pinus* and other conifers, leading to root rot, stem cankers and needle blight (Jackson 1938, Cox 1953, Thies & Patton 1970, Sobers & Alfieri 1972, Cordell & Skilling 1975, Darvas et al. 1978, Crous et al. 1991, Crous 2002). Most of these reports implicated *Ca. morganii* and *Ca. pteridis* (as *Cy. macrosporum* or *Cy. pteridis*) as the primary pathogens (Thies & Patton 1970, Ahmad & Ahmad 1982). However, as knowledge of these fungi has grown, together with refinement of their taxonomy applying DNA sequence comparisons (Crous et al. 2004, 2006), several additional *Cylindrocladium* spp. have been identified as causal agents of disease on different conifer species. These include *Ca. acicola*, *Ca. colhounii*, *Ca. kyotensis* (= *Cy. floridanum*), *Ca. pteridis, Cy. canadense*, *Cy. curvisporum, Cy. gracile* and *Cy. pacificum* (Hodges & May 1970, Crous 2002, Gadgill & Dick 2004, Taniguchi et al. 2008).

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In a recent survey, wilting, collar and root rot symptoms were observed in Colombian nurseries generating *Pinus* spp. from cuttings. Isolations from these diseased plants consistently yielded *Cylindrocladium* anamorphs of *Calonectria* spp., and hence the aim of this study was to identify them, and to determine if they were the causal agents of the disease in Colombian nurseries.

MATERIAL AND METHODS

Isolates

Pinus maximinoi and *P. tecunumanii* rooted cutting plants showing symptoms of collar and root rot (Fig. 1) were collected from a nursery close to Buga in Colombia. Isolations were made directly from lesions on the lower stems and roots on fusarium selective medium (FSM; Nelson et al. 1983) and malt extract agar (MEA, 2 % w/v; Biolab, Midrand, South Africa). After 5 d of incubation at 25 °C, fungal colonies of *Calonectria* spp. were transferred on to MEA and incubated further for 7 d. For each isolate, single conidial cultures were prepared on MEA, and representative strains are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Taxonomy

For morphological identification of *Calonectria* isolates, single conidial cultures were prepared on MEA and synthetic nutrient-poor agar (SNA; Nirenburg 1981). Inoculated plates were incubated at room temperature and examined after 7 d. Gross morphological characteristics were assessed by mounting fungal structures in lactic acid. Thirty measurements at × 1 000 magnification were made for each isolate. The 95 % confidence levels were determined for the pooled measurements of the respective species studied and extremes for structure sizes are given in parentheses. Optimal growth temperatures were determined between 6–36 °C at 6 °C intervals in the dark on MEA for each isolate. Colony reverse colours were determined

Fig. 1 Collar and root rot on *Pinus maximinoi* and *P. tecunumanii*. a. Girdled stem of *P. maximinoi*; b. exposed *P. maximinoi* root collar showing discolouration and resin exudation; c, d. exposed *P. tecunumanii* root collars showing girdling and discolouration of the cambium.

after 7 d on MEA at 24 °C in the dark, using the colour charts of Rayner (1970) for comparison.

DNA phylogeny

Calonectria isolates were grown on MEA for 7 d. Mycelium was then scraped from the surfaces 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). A fragment of the β-tubulin gene region was amplified and sequenced using primers T1 (O'Donnell & Cigelnik 1997) and CYLTUB1R (Crous et al. 2004) and a fragment for the histone H3 gene region was sequenced using primers CYLH3F and CYLH3R (Crous et al. 2004). The PCR reaction mixture used to amplify the different loci consisted of 2.5 units FastStart *Taq* polymerase (Roche Applied Science, USA), 10 × PCR buffer, 1–1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 µm of each primer and 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 (v3.1, Applied Biosystems, USA) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems) were used. All PCRs and sequencing reactions were performed on an Eppendorf Mastercycler Personal PCR (Eppendorf AG, Germany) with cycling conditions as described in Crous et al. (2006) for each locus. Sequences generated were added to other sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov) and were assembled and aligned using Sequence Navigator v1.0.1 (Applied Biosystems) and MAFFT v5.11 (Katoh et al. 2005), respectively. The aligned sequences were then manually corrected where needed. PAUP (Phylogenetic Analysis Using Parsimony, v4.0b10; Swofford 2002) was used to analyse the DNA sequence datasets. A partition homogeneity test (Farris et al. 1994) and a 70 % reciprocal bootstrap method (Mason-Gamer & Kellogg 1996) were applied to evaluate the feasibility of combining the datasets. Phylogenetic relationships were estimated by heuristic searches based on 1 000 random addition sequences and tree bisection-reconnection, 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 19 partial gene sequences per gene, representing eight *Calonectria* spp. (Table 1) closely related to the isolates studied. *Calonectria colombiensis* was used as the outgroup taxon. All sequences were deposited in GenBank and the alignments in TreeBASE (http://treebase.org).

A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v3.1.1 (Ronquist & Huelsenbeck 2003). Models of nucleotide substitution for each gene were determined using MrModeltest (Nylander 2004) and included for each gene partition. Four MCMC chains were run simultaneously from

random trees for one million generations and sampled every 100 generations. The first 800 trees were discarded as the burn-in phase of each analysis and posterior probabilities determined from the remaining trees.

Pathogenicity tests

In order to test the pathogenicity of the *Calonectria* spp. collected in this study, profusely sporulating isolates CMW 25293, representing *Ca. brachiatica*, CMW 25296 and CMW 25297, both representing *Ca. brassicae*, were used for inoculations onto rooted cuttings of *P. maximinoi*. Isolate CMW 25299, representing *Ca. brassicae* and isolates CMW 25302 and CMW 25307 representing *Ca. brachiatica* were used for inoculations onto rooted cuttings of *P. tecunumanii*. Trees used for inoculation were between 0.5–1 m in height and 10–50 mm diam at the root collar. Trees were maintained in a greenhouse under controlled conditions prior to inoculation, so that they could become acclimatised and to ensure that they were healthy. Sixty trees for each *Pinus* spp. were used and an additional 60 trees were used as controls. This resulted in a total of 180 trees in the pathogenicity tests.

Inoculations were preformed in the greenhouse by making a 5 mm diam wound on the main stems of plants with a cork borer to expose the cambium. The cambial discs were replaced with an MEA disc overgrown with the test fungi taken from 7 d old cultures. The inoculum discs were placed, mycelium side facing the cambium and the inoculation points were sealed with Parafilm to reduce contamination and desiccation. Control trees were treated in a similar fashion but inoculated with a sterile MEA plug.

Six weeks after inoculation, lesion lengths on the stems of the plants were measured. The results were subsequently analysed using SAS Analytical Programmes v2002. Re-isolations were made from the edges of lesions on the test trees to ensure the presence of the inoculated fungi.

RESULTS

DNA phylogeny

For the β-tubulin gene region, ± 580 bases were generated for each of the isolates used in the study (Table 1). The adjusted alignment included 19 taxa with the outgroup, and 523 characters including gaps after uneven ends were removed from the beginning of each sequence. Of these characters, 459 were constant and uninformative. For the analysis, only the 64 parsimony informative characters were included. Parsimony analysis of the aligned sequences yielded five most parsimonious trees (TL = 231 steps; CI = 0.870; RI = 0.799; RC = 0.695; results not shown). Sequences for the histone gene region consisted of ± 460 bases for the isolates used in the study and the adjusted alignment of 19 taxa including the outgroup, consisted of 466 characters including gaps. Of these characters, 391 were excluded as constant and parsimony uninformative and 79 parsimony informative characters included. Analysis of the aligned data yielded one most parsimonious tree (TL = 290 steps; CI = 0.845; RI = 0.807; RC = 0.682; results not shown).

The partition homogeneity test showed that the β-tubulin and histone dataset could be combined (P = 0.245). The 70 % reciprocal bootstrap method indicated no conflict in tree topology among the two partitions, resulting in a combined sequence dataset consisting of 993 characters including gaps for the 19 taxa (including outgroup). Of these, 850 characters were constant and parsimony uninformative and excluded from the analysis. There were 143 characters in the analysis that were parsimony informative. Parsimony analysis of the combined alignments yielded one most parsimonious tree (TL = 526 steps; CI = 0.848; RI = 0.791; RC = 0.670), which is presented in Fig. 2 (TreeBase SN 4332).

All the isolates obtained from the *Pinus* spp. used in this study grouped in the *Ca. brassicae* species complex with a bootstrap (BP) value of 96 and a low Bayesian posterior probability (PP) of 0.70. This clade was further subdivided into two clades. The first clade (BP = 64, PP below 0.70) representing *Ca. brassicae*, included the type of *Cy. gracile* and *Cy. clavatum*. It also included three isolates (CMW 25297, CMW 25296 and CMW 25299) from *P. maximinoi* and *P. tecunumanii*. The second clade (BP = 98, PP = 0.82) accommodated *Calonectria* isolates (CMW 25293, CMW 25298, CMW 25302 and CMW 25307), representing what we recognise as a distinct species. The consensus tree obtained with Bayesian analysis showed topographical similarities with the most parsimonious tree as indicated in Fig. 2.

Pathogenicity tests

All plants inoculated with *Calonectria* spp. in this study developed lesions. Lesions included discolouration of the vascular tissue with abundant resin formation, 6 wk after inoculation.

Table 1 Strains of *Calonectria* (*Cylindrocladium*) species included in the phylogenetic analyses (TreeBase SN 4332).

Species	Isolate number[1]	β-tubulin[2]	Histone H3[2]	Host	Origin	Collector
Ca. avesiculata (*Cy. avesiculatum*)	CBS 313.92[T]	AF333392	DQ190620	*Ilex vomitoria*	USA	S.A. Alfieri
Ca. brachiatica sp. nov.	CMW 25293	FJ716710	FJ716714	*P. maximinoi*	Colombia	M.J. Wingfield
	CMW 25298 (= CBS 123700)[T]	FJ696388	FJ696396	*P. maximinoi*	Colombia	M.J. Wingfield
	CMW 25302	FJ716708	FJ716712	*P. tecunumanii*	Colombia	M.J. Wingfield
	CMW 25307	FJ716709	FJ716713	*P. tecunumanii*	Colombia	M.J. Wingfield
Ca. brassicae comb. nov.	CBS 111869[T]	AF232857	DQ190720	*Argyreia* sp.	South East Asia	
	CBS 111478	DQ190611	DQ190719	Soil	Brazil	A.C. Alfenas
	CMW 25296	FJ716707	FJ716711	*P. maximinoi*	Colombia	M.J. Wingfield
	CMW 25297; CBS123702	FJ696387	FJ696395	*P. maximinoi*	Colombia	M.J. Wingfield
	CMW 25299; CBS123701	FJ696390	FJ696398	*P. tecunumanii*	Colombia	M.J. Wingfield
Ca. clavata (*Cy. flexuosum*)	CBS 114557[T]	AF333396	DQ190623	*Callistemon viminalis*	USA	N.E. El-Gholl
	CBS 114666[T]	DQ190549	DQ190624		USA	N.E. El-Gholl
Cy. clavatum (= *Cy. gracile*)	CBS111776[T]	AF232850	DQ190700	*Pinus caribaea*	Brazil	C.S. Hodges
Ca. colombiensis (*Cy. colombiensis*)	CBS 12221	AY725620	AY725663	Soil	Colombia	M.J. Wingfield
Cy. ecuadoriae	CBS 111406[T]	DQ190600	DQ190705	Soil	Ecuador	M.J. Wingfield
Ca. gracilipes (*Cy. graciloideum*)	CBS 111141[T]	DQ190566	DQ190644	*Eucalyptus* sp.	Colombia	M.J. Wingfield
	CBS 115674	AF333406	DQ190645	Soil	Colombia	M.J. Wingfield
Ca. gracilis (*Cy. pseudogracile*)	CBS 111284	DQ190567	DQ190647	*Manilkara* sp.	Brazil	P.W. Crous
	CBS 111807[T]	AF232858	DQ190646		Brazil	

[1] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW: culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

[2] GenBank accession numbers.

[T] ex-type culture.

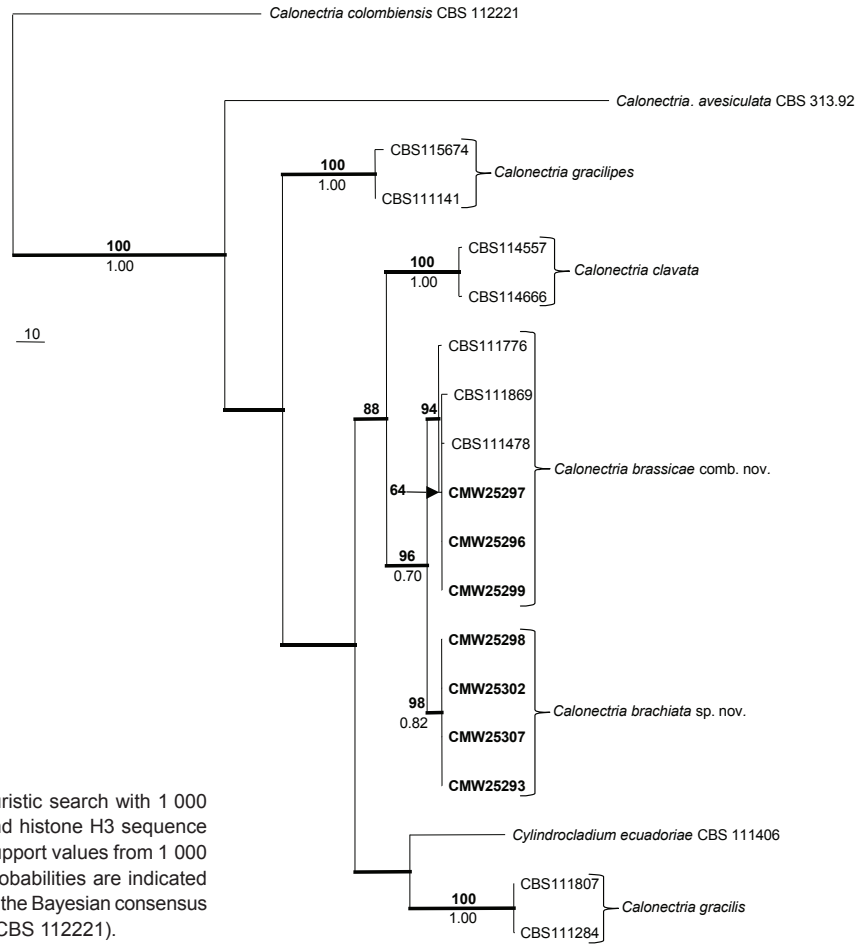

Fig. 2 The most parsimonious tree obtained from a heuristic search with 1 000 random addition sequences of the combined β-tubulin and histone H3 sequence alignments. Scale bar shows 10 changes and bootstrap support values from 1 000 replicates are shown at the nodes. Bayesian posterior probabilities are indicated below the branches. **Bold** lines indicate branches present in the Bayesian consensus tree. The tree was rooted with *Calonectria colombiensis* (CBS 112221).

Lesions on the control trees were either non-existent or small, representing wound reactions. There were significant ($p < 0.0001$) differences in lesion lengths associated with individual isolates used on *P. maximinoi* (Fig. 3). Comparisons of the lesion lengths clearly showed that *Ca. brassicae* (CMW 25297) produced the longest average lesions (av. = 30.04 mm) compared to the undescribed *Calonectria* sp. (CMW 25293) (av. = 14.41 mm). The other *Ca. brassicae* isolate (CMW 25296) produced an average lesion length of 15.30 mm. Lesions on the control trees were an average of 8.84 mm and significantly ($p < 0.0001$) smaller than those on any of the trees inoculated with the test fungi (Fig. 3).

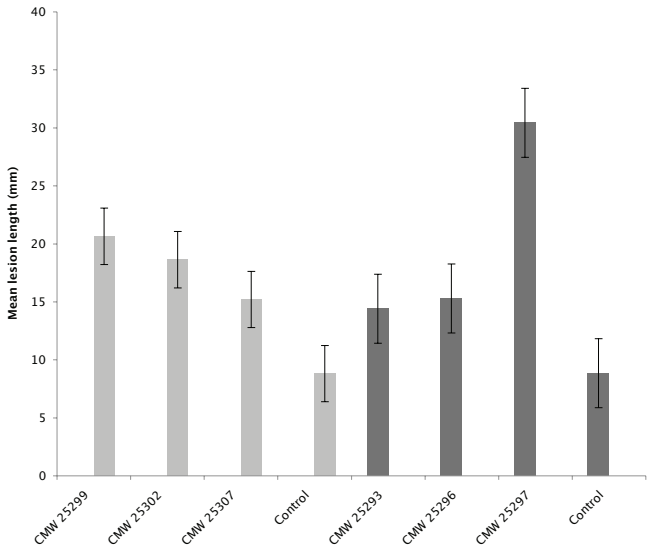

Fig. 3 Histogram showing mean lesion lengths induced by each isolate on *P. maximinoi* (dark grey) and *P. tecunumanii* (light grey). *Calonectria brassicae* is represented by CMW 25296, CMW 25297 and CMW 25299; *Ca. brachiatica* is represented by CMW 25293, CMW 25302 and CMW 25307.

Results of inoculations on *P. tecunumanii* were similar to those on *P. maximinoi*. Thus, *Ca. brassicae* (CMW 25299) (av. = 20.64 mm) produced the longest lesions compared with the undescribed *Calonectria* sp. (CMW 25302; av. = 18.63 mm and CMW 25307; av. = 15.20 mm). The lesions on the *P. tecunumanii* control trees were also significantly ($p < 0.0001$) smaller (av. = 8.82 mm) than those on any of the trees inoculated with the test fungi. Re-isolations from the test trees consistently yielded the inoculated fungi and no *Calonectria* spp. were isolated from the control trees.

Taxonomy

Isolates CMW 25296, CMW 25297 and CMW 25299 clearly represent *Ca. brassicae* based on morphological observations (Crous 2002) and comparisons of DNA sequence data. Isolates CMW 25293, CMW 25298, CMW 25302 and CMW 25307 represent an undescribed species closely related to *Ca. brassicae* but morphologically distinct. Species of *Cylindrocladium* (1892) represent anamorph states of *Calonectria* (1867) (Rossman et al. 1999), and therefore this fungus is described as a new species of *Calonectria,* which represents the older generic name for these holomorphs:

Calonectria brachiatica L. Lombard, M.J. Wingf. & Crous, *sp. nov*. — MycoBank MB512998; Fig. 4

Stipa extensiones septatum, hyalinum, 134–318 µm, in vesiculam clavatum, 5–7 µm diam terminans. Conidia cylindrica, hyalina, 1–2-septata, utrinque obtusa, (37–)40–48(–50) × 4–6 µm.

Teleomorph. Unknown.

Etymology. Name refers to the stipe extensions on the conidiophore.

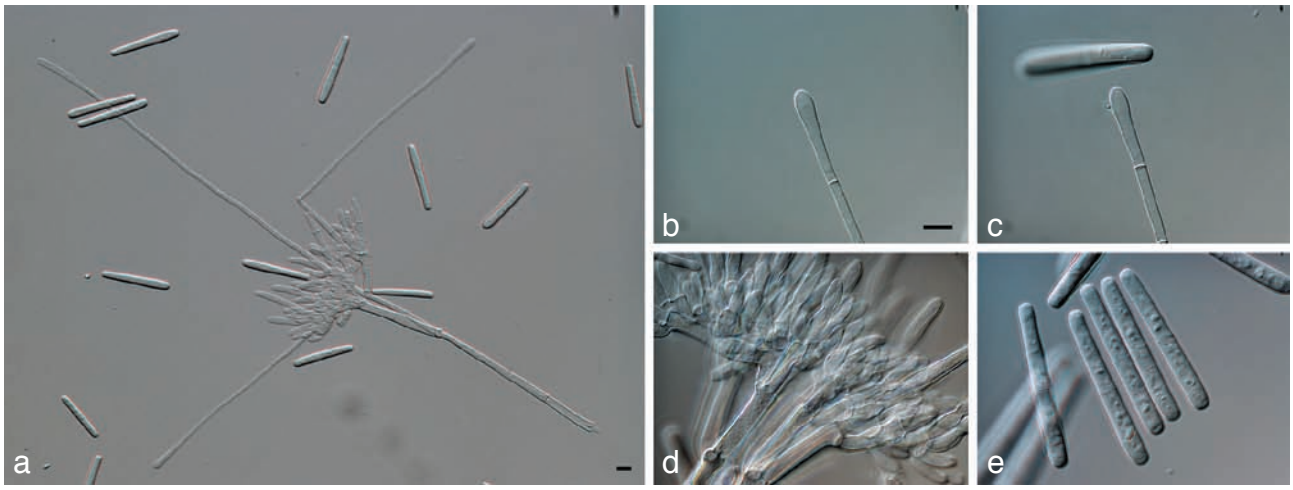

Fig. 4 *Calonectria brachiatica*. a. Macroconidiophore with lateral branching stipe extensions; b, c. clavate vesicles; d. fertile branches; e. macroconidia. — Scale bars = 10 µm.

Conidiophores with a stipe bearing penicillate suites of fertile branches, stipe extensions and terminal vesicles; stipe septate, hyaline, smooth, 32–67 × 6–8 µm; stipe extensions septate, straight to flexuous, 134–318 µm long, 4–5 µm wide at the apical septum, terminating in a clavate vesicle, 5–7 µm diam; lateral stipe extensions (90° to the axis) also present. *Conidiogenous apparatus* 40–81 µm long, and 35–84 µm wide; primary branches aseptate or 1-septate, 15–30 × 4–6 µm; secondary branches aseptate, 10–23 × 3–5 µm; tertiary branches and additional branches (–5), aseptate, 10–15 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 10–15 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (37–)40–48(–50) × 4–6 µm (av. = 44 × 5 µm), 1(–2)-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Mega-* and *microconidia* not seen.

Cultural characteristics — Colonies fast growing with optimal growth temperature at 24 °C (growth at 12–30 °C) on MEA, reverse amber to sepia brown after 7 d; abundant white aerial mycelium with moderate to extensive sporulation; chlamydospores extensive throughout the medium.

Specimens examined. Colombia, Valle del Cauca, Buga, from *Pinus maximinoi*, July 2007, *M.J. Wingfield*, holotype PREM 60197, culture ex-type CMW 25298 = CBS 123700; Buga, from *P. tecunumanii*, July 2007, *M.J. Wingfield*, culture CMW 25303 = CBS 123699; Buga, from *P. tecunumanii,* July 2007, *M.J. Wingfield*, PREM 60198, culture CMW 25341 = CBS 123703.

Notes — The anamorph state of *Ca. brachiatica* can be distinguished from *Cy. gracile*, *Cy. pseudogracile* and *Cy. graciloideum* by its shorter macroconidia. Another characteristic distinguishing *Ca. brachiatica* is the formation of lateral branches not reported for *Cy. gracile* or other closely related species.

Calonectria brassicae (Panwar & Bohra) L. Lombard, M.J. Wingf. & Crous, *comb. nov*. — MycoBank MB513423; Fig. 5

Basionym. *Cylindrocladium brassicae* Panwar & Bohra, Indian Phytopathol. 27: 425. 1974.

= *Cylindrocarpon gracile* Bugnic., Encycl. Mycologique 11: 162. 1939.

≡ *Cylindrocladium gracile* (Bugnic.) Boesew., Trans. Brit. Mycol. Soc. 78: 554. 1982.

= *Cylindrocladium clavatum* Hodges & L.C. May, Phytopathology 62: 900. 1972.

Notes — Both the names *Ca. clavata* and *Ca. gracilis* and are already occupied, hence the oldest available epithet is that of *Cy. brassicae* (Crous 2002).

DISCUSSION

Results of this study show that *Calonectria* spp. are important pathogens in pine cutting nurseries in Colombia. In this case, two species were discovered, the one newly described here as *Ca. brachiatica* and the other representing *Ca. brassicae* (Fig. 5). Both of the species were pathogenic on *P. maximinoi* and *P. tecunumanii*.

The description of *Ca. brachiatica* from *P. maximinoi* and *P. tecunumanii* adds a new species to the *Ca. brassicae* species complex, which already includes six other *Calonectria* spp. (Crous 2002, Crous et al. 2006). This species can be distinguished from the other species in the complex by the formation of lateral branches on the macroconidiophores and the presence of a small number of 2-septate macroconidia. Macroconidial dimensions (av. = 44 × 5 µm) are also smaller then those of *Ca. brassicae* (av. = 53 × 4.5 µm; Fig. 5).

A recent study of *Calonectria* species with clavate vesicles by Crous et al. (2006) attempted to resolve the taxonomic status of these species, and added two new species to the group. Crosses among isolates of *Ca. brachiatica* and isolates of *Ca. brassicae*, did not result in sexual structures in the present study, and teleomorphs are rarely observed in this species complex.

Hodges & May (1972) reported *Ca. brassicae* (as *Cy. clavatum*) from several *Pinus* spp. in nurseries and plantations in Brazil. Subsequent studies based on comparisons of DNA sequence data revealed *Cy. clavatum* to be a synonym of *Cy. gracile* (Crous et al. 1995, 1999, Schoch et al. 2001). *Calonectria brassicae* (*as Cy. gracile*) is a well-known pathogen of numerous plant hosts in subtropical and tropical areas of the world. However, in Colombia, this plant pathogen has been isolated only from soil (Crous 2002, Crous et al. 2006). This study thus represents the first report of *Ca. brassicae* infecting *Pinus* spp. in Colombia.

Pathogenicity tests with isolates of *Ca. brachiatica* and *Ca. brassicae* clearly showed that they are able to cause symptoms similar to those observed in naturally infected plants. Both *P. maximinoi* and *P. tecunumanii* were highly susceptible to infection by *Ca. brassicae*. This supports earlier work of Hodges & May (1972) in Brazil, where they reported a similar situation. In their study, seven *Pinus* spp. were wound-inoculated with *Ca. brassicae* and this resulted in mortality of all test plants within 2 wk. Although they did not include *P. maximinoi* and *P. tecunumanii* in the study, they concluded that the pathogen

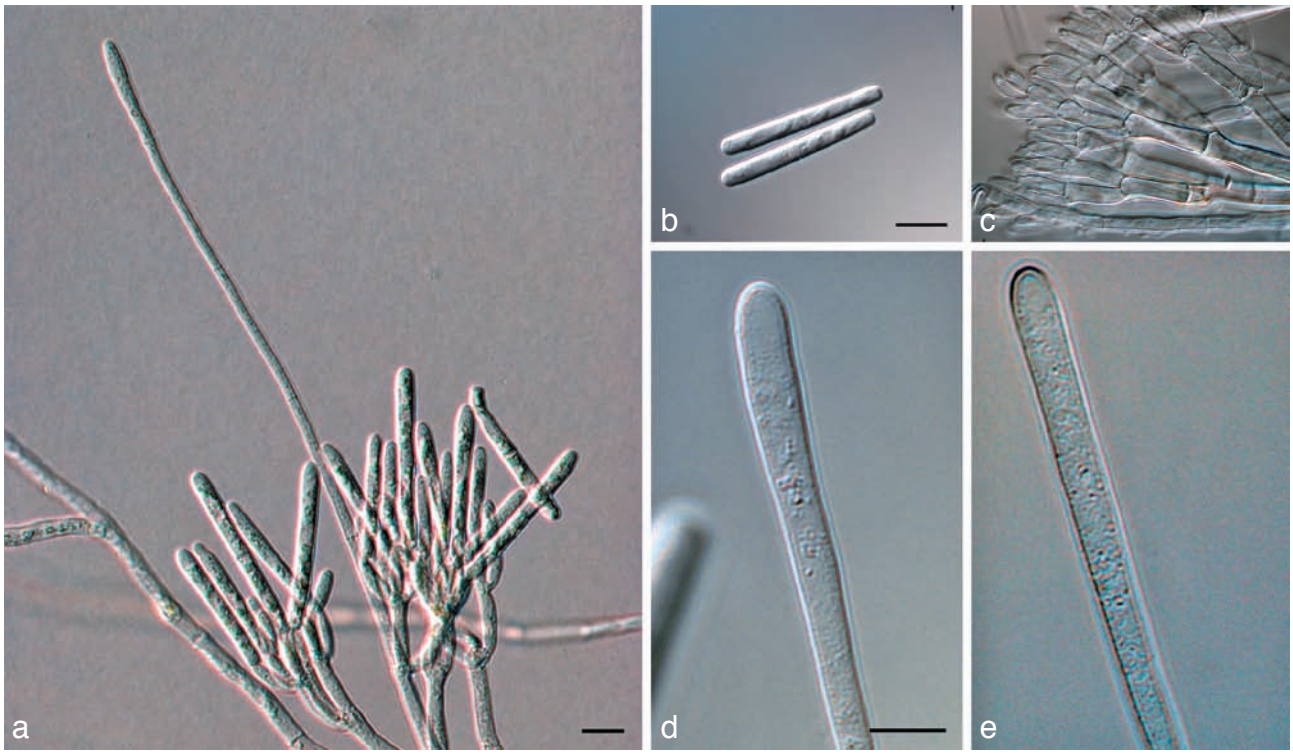

Fig. 5 *Calonectria brassicae*. a. Macroconidiophore on SNA; b. macroconidia; c. fertile branches; d, e. clavate vesicles. — Scale bars = 10 µm.

is highly virulent and regarded it as unique in causing disease symptoms in established plantations of *Pinus* spp. No disease symptoms associated with *Ca. brachiatica* or *Ca. brassicae* were seen in established plantations in the present study and we primarily regard these fungi as nursery pathogens, of which the former species is more virulent than the latter.

The use of SNA (Nirenburg 1981) rather than carnation leaf agar (CLA; Fisher et al. 1982) for morphological descriptions of *Calonectria* spp. represents a new approach employed in this study. Previously, species descriptions for *Calonectria* have typically been conducted on carnation leaf pieces on tap water agar (Crous et al. 1992). However, carnation leaves are not always readily available for such studies and SNA, a low nutrient medium, also used for the related genera *Fusarium* and *Cylindrocarpon* spp. identification (Halleen et al. 2006, Leslie & Summerell 2006), provides a useful medium for which the chemical components are readily available. Another advantage of using SNA is its transparent nature, allowing direct viewing through a compound microscope as well as on mounted agar blocks for higher magnification (Leslie & Summerell 2006). In this study, it was found that the *Calonectria* isolates sporulate profusely on the surface of SNA and comparisons of measurements for structures on SNA and those on CLA showed no significant difference. However, CLA remains important to induce the formation of teleomorph structures in homothallic isolates or heterothallic isolates for which both mating types are present.

KEY TO *CALONECTRIA* SPECIES WITH CLAVATE VESICLES AND PREDOMINANTLY 1-SEPTATE MACROCONIDIA
(To be inserted in Crous 2002, p. 56, couplet no. 2)

2. Stipe extension thick-walled; vesicle acicular to clavate . . . . . *Cy. avesiculatum*
2. Stipe extension not thick-walled; vesicle clavate 3
3. Teleomorph unknown 4
3. Teleomorph readily formed 7
4. Macroconidia always 1(–2)-septate 5
4. Macroconidia 1(–3)-septate 6
5. Macroconidia 1-septate, (38–)40–55(–65) × (3.5–)4–5(–6) µm, av. = 53 × 4.5 µm; lateral stipe extensions absent . . . . . *Ca. brassicae*
5. Macroconidia 1(–2)-septate, (37–)40–48(–50) × 4–6 µm, av. = 44 × 5 µm; lateral stipe extensions present . . . . . *Ca. brachiatica*
6. Macroconidia (48–)57–68(–75) × (6–)6.5(–7) µm, av. = 63 × 6.5 µm . . . . . *Cy. australiense*
6. Macroconidia (45–)48–55(–65) × (4–)4.5(–5) µm, av. = 51 × 4.5 µm . . . . . *Cy. ecuadoriae*
7. Macroconidial state absent; megaconidia and microconidia present . . . . . *Ca. multiseptata*
7. Macroconidial state present 8
8. Teleomorph homothallic 9
8. Teleomorph heterothallic 10
9. Perithecia orange; macroconidia av. size = 45 × 4.5 µm . . . . . *Ca. gracilipes*
9. Perithecia red; macroconidia av. size = 56 × 4.5 µm . . . . . *Ca. gracilis*
10. Perithecia orange; macroconidia av. size = 32 × 3 µm . . . . . *Ca. clavata*
10. Perithecia red-brown; macroconidia av. size = 30 × 3 µm . . . . . *Ca. pteridis*

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