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The *Cylindrocladium candelabrum* species complex includes four distinct mating populations

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Abstract: *Cylindrocladium candelabrum*-like isolates were collected from a wide variety of geographic locations and compared based on their morphology, sexual compatibility and the nucleotide sequences of their rDNA ITS regions. All isolates included in this study mated to produce *Calonectria* teleomorphs with viable progeny. Four distinct mating populations were identified, each representing a genetically isolated, biallelic, heterothallic population. Several representative isolates of each mating population, reflecting geographic diversity, were chosen for sequence comparisons. The internal transcribed spacer (ITS) regions 1 and 2 that flank the 5.8S rDNA gene, as well as the gene itself, were sequenced and compared. All isolates representing the same group yielded similar sequences, but small, consistent differences were found between the groups. Based on these results we recognise *Calonectria scoparia* (anamorph *Cylindrocladium candelabrum*), and describe three new species, namely *Calonectria pauciramosa* (anamorph *Cylindrocladium pauciramosum*), *Calonectria insularis* (anamorph *Cylindrocladium insularae*) and *Calonectria mexicana* (anamorph *Cylindrocladium mexicanum*).

Key Words: *Calonectria,* ITS sequence analysis, mating studies, systematics

INTRODUCTION

Cylindrocladium scoparium Morgan, the type species of *Cylindrocladium* Morgan (*Cy.*) (Morgan 1892), has been associated with a wide range of plant disease problems in over 30 families throughout the world (Booth and Gibson 1973, French and Menge 1979, Peerally 1991, Waipara et al 1996). This species is, however, the most commonly incorrectly identified taxon in the genus. After several years of extensive collection by the authors, *Cy. scoparium* s. s. has been confirmed from only North America, but has possibly also been introduced into Europe (Overmeyer et al 1996).

Cylindrocladium scoparium, still incorrectly treated by many researchers as synonymous with *Cy. floridanum* Sobers & C. P. Seymour, has been the subject of much controversy. Victor et al (1997) used morphology, sexual compatibility, RAPD markers and A+T-rich total DNA polymorphisms to compare *Cy. scoparium* (teleomorph *Calonectria morganii* Crous et al), *Cy. candelabrum* Viégas (teleomorph *Ca. scoparia* Peerally), *Cy. ovatum* El Gholl et al (teleomorph *Ca. ovata* D. Victor and Crous) and *Cy. floridanum* (teleomorph *Ca. kyotensis* Terash.). This study showed that these species represent distinct taxa. Furthermore, evidence was presented to show that more than one species possibly exists in the *Cy. floridanum* complex. Additionally, based on DNA fingerprinting with human minisatellite DNA as a probe, Jeng et al (1997) showed the presence of three groups of isolates in collections of *Cy. floridanum* from Canada and the USA.

Among the small-spored species of *Cylindrocladium, Cy. scoparium* has also commonly been confused with taxa such as *Cy. ovatum* and *Cy. candelabrum*. All three of the latter species are heterothallic. In a recent study Crous et al (1998) confirmed the biallelic, heterothallic nature of *Cy. ovatum*. In earlier studies, however, very low mating percentages were obtained for *Cy. candelabrum* and *Cy. scoparium* (Crous et al 1993a, Overmeyer et al 1996), suggesting that further research was required to elucidate their mating systems.

Cylindrocladium candelabrum, which was originally described from leaves of a *Luma* sp. in Brazil, was characterized by Viégas (1946) as having narrowly ellipsoidal vesicles and 1-septate conidia, 40–88 × 5–6 μm. Crous et al (1993a) reexamined the type specimen (IACM 440), and found it to be almost completely devoid of material, but the few conidia that

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were observed were 46–70 × 3.5–5 μm, and the vesicles were ellipsoidal to narrowly obpyriform. A neotype (PREM 51045) was subsequently designated, and two isolates PPRI 4153 and 4163 identified as the two mating tester strains. The species concept of *Cy. candelabrum* was complicated by Peerally (1991) who considered it synonymous with *Cy. ellipticum* Alfieri, Seymour & Sobers. The latter species was later shown to be a synonym of *Cy. scoparium* (Crous et al 1993a). To readily distinguish these species, *Cy. scoparium* was circumscribed as having ellipsoidal to pyriform vesicles (widest above the middle), while those of *Cy. candelabrum* were ellipsoidal to obpyriform (widest below the middle). However, a high degree of plasticity was observed amongst *Cy. candelabrum*-like isolates. This was particularly true in their vesicle shape, conidiophore branching pattern and conidial dimensions. Due to the low mating type frequency of isolates in previous studies, no clear indication was obtained on the nature and relevance of this variation amongst *Cy. candelabrum* isolates, and the species was accepted as being highly variable.

Molecular tools have become increasingly useful in providing additional evidence that has supported the interpretation of morphological variation. Several techniques including protein profiles (Crous et al 1993a), RAPD (Victor et al 1997) and RFLP (Crous et al 1997b), have been applied to the taxonomy of *Cylindrocladium* spp. The nucleotide sequences of the ribosomal DNA (rDNA) region contain intermittent functional and nonfunctional regions (Furlong et al 1983). The more conserved rDNA genes allow for comparisons between higher taxa. For example, Rehner and Samuels (1995) compared the nucleotide sequences of the 28S rDNA gene from a wide range of hypocrealean taxa, including *Cy. scoparium* and *Cy. floridanum*. More variable areas are provided by intergenic regions such as the internal transcribed spacers (ITS1 and ITS2) that flank the 5.8S rDNA gene. Various researchers have used these sequences to resolve intra- and interspecies phylogenies (Nazar et al 1991, Sreenivasprasad et al 1994, Bryan et al 1995, Jeng et al 1996, Witthuhn et al 1998).

Recently Jeng et al (1997) published ITS1, ITS2 and 5.8S rDNA sequences of *Cy. scoparium* and *Cy. floridanum*. In these comparisons, one 6-bp deletion and three point mutations were found in the ITS2 region. This indicated the potential of this region to be used as a tool to differentiate between morphologically similar *Cy. candelabrum*-like species. Accordingly, the present study was undertaken to investigate the application of a biological species concept as well as a phylogenetic species concept to isolates provisionally accommodated in the *Cy. candelabrum* species complex. Using these data, it was possible to evaluate the value of morphological characters in *Cylindrocladium*.

MATERIALS AND METHODS

Isolates.—*Cylindrocladium candelabrum* isolates were either obtained from symptomatic material or they were baited from soil samples. Soil samples were collected and treated as explained in Crous et al (1997a). Type specimens were lodged at the National Collection of Fungi in Pretoria (PREM), and ex-type cultures maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa (STE-U).

Sexual compatibility.—One hundred single conidial *Cy. candelabrum*-like isolates (listed under the results), originating from various geographic locations were mated in all possible combinations. This was achieved by removing 3 mm diam agar plugs from the periphery of actively growing cultures and placing them on CLA plates as described by Crous et al (1993a). Two different isolates were placed in a Petri dish with carnation leaves between them. Following this, plates were packed in stacks of 10, sealed in plastic bags and incubated on the laboratory bench at 22 C. Protoperithecia appeared after 2 wk and successful matings were determined after 2 mo of incubation. Successful matings were regarded as those isolate combinations that produced perithecia with fertile, extruding ascospores. Mating groups were subsequently distinguished and strains that resulted in prolific matings were selected from each group. For each mating group identified, ascospores were obtained from two matings, involving four separate isolates. Seven single ascospores were sub-cultured for each mating group, and these were crossed in all possible combinations in order to reconfirm the biallelic, heterothallic nature of each mating population. Two isolates of opposing mating type were selected as tester strains from these isolates, and these were subsequently mated with the tester strains of the other groups to reconfirm that no mating was occurring between groups.

Sequence comparisons.—Four isolates, two isolates per mating type, of each mating group (species), representing a wide geographic distribution (TABLE I), were selected for sequencing. Single conidial isolates were grown on MEA plates and plugs were transferred into 500 mL Erlenmeyer flasks containing 100 mL liquid MEA broth. Flasks were shaken at 25 C and 125 rpm for approximately 7 d. Mycelium was collected by filtration (Whatman no. 1 filter paper) and DNA was extracted as described by Crous et al (1993b). Both strands of the ITS1 and ITS2 intergenic spacers as well as the 5.8S ribosomal gene were sequenced and compared. Sequences were deposited at GenBank (AF059280–AF059283). DNA was amplified using the primers ITS1 (5′-dTCCGTAGGTGAACCTGCGG) and ITS4 (5′-dTCCTCCGCTTATTGATATGC) (White et al 1990). The region amplified was the 5.8S ribosomal gene and the two internal transcribed spacers (ITS1 and ITS2) flanking the gene. PCR amplifications were performed on a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, UK). Reactions comprised of 1 μL Expand High Fidelity DNA polymer-

TABLE I. Isolates selected for sequencing

Species	STE-U No.	Origin
Cy. pauciramosum (Group 1)	951	Mexico
	971	South Africa
	1160	Colombia
	1691	Australia
Cy. candelabrum (Group 2)	1674	Brazil
	1675	Brazil
	1676	Brazil
	1678	Brazil
Cy. insularae (Group 3)	766	Madagascar
	768	Madagascar
	616	Brazil
	954	Mexico
Cy. mexicanum (Group 4)	927	Mexico
	928	Mexico
	941	Mexico
	966	Mexico

ase (Boehringer Mannheim, Mannheim, Germany) and 1 μL reaction buffer containing 1.5 mM $MgCl_2$ (Boehringer Mannheim), with $MgCl_2$ added to make up the final buffer concentration to 5.5 mM. Liquid paraffin oil was overlaid to prevent evaporation. Other reagents added to the final volume of 100 μL were 250 μM of each NTP, 0.5 μM of each primer and 25 ng DNA. PCR conditions were a denaturing step at 94 C for 1 min followed by 10 cycles of 56 C for 30 s, 72 C for 2 min and 94 C for 15 s. This was followed by a further 20 cycles at the same settings except for a 20 s time increase at 72 C. PCR products were purified using Wizard PCR Preps (Promega Corporation, Madison, Wisconsin). Both strands of the PCR product were sequenced using the ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut). A Dye Terminator Cycle Sequencing Ready Reaction Kit containing AmpliTaq DNA Polymerase (Perkin-Elmer) was used for the sequencing reactions. The reactions were carried out with a concentration of 20 to 40 ng of DNA template and 3.2 pmol primer in a total volume of 10 μL. The cycle sequencing reaction was done by PCR under conditions of 96 C for 30 s, 50 C for 15 s, and 60 C for 4 min. This was repeated for 25 cycles. DNA was finally purified using Centri-Sep Spin columns (Princeton Separations, Adelphia, New Jersey) and loaded onto the sequencing gel. Phylogenetic analysis of the ITS1 and ITS2 DNA sequences was performed by using the PAUP (Phylogenetic Analysis Using Parsimony) 3.1.1 program (Swofford 1993). The branch and bound algorithm, with gaps treated as a fifth character, was used. Confidence intervals were determined using a 100 bootstrap replications. All uninformative characters were ignored. Sequences of *Cy. scoparium* and *Cy. floridanum,* previously published by Jeng et al (1997), were used for comparison. In addition to this, a sequence of *Fusarium subglutinans,* deposited by Waalwijk et al (1996), was obtained (EMBL accession number X94167) and used as outgroup.

Morphological comparisons.—Isolates were cultured on 2% malt extract agar (MEA) (Oxoid), plated onto carnation-leaf agar (CLA) (Crous et al 1992), incubated at 25 C under near-ultraviolet light, and examined after 7 d. Only material occurring on carnation leaves was examined. Mounts were prepared in lactophenol, examined under nomarski and phase contrast, and measurements made at ×1000. Wherever possible, each measurement represents at least 30 observations, and extremes are given in parentheses. Cardinal temperature requirements for growth and cultural characteristics were determined after 6 d on MEA using procedures described by Crous and Wingfield (1994), and colony colors coded according to Rayner (1970). Cultures of *Cy. candelabrum* were identified using the keys of Crous and Wingfield (1994).

RESULTS

Sexual compatibility.—All matings between the selected isolates resulted in perithecia containing fertile ascospores, except where STE-U 216 was concerned (FIG. 1). Whether this isolate constitutes another mating population, or has lost the ability to mate, remains unresolved. Control inoculations indicated that all isolates used were self sterile. Isolates of the same mating type yielded no perithecia when mated, confirming the biallelic, heterothallic mating system commonly found in ascomycetes (Yoder et al 1986). Four distinct mating populations (Groups 1–4) were observed. No successful matings were observed between the different mating groups, and subsequent crossings between ascospore progeny of prolific mating strains confirmed the distinctiveness of the mating groups (results not shown).

Sequence analysis.—No differences were detected between isolates for their 5.8S sequences. The four isolates selected per mating group (TABLE I), revealed ITS sequences that were 100% similar within each group, irrespective of geographic location. For the purpose of comparison a single sequence, representing the four isolates from one species, was subsequently used to compare isolates of the four mating populations. A number of single and double base pair substitutions and deletions were found between all the species in the ITS1 and ITS2 regions. Previous work done by Jeng et al (1997) showed one 6-bp deletion and 3 single base substitutions when the sequences of *Cy. floridanum* and *Cy. scoparium* were compared. None of the other species sequenced contained a 6-bp deletion found in the *Cy. floridanum* ITS2 region. Additional differences were observed in the ITS1 region of the four species in the *Cy. candelabrum* complex. Single bp substitutions in the ITS2 region could distinguish *Cy. floridanum* from the other species' sequences, while a similar single

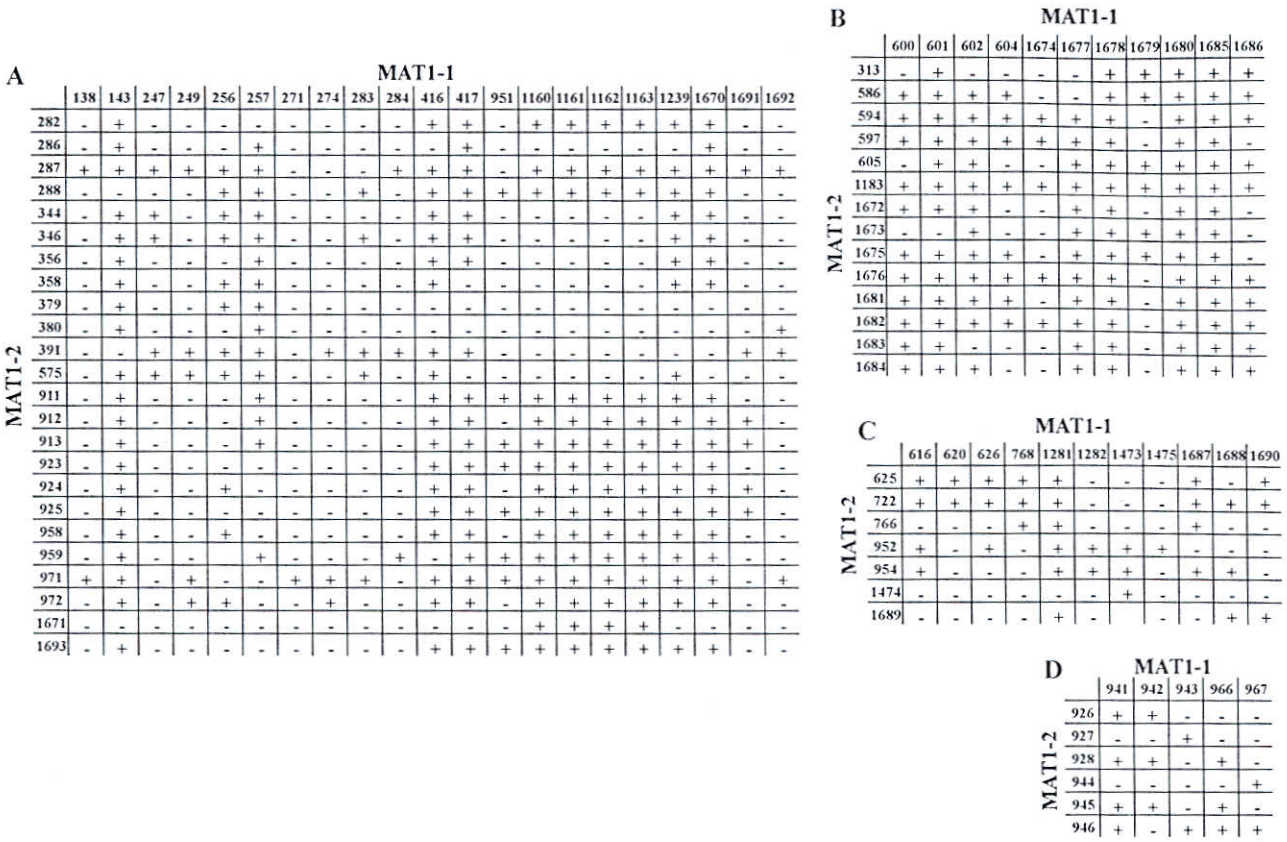


FIG. 1. Results of mating studies. Although matings were performed in all possible combinations, results are only presented between the isolates of the two different mating types (MAT1-1 and MAT1-2) in each mating population. Horizontally numbered isolates form part of the MAT1-1 mating types and vertically numbered isolates are MAT1-2 mating types. When ascospores were produced the matings are indicated as successful (+). Unsuccessful matings are indicated with (−).

base difference could differentiate the four species in the *Cy. candelabrum* complex from *Cy. scoparium* and *Cy. floridanum*. Additional single base deletions and substitutions distinguished all species on the basis of sequence dissimilarity. Accordingly, a phylogenetic tree was produced using PAUP analysis (Swofford 1993). FIGURE 2 shows one of the four most parsimonious trees obtained by branch and bound analysis of the informative sites of the ITS1, 5.8S and ITS2 DNA regions for the six species mentioned above. All four most parsimonious trees indicated a closer relationship between the sequences of *Cy. insularae* and those of *Cy. scoparium* and *Cy. floridanum*. The exact relationships between the other species were ambiguous.

Morphological comparisons.—Several morphological characters were studied. This included the shape and diameter of the terminal vesicles extending from the conidiophore stipes, conidial size, conidiophore branching pattern, ascospore shape, size, perithecial color, anatomy, morphology, and cultural characteristics.

The morphological similarities of the anamorph and teleomorph states corresponded well with the results obtained in the mating studies, and grouped isolates into four distinct groups. The four groups identified based on these features were further supported by their distinct DNA sequences, which led us to conclude that they represent four biological species, which are subsequently described below.

Calonectria pauciramosa C. L. Schoch et Crous, sp. nov. FIGS. 3–10

Anamorph. **Cylindrocladium pauciramosum** C. L. Schoch et Crous, sp. nov.

Perithecia subglobosa ad ovoidea, 250–400 μm alta, 170–300 μm lata, crocea ad rubro-brunnea, pariete exteriore verrucosa, ostiolo papillato. Asci clavati, in stipitem longum tenuem gradatim angustatae, 70–140 × 8–25 μm, 8-spori. Ascosporae hyalinae, fusiformes, 1-septatae, nihil vel leviter ad septum constrictae, (30–)33–38(–40) × 6–7(–8) μm. Filum septatum, hyalinum (120–)180(–230) μm, in vesiculam obpyriformam ad late ellipsoidam (5–)7–9(–11) μm diam terminans. Conidia cylindrica, hyalina, 1-septata, apicibus obtusis, (30–)45–55(–60) × (3.5–)4–5 μm. Microconidiophora ignota.

HOLOTYPES. BRAZIL × SOUTH AFRICA. BRAZIL. BA-

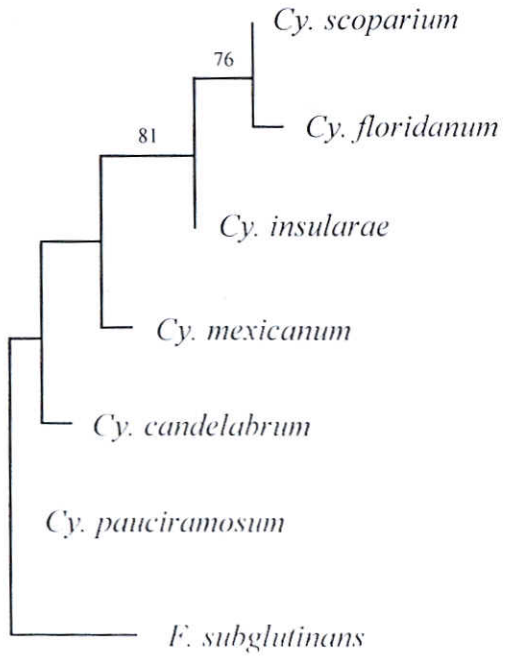
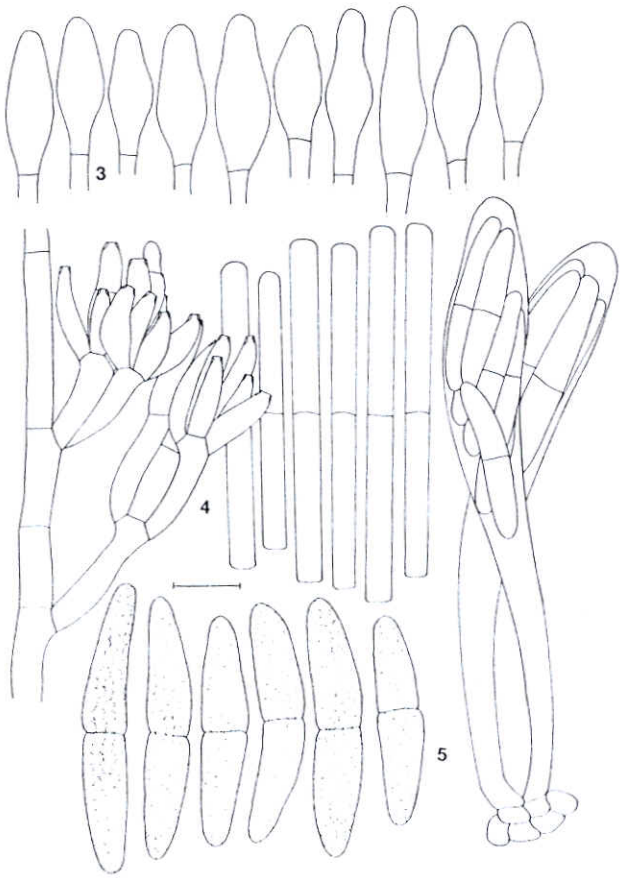


FIG. 2. One of four most parsimonious trees generated with a branch and bound algorithm in PAUP 3.1.1 from aligned sequences of the 5.8S gene and flanking ITS1, ITS2 regions (15 steps, CI = 0.8, RI = 0.786). Bootstrap values above 50% are shown. A *Fusarium subglutinans* sequence (EMBL accession number X94167) was used as outgroup.



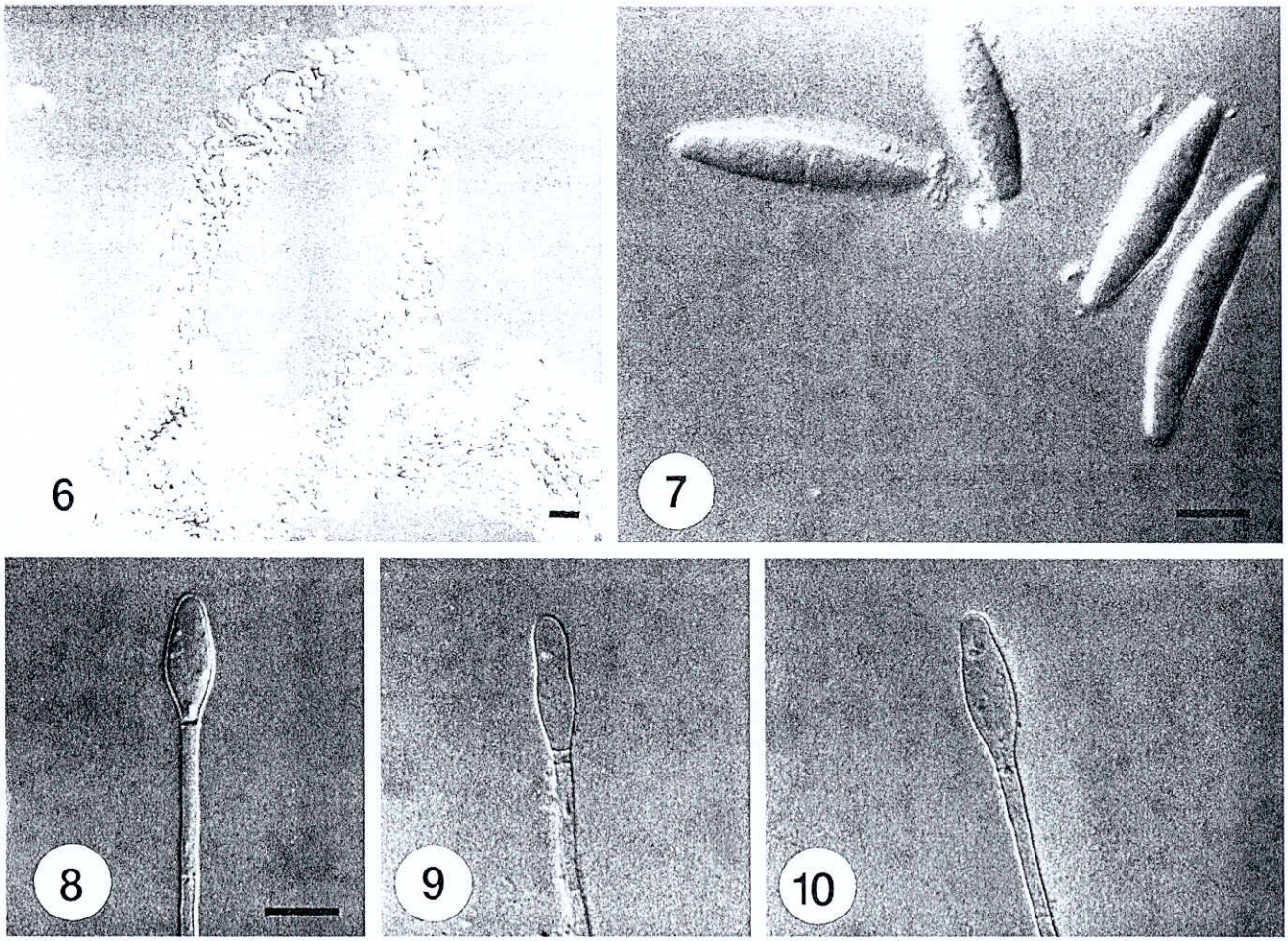
FIGS. 3–5. *Calonectria pauciramosa* and its anamorph *Cylindrocladium pauciramosum*. 3. Terminal vesicles on stipe extensions. 4. Conidiophore and conidia. 5. Asci and ascospores. Bar = 10 μm.

HA: Nursery, *Eucalyptus* sp., Jul. 1990, *A. C. Alfenas*; Knysna, soil, Nov. 1994, *P. W. Crous*, heterothallic mating of STE-U 1670 (PREM 55753 anamorph) × STE-U 971 (PREM 55752 anamorph HOLOTYPE), Apr. 1997 *C. L. Schoch* (PREM 55754 teleomorph HOLOTYPE).

Etymology. Refers to the relatively low number of conidiophore branches in the species.

Perithecia orange to red-brown, subglobose to ovoid, 250–400 μm high, 170–300 μm wide, turning dark red in 3% KOH; ostiole papillate. Perithecia rough-walled, wall consisting of two layers: outside layer of *textura globulosa*, 20–50 μm wide; inner layer of *textura angularis*, 5–10 μm wide, outer cells 40–55 × 15–35 μm; hymenial layer of *textura prismatica*, hyaline, 5–10 μm wide; perithecial base up to 100 μm wide, consisting of dark red, angular cells. *Asci* 8-spored, clavate, 70–140 × 8–25 μm, tapering to a long thin stalk. *Ascospores* aggregated in the upper third of the ascus, hyaline, fusoid with rounded ends, straight to slightly curved, 1-septate, not or slightly constricted at the septum, (30–)33–38(–40) × 6–7(–8) μm. *Macroconidiophores* comprised of a stipe, a sterile elongation and a penicillate arrangement of fertile branches. Stipe septate, (120–)180(–230) μm long, terminating in an obpyriform to broadly ellipsoidal vesicle, (5–)7–9(–11) μm diam; primary branches aseptate or 1-septate, 12–45 × 5–6 μm; secondary branches aseptate, 15–20 × 5 μm, and tertiary branches aseptate, 12–15 × 5 μm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 10–13 × 2.5–4 μm, apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (30–)45–55(–60) × (3.5–)4–5 μm, 1-septate, lacking a visible abscission scar, held in cylindrical clusters by colorless slime. *Microconidiophores* not observed. *Chlamydospores* dark brown, thickened, formed in extensive numbers throughout the medium, and aggregated to form microsclerotia.

Cultures. Colony color (underneath) 13i fulvous, (surface) 13i sienna with abundant white aerial mycelia. Colony margin irregular, with extensive chlamydospores and sparse sporulation on aerial mycelia. Colonies obtaining a radius of 17–20 mm diam on MEA after 6 d in the dark at 25 C. Cardinal temperatures for growth were min above 5 C, max below 35 C, opt 25 C. This is both a high and low temperature species, growing below 5 C, and above 30 C.



FIGS. 6–10. *Calonectria pauciramosa* and its anamorph *Cylindrocladium pauciramosum*. 6. Vertical section through a perithecium. 7. Ascospores. 8–10. Terminal vesicles. Bars = 10 μm.

Substrate. *Acacia cyclops*, *Azalea* sp., *Eucalyptus* spp., *Fragraria* sp., *Protea* sp., *Rhododendron* sp., soil.

Distribution. Australia, Brazil, Colombia, Mexico, South Africa.

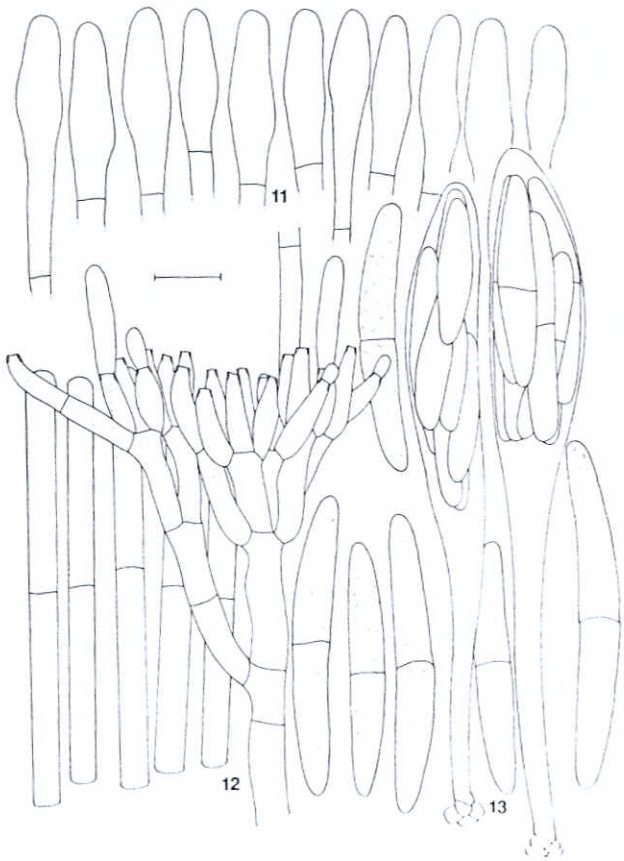
Additional cultures examined. AUSTRALIA. QUEENSLAND: Locality unknown, strawberry, 1991, *D. Hutton* (N167/91 = STE-U 1691; N335/91 = STE-U 1692). BRAZIL. BAHIA: Vivieros, *Eucalyptus* sp., Jul. 1990, *A. C. Alfenas* (UFV 25 = STE-U 1670; UFV 27 = STE-U 1671). SANTA CATARINA: Florianópolis, soil, Apr. 1994, *M. J. Wingfield* (STE-U 911–913, 923–925). COLOMBIA. CÓRDOBA: La Selva, Jun. 1995, *M. J. Wingfield* (STE-U 1160–1163). MEXICO. VERACRUZ: Catemaco, Laguna Encantada, soil, Apr. 1994, *M. J. Wingfield* (STE-U 951). SOUTH AFRICA. KWAZULU-NATAL: Kwambonambi, *Eucalyptus grandis* seedlings, Feb. 1990, *P. W. Crous* (STE-U 247, 249, 256, 257, 271, 274, 344, 346); *Eucalyptus grandis*, Oct. 1995, *P. W. Crous* (STE-U 1239); Pietermaritzburg, *Eucalyptus nitens*, Mar. 1990, *P. W. Crous* (STE-U 391); WESTERN CAPE: *Acacia cyclops*, Jul. 1990, *M. Morris* (CMM 953 = STE-U 1693); Cedarberge, *Azalea* bushes, Feb. 1993, *S. Lamprecht* (STE-U 575); Knysna, soil, Nov. 1994, *P. W. Crous* (STE-U 971, 972); MPUMALANGA: Kruisfontein, *Eucalyptus grandis* trunk, Sept. 1989, *P. W. Crous* (STE-U 138, 143); Sabie, soil, Feb. 1990, *P. W. Crous* (STE-U 356, 358); Klipkraal, *Eucalyptus grandis* seedlings, Feb. 1990, *P. W. Crous* (STE-U 286–288); Witrivier, *Azalea* sp., May 1990, *S. Lamprecht* (STE-U 379, 380); NORTHERN PROVINCE: Piet Retief, pine cuttings, Nov. 1994, *P. W. Crous* (STE-U 958, 959); Tzaneen, *Eucalyptus grandis* seedlings, Feb. 1990, *P. W. Crous* (STE-U 282–284). *Eucalyptus grandis* cuttings, Jun. 1990, *S. de Buisson* (STE-U 416, 417).

Calonectria scoparia Peerally, Mycotaxon 40: 341 (1991). FIGS. 11–17

Anamorph. *Cylindrocladium candelabrum* Viégas, Bragantia 6: 370 (1946).

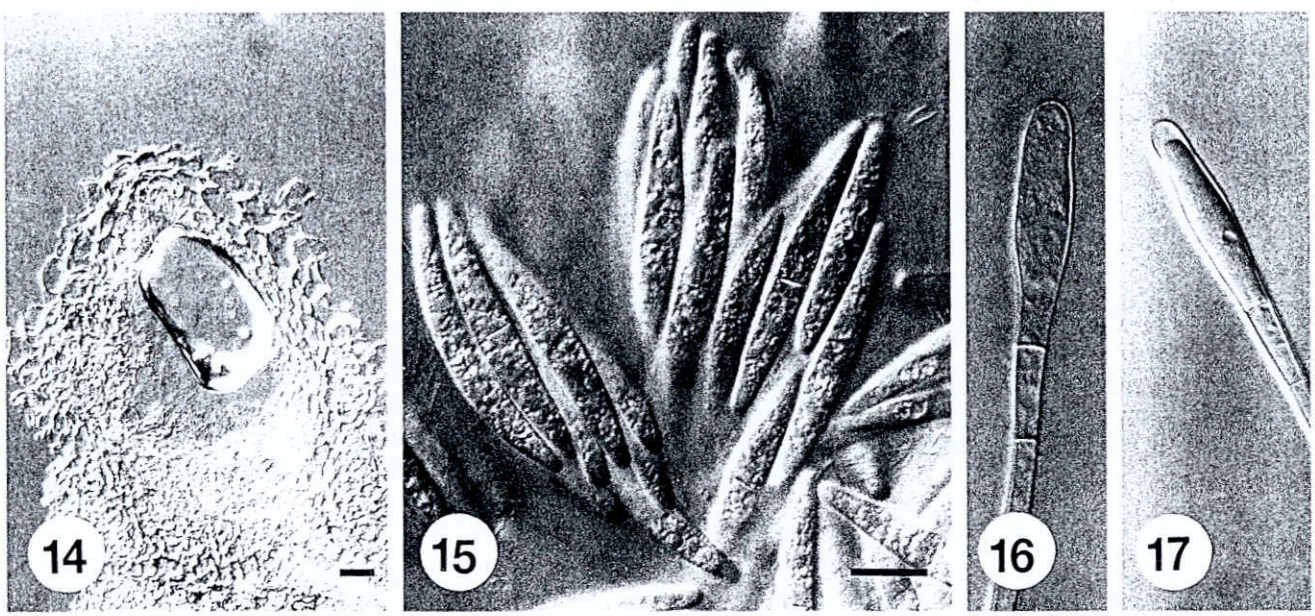
HOLOTYPES. BRAZIL. BAHIA. Picadao, Conceicao de Barra, *Eucalyptus grandis*, Apr. 1992, *A. C. Alfenas & F. A. Ferreira* (PREM 51045 NEOTYPE of teleomorph; Crous et al 1993a); Copener, *Eucalyptus* sp., *A. C. Alfenas*, PREM 51044 (NEOTYPE of anamorph; Crous et al 1993a), culture ex-type PPRI 4135.

Perithecia red-brown, subglobose to ovoid, 350–450 μm high, 300–350 μm wide, turning dark red in 3%



FIGS. 11–13. *Calonectria scoparia* and its anamorph *Cylindrocladium candelabrum*. 11. Terminal vesicles on stipe extensions. 12. Conidiophore and conidia. 13. Asci and ascospores. Bar = 10 μm.

KOH, frequently in clusters of 3–4; ostiole papillate. Perithecia rough-walled, wall consisting of two layers: outside layer of *textura globulosa*, 50–100 μm wide; inner layer of *textura angularis*, 5–10 μm wide, outer cells 35–45 × 18–30 μm; hymenial layer of *textura prismatica*, hyaline, 5–10 μm wide; perithecial base up to 150 μm wide, consisting of dark red, angular cells. *Asci* 8-spored, clavate, 70–130 × 7–15 μm, tapering to a long thin stalk. *Ascospores* aggregated in the upper third of the ascus, hyaline, fusoid with rounded ends, straight to slightly curved, 1-septate, not to slightly constricted at the septum, (40–)45–50(–60) × 5–6 μm; becoming 3-septate once discharged. *Macroconidiophores* comprised of a stipe, a sterile elongation and a penicillate arrangement of fertile branches. Stipe septate, (100–)170(–220) μm long, terminating in an ellipsoidal to narrowly obpyriform vesicle, (5–)6–7(–8) μm diam; primary branches aseptate or 1-septate, 20–45 × 4–5 μm; secondary branches aseptate, 15–25 × 4–5 μm, tertiary branches aseptate, 15–20 × 4–5 μm, and quaternary branches aseptate, 10–15 × 4–5 μm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 10–20 × 3–4 μm, apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (45–)58–68(–80) × 4–5(–6) μm, 1-septate, lacking a visible abscission scar, held in cylindrical clusters by colorless slime. *Microconidiophores* not observed. *Chlamydospores* dark brown, thickened, formed in extensive numbers throughout the medium, and aggregated to form microsclerotia.



FIGS. 14–17. *Calonectria scoparia* and its anamorph *Cylindrocladium candelabrum*. 14. Vertical section through a perithecium. 15. Ascospores. 16, 17. Terminal vesicles. Bars = 10 μm.

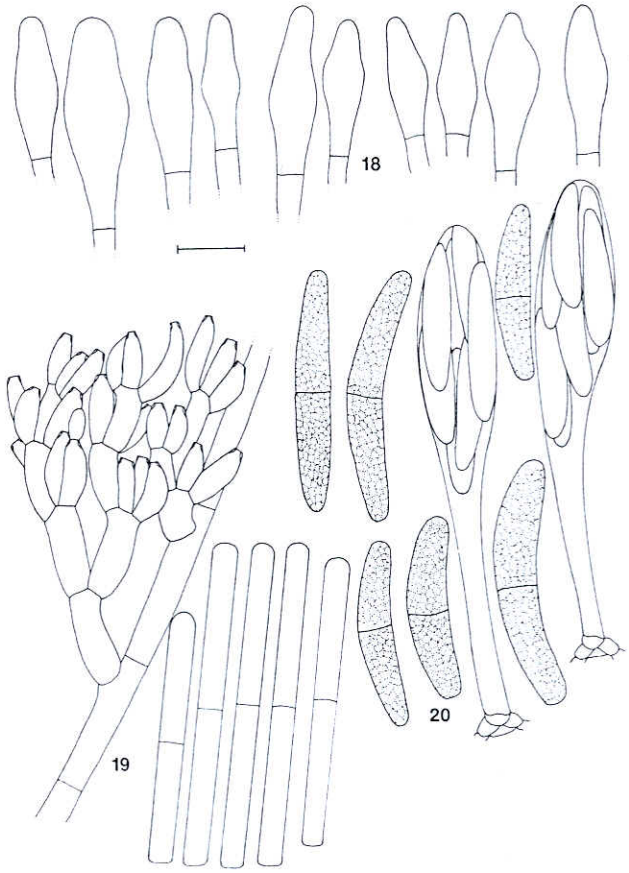
Cultures. Colony color (underneath) 13i fulvous, (surface) 13i sienna. Colony margin irregular with sparse to moderate aerial mycelia, and extensive chlamydospores. Colonies obtaining a radius of 12–17 mm diam on MEA after 6 d in the dark at 25 C. Cardinal temperatures for growth were min above 5 C, max below 35 C, opt 25 C. This is both a high and low temperature species, with medium sporulation on aerial mycelium.

Substrate. Eucalyptus spp., *Luma* sp., soil.

Distribution. Brazil, Venezuela.

Additional specimens deposited. BRAZIL. BAHIA: Vivieros, soil, heterotallic mating of STE-U 1675 (PREM 55755 anamorph) × STE-U 1677 (PREM 55756 anamorph), Apr. 1997, *C. L. Schoch,* (PREM 55757 teleomorph).

Additional cultures examined. BRAZIL. AMAZONAS: Locality unknown, *Eucalyptus* sp., 1991, *A. C. Alfenas* (UFV 117 = STE-U 1675; UFV 118 = STE-U 1676; UFV 121 = STE-U 1677; UFV 122 = STE-U 1678; UFV 126 = STE-U 1679; UFV 128 = STE-U 1680; UFV 129 = STE-U 1681; UFV 130 = STE-U 1682; UFV 132 = STE-U 1683); *Eucalyptus* sp., 1991, *J. C. Dianese* (D1038 = STE-U 1684); Belém, *Eucalyptus* sp., Feb. 1990, *M. J. Wingfield* (STE-U 313); BAHIA: Copener, *Eucalyptus* sp., Jul. 1990, *A. C. Alfenas* (UFV 63 = STE-U 1674); Vivieros, *Eucalyptus* sp., Jul. 1990, *ACA:* (UFV 29 = STE-U 1672); MINAS GERAIS: Ipatinga, *Eucalyptus* sp., Jul. 1990, *A. C. Alfenas* (UFV 45 = STE-U 1673); Bocaiúva, *Eucalyptus* sp., Jul. 1990, *A. C. Alfenas* (UFV 170 = STE-U 1685); Bom Despacho, *Eucalyptus* sp., Jul. 1990, *A. C. Alfenas* (UFV 172 = STE-U 1686); SÃO PAULO: São Paulo, *Eucalyptus* cuttings, Mar. 1993, *P. W. Crous* (STE-U 586, 594, 597, 600–602, 604, 605). VENEZUELA. Locality unknown, soil, Jun. 1995, *M. J. Wingfield,* (STE-U 1183).



FIGS. 18–20. *Calonectria insularis* and its anamorph *Cylindrocladium insularae.* 18. Terminal vesicles on stipe extensions. 19. Conidiophore and conidia. 20. Asci and ascospores. Bar = 10 μm.

Calonectria insularis C. L. Schoch et Crous, sp. nov. FIGS. 18–24

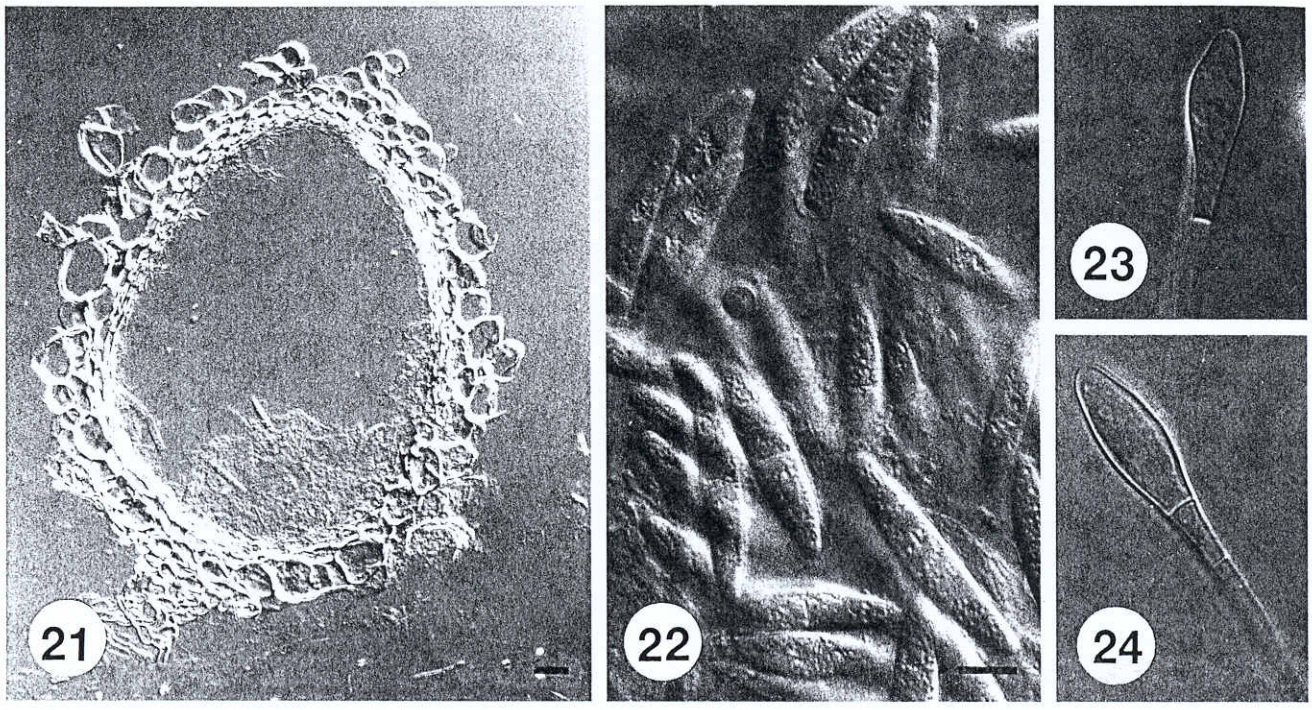
Anamorph. **Cylindrocladium insularae** C. L. Schoch et Crous, sp. nov.

Perithecia subglobosa ad ovoidea, 350–450 μm alta, 300–350 μm lata, crocea ad rubra, pariete exteriore verrucosa, ostiolo papillato. Asci clavati, in stipitem longum tenuem gradatim angustatae, 70–120 × 7–18 μm, 8-spori. Ascosporae hyalinae, fusiformes, 1-septatae, ad septum nihil constrictae, (27–)30–36(–42) × 5–6(–7) μm. Ascosporae evolentes usque ad constrictae dismissae ab asco. Filum septatum, hyalinum (110–)160(–250) μm, in vesiculam obpyriformam ad late ellipsoidam (4–)7–10(–13) μm diam terminans. Conidia cylindrica, hyalina, 1-septata, apicibus obtusis, (33–)40–50(–60) × 3.5–4 μm. Microconidiophora ignota.

HOLOTYPES. MADAGASCAR: Tamatave, soil, Apr. 1997, *P. W. Crous,* heterothallic mating of STE-U 766 (PREM 55758 anamorph HOLOTYPE) × STE-U 768 (PREM 55759 anamorph), Apr. 1997, *C. L. Schoch,* (PREM 55760 teleomorph HOLOTYPE).

Etymology. In reference to its geographic distribution.

Perithecia orange to red, subglobose to ovoid, 350–450 μm high, 300–350 μm wide, turning dark red in 3% KOH; ostiole papillate. Perithecia rough-walled, wall consisting of two layers: outside layer of *textura globulosa,* 40–80 μm wide; inner layer of *textura angularis,* 5–10 μm wide, outer cells 25–45 × 20–35 μm; hymenial layer of *textura prismatica,* hyaline, 5–10 μm wide; perithecial base up to 100 μm wide, consisting of dark red, angular cells. *Asci* 8-spored, clavate, 70–120 × 7–18 μm, tapering to a long thin stalk. *Ascospores* aggregated in the upper third of the ascus, hyaline, fusoid with rounded ends, straight to slightly curved, 1-septate, not constricted at the septum, becoming constricted once discharged, (27–)30–36(–42) × 5–6(–7) μm. *Macroconidiophores* comprised of a stipe, a sterile elongation and a penicillate arrangement of fertile branches. Stipe septate, (110–)160(–250) μm long, terminating in an obpyriform to broadly ellipsoidal vesicle, (4–)7–10(–13) μm diam; primary branches aseptate or 1-septate, 10–45 × 4–5 μm; secondary branches aseptate, 10–25 × 4–5 μm, tertiary branches aseptate, 10–17 × 4–5 μm, and



FIGS. 21–24. *Calonectria insularis* and its anamorph *Cylindrocladium insularae*. 21. Vertical section through a perithecium. 22. Ascospores. 23, 24. Terminal vesicles. Bars = 10 μm.

quaternary branches aseptate, 10–12 × 4–5 μm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 9–14 × 3–5 μm, apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (33–)40–50(–60) × 3.5–4 μm, 1-septate, lacking a visible abscission scar, held in cylindrical clusters by colorless slime. *Microconidiophores* not observed. Dark brown, thickened *chlamydospores* formed in extensive numbers throughout the medium, and aggregated to form microsclerotia.

Cultures. Same characteristics as *Cy. pauciramosum* with colonies obtaining a radius of 18–23 mm diam on MEA after 6 d in the dark at 25 C. Cardinal temperatures for growth were min above 15 C, max above 35 C, opt 25–30 C. This is a high temperature species.

Substrate. Acacia sp., *Auracaria heterophylla, Medicago sativa, Persea americana, Pisum sativum, Eucalyptus* sp., soil.

Distribution. Brazil, Hawaii, Indonesia, Madagascar, Malaysia, Mauritius, Mexico.

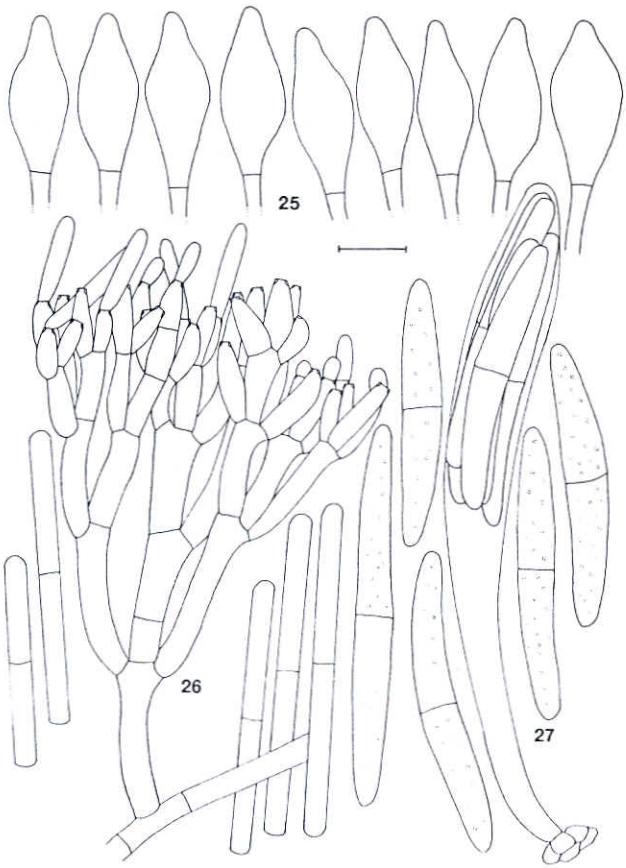
Additional cultures examined. BRAZIL. AMAZONAS: Belém, soil, Apr. 1993, *M. J. Wingfield* (STE-U 616, 620, 625, 626). INDONESIA. SUMATRA: Sei Kobaro, *Acacia mangium* rhizosphere, Jan. 1994, *A. C. Alfenas* (STE-U 722). MADAGASCAR. Tamatave, soil, Apr. 1994, *P. W. Crous* (STE-U 766, 768). MALAYSIA. MALAY PENINSULA: Kemasik, *Acacia* sp., Dec. 1995, *M. J. Wingfield* (STE-U 1281, 1282). MAURITIUS. Rivière Noire, soil, Apr. 1996, H. Smith (STE-U 1473, 1474). Pampalmousses, soil, Apr. 1996, H. Smith (STE-U 1475). MEXICO. VERACRUZ: Concjos, Puente Nacional, soil, Apr. 1994, *M. J. Wingfield* (STE-U 952, 954). USA. HAWAII: Locality unknown, *Medicago sativa*, 1981, *M. Aragaki* (A 890 = STE-U 1687); *Auracaria heterophylla*, 1987, *M. Aragaki* (A 1570 = STE-U 1688); *Pisum sativum*, 1988, *M. Aragaki* (A 1823 = STE-U 1689); *Persea americana*, 1988, *M. Aragaki* (A 1853 = STE-U 1690).

Calonectria mexicana C. L. Schoch et Crous, sp. nov. FIGS. 25–34

Anamorph. **Cylindrocladium mexicanum** C. L. Schoch et Crous, sp. nov.

Perithecia subglobosa ad ovoidea, 400–450 μm alta, 350–450 μm lata, crocea ad rubra, pariete exteriore verrucosa, ostiolo papillato. Asci clavati, in stipitem longum tenuem gradatim angustatae, 70–120 × 10–20 μm, 8-spori. Ascosporae hyalinae, fusiformes, 1-septatae, nihil vel leviter constrictae ad septum, (35–)40–55(–65) × 5–6(–7) μm. Ascoporae evolentes usque ad tres septa dismissae ab asco. Filum septatum, hyalinum (160–)180(–250) μm, in vesiculam late ellipsoidam apicibus papillatis (7–)8–10(–12) μm diam terminans. *Conidia* cylindrica, hyalina, 1-septata, apicibus obtusis, (35–)40–48(–52) × 3–4(–4.5) μm. Microconidiophora ignota.

HOLOTYPES. MEXICO. YUCATAN: Uxmal, soil., Apr. 1994, *M. J. Wingfield*; HOLPECHÉN: Campeche, soil., Apr. 1994, *M. J. Wingfield*, heterothallic mating of STE-U 927 (PREM 55761 anamorph HOLOTYPE) × STE-U 941 (PREM 55762 anamorph), Apr. 1997, *C. L. Schoch* (PREM 55763 teleomorph HOLOTYPE).



FIGS. 25–27. *Calonectria mexicana* and its anamorph *Cylindrocladium mexicanum*. 25. Terminal vesicles on stipe extensions. 26. Conidiophore and conidia. 27. Asci and ascospores. Bar = 10 μm.

Etymology. In reference to its country of origin.

Perithecia orange to red, subglobose to ovoid, 400–450 μm high, 350–450 μm wide, turning dark red in 3% KOH; ostiole papillate. Perithecia rough-walled, wall consisting of two layers: outside layer of *textura globulosa,* 35–90 μm wide; inner layer of *textura angularis,* 5–15 μm wide, outer cells 20–35 × 20–30 μm; hymenial layer of *textura prismatica,* hyaline, 5–10 μm wide; perithecial base up to 100 μm wide, consisting of dark red, angular cells. *Asci* 8-spored, clavate, 70–120 × 10–20 μm, tapering to a long thin stalk. *Ascospores* aggregated in the upper third of the ascus, hyaline, fusoid with rounded ends, straight to slightly curved, 1-septate, not or slightly constricted at the septum, (35–)40–55(–65) × 5–6(–7) μm; becoming 3-septate once discharged. *Macroconidiophores* comprised of a stipe, a sterile elongation and a penicillate arrangement of fertile branches. Stipe septate, (160–)180(–250) μm long, terminating in a broadly ellipsoidal vesicle with a papillate apex, (7–)8–10(–12) μm diam; primary branches aseptate or 1-septate, 17–45 × 4–6 μm; secondary branches aseptate, 15–25 × 4–5 μm, tertiary branches aseptate, 11–17 × 3–5 μm, and quaternary branches aseptate, 10–15 × 2.5–4 μm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 7–16 × 3–4 μm, apex with minute periclinal thickening and inconspicuous collarette. *Conidia* cylindrical, rounded at both ends, straight, (35–)40–48(–52) × 3–4(–4.5) μm, 1-septate, lacking a visible abscission scar, held in cylindrical clusters by colorless slime. *Microconidiophores* not observed. *Chlamydospores* dark brown, thickened, formed in extensive numbers throughout the medium, and aggregated to form microsclerotia.

Cultures. Colony color (underneath) 13b–13i (orange to sienna), (surface) similar as underneath with moderate white aerial mycelia. Colony margin irregular with extensive chlamydospores and sparse sporulation on aerial mycelium. Colonies obtaining a radius of 17–20 mm diam on MEA after 6 d in the dark at 25 C. Cardinal temperatures for growth were min above 10 C, max above 35 C, opt 25–30 C. This is both a high and low temperature species.

Substrate. Soil.

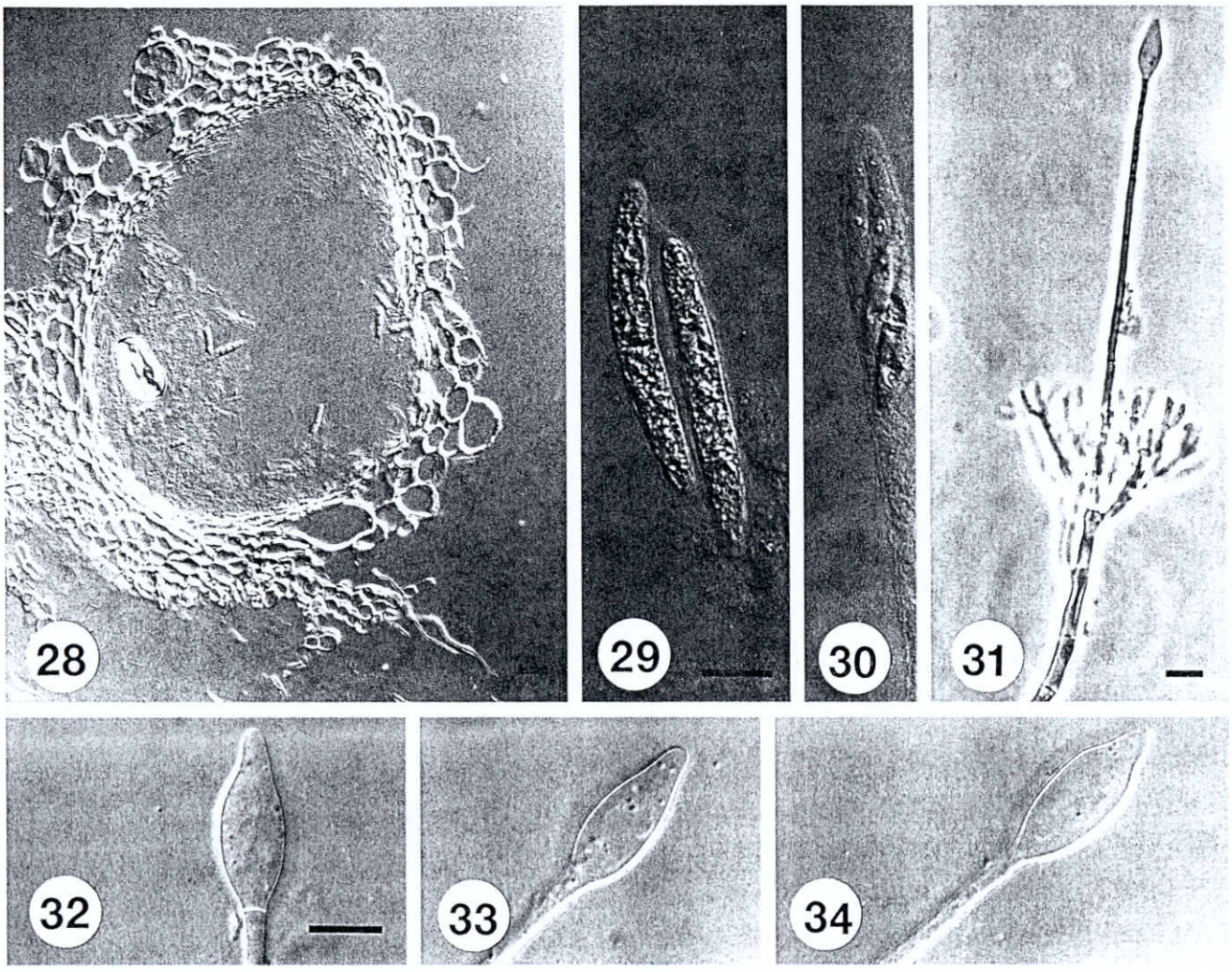
Distribution. Mexico.

Additional cultures examined. MEXICO. CAMPECHE: Holpechén, soil, Apr. 1994, *M. J. Wingfield* (STE-U 941–943, 966, 967); YUCATAN: Uxmal, soil, Apr. 1994, *M. J. Wingfield* (STE-U 926–928, 944–946).

DISCUSSION

This study was initiated in order to investigate the morphological variability observed within the *Cy. candelabrum* species complex. Mating studies revealed the existence of four distinct mating populations in this complex. These findings were further supported by differences in morphology, and sequence data. In accordance with the biological species concept, different species were therefore proposed for each mating population.

Previous mating studies between isolates of *Cy. scoparium* and *Cy. candelabrum* showed these species to be genetically isolated (Crous et al 1993a). Within the *Cy. candelabrum*-complex, however, prominent differences were observed when perithecia of South African × South African, or South African × Brazilian matings were compared with some Brazilian × Brazilian matings. In light of the distribution data of some of these species (*Cy. pauciramosum* and *Cy. candelabrum*) as circumscribed in the present study, it is obvious that the variation observed by Crous et al (1993a) can now be ascribed to different biological species. In light of the results presented here, previous mating groups observed in *Cy. candelabrum* (as *Cy. scoparium*; Ribeiro 1978), suggest that yet other,



FIGS. 28–34. *Calonectria mexicana* and its anamorph *Cylindrocladium mexicanum*. 28. Vertical section through a perithecium. 29. Ascospores. 30. Asci. 31. Conidiophore with extending stipe and terminal vesicle. 32–34. Terminal vesicles. Bars = 10 μm.

undescribed biological species could exist in this complex. Recent molecular work done in another homothallic species complex, *Cy. floridanum* (Victor et al 1997), suggests that this aggregation of distinct biological taxa in species complexes is much more common in *Cylindrocladium* than expected earlier.

The high proportion of successful matings obtained in the present study, and recently by Crous et al (1998) in *Cy. ovatum,* can possibly be ascribed to the fact that these matings were conducted at 22 C, compared to previous studies that used 15 and 25 C as optimum temperature. Within each species, however, isolates showed varying degrees of success in mating with opposing mating types. For example, in *Cy. pauciramosum* STE-U 138 mated only with two other opposing mating type strains, while in *Cy. candelabrum* STE-U 1678 mated successfully in all instances. Age of isolates as well as differences in their female fertility could account for this variation. It appears that *Cy. pauciramosum* and *Cy. insularae* are largely allopatric in character, with isolates available from various localities.

Sequence data can quantify relatedness among taxa, and is commonly used to clarify different taxonomic questions (Viljoen et al 1993, Rehner and Samuels 1995). The sequences of the ITS1 and 2 flanking regions of the 5.8S ribosomal gene indicated small, but consistent differences between the species proposed in this study. Although a high degree of sequence variation in this region has been reported before (Chambers et al 1986), a low amount of variation was observed between the *Cylindrocladium* species examined in the present study. Within a biological species no variation could be observed at all. Even in the case of *Cy. insularae,* identical sequences were observed for isolates from disparate geographic

areas like Madagascar, Mexico and Brazil. When compared to a similar situation in *Gibberella fujikuroi,* where several mating populations exist between isolates with similar morphological features (Leslie 1995), the high relatedness in the *Cy. candelabrum* complex becomes more evident. However sequences of the 5.8S gene and ITS1 and 2 flanking regions proved problematic in differentiating the different mating populations in the *Gibberella fujikuroi* complex (Waalwijk et al 1996). Although the species in this study could be differentiated using sequence results, further consideration will have to be given to other, more variable DNA regions. Studies conducted in the hypocrealean genus *Fusarium* (O'Donnell 1996), could prove useful in this regard.

Crous et al (1997a) recently provided a key to *Calonectria* spp. with *Cy. candelabrum*-like anamorphs that have 1-septate conidia. The following key can therefore be used in conjunction to identify the species occurring in the *Cy. candelabrum* complex.

KEY TO SPECIES IN THE *CYLINDROCLADIUM CANDELABRUM* COMPLEX

1. Vesicles broadly ellipsoidal with a papillate apex, (7–)8–10(–12) µm ... *Cy. mexicanum* (teleo. *Ca. mexicana*)
1. Vesicles not as above 2
2. Vesicles ellipsoidal to narrowly obpyriform, (5–)6–7(–8) µm, perithecia, red-brown, ascospores (40–)45–50(–60) × 5–6 µm, conidia (45–)58–68(–80) × 4–5(–6) µm ..... *Cy. candelabrum* (teleo. *Ca. scoparia*)
2. Vesicles obpyriform to ellipsoidal (4–)7–10(–13), perithecia orange to red or orange to red-brown, ascospores shorter than 42 µm, conidia shorter than 60 µm 3
3. Conidiophores lacking quaternary branches, perithecia orange to red-brown, ascospores 6–7(–8) µm wide ..... *Cy. pauciramosum* (teleo. *Ca. pauciramosa*)
3. Conidiophores commonly with quaternary branches, perithecia orange to red, ascospores 5–6(–7) µm wide ..... *Cy. insularae* (teleo. *Ca. insularis*)

ACKNOWLEDGMENTS

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