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New species of *Calonectria* and *Cylindrocladium* isolated from soil in the tropics

Pedro W. Crous[1]
Godwin R.A. Mchau

Department of Plant Pathology, University of Stellenbosch, P. Bag X1, Matieland 7602, South Africa

Willem H. Van Zyl

Department of Microbiology, University of Stellenbosch, P. Bag X1, Matieland 7602, South Africa

Michael J. Wingfield

Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein 9300, South Africa

Abstract: Several *Calonectria* (*Ca.*) and *Cylindrocladium* (*Cy.*) species were recovered from alfalfa-baited soil samples gathered in Colombia and Venezuela. Perithecia of *Calonectria naviculata* sp. nov. formed in culture when Venezuelan strains of *Cy. naviculatum* were crossed with ex-type Brazilian strains. *Calonectria gracilipes* sp. nov. (anam. *Cy. gracilioideum* sp. nov.), a homothallic species, was isolated from Colombian soils. *Cylindrocladium graciloideum*, *Cy. gracile*, *Cy. pteridis* and *Cy. pseudogracile* form a complex of morphologically similar species characterized by clavate vesicles and primarily 1-septate conidia. Each species could, however, be distinguished using RFLP banding patterns. A key to *Calonectria* spp. having *Cylindrocladium* anamorphs with 1-septate conidia is also presented.

Key Words: Hypocreales, mating studies, soil, systematics

INTRODUCTION

The hyphomycete genus *Cylindrocladium* Morgan represents species with hyaline, smooth, cylindrical conidia, and penicillate conidiophores with septate stipe extensions terminating in vesicles of characteristic shape. Where known, teleomorphs of *Cylindrocladium* spp. (*Cy.*) are best accommodated in *Calonectria* De Not. (*Ca.*).

More than 20 species of *Cylindrocladium* are recognized (Crous and Wingfield, 1994; Crous et al., 1995, 1997; Victor et al., 1997). Of these, *Cy. gracile* (Bugnic.) Boesew. (= *Cy. clavatum* Hodges & May; Crous et al., 1995) has been described under various epithets, and has also been incorrectly linked to different *Calonectria* teleomorphs. In contrast to this species, *Cy. naviculatum* Crous & M.J. Wingf. is presently known only from its type collection, isolated from soil in the Amazonas Province, Brazil (Crous et al., 1994). In the present study, several soil samples were collected from *Eucalyptus* plantations in South America (Colombia and Venezuela). Some of the samples rendered isolates of *Cylindrocladium* spp., including *Cy. naviculatum* and a species morphologically similar to *Cy. gracile*. When cultured, two previously undescribed *Calonectria* states were induced for these species. This report describes the hitherto unknown teleomorph of *Cy. naviculatum*, and characterizes the *Calonectria* species isolated from Colombian soil.

MATERIALS AND METHODS

Isolation and identification.—Fifteen soil samples were randomly collected from different eucalypt plantations in Colombia and Venezuela. Each sample consisted of two petri dishes filled with moist soil collected in the upper 15 cm soil layer in a 1 m radius. Dished were sealed, and transported to the laboratory, where soil samples were moistened, after which surface-disinfested (1 min in 1% NaOCl) alfalfa seeds were scattered onto the soil surface in each dish. After 14 d, the germinating seedlings were removed from the dishes, submerged in 1% NaOCl for 30 s, rinsed in sterile H_2O, and plated onto 2% malt extract agar (Biolab), amended with streptomycin sulphate (0.05 g/L) (MEA). Petri dishes were incubated for 7 d at 25 C under continuous near-ultraviolet light, after which single conidia from sporulating *Cylindrocladium* isolates were plated onto carnation leaf agar (CLA) (Crous et al., 1992). Cardinal temperature requirements for growth and cultural characteristics were determined after 6 d on MEA using the procedures of Crous et al. (1994). Cultures of *Cylindrocladium* were identified using the keys of Crous and Wingfield (1994). Wherever possible, 30 measurements were made of structures mounted in lactophenol, and extremes given in parentheses. Type

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[1] Corresponding author, email: pwc@maties.sun.ac.za

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 at the University of Stellenbosch, South Africa (STE-U).

Sexual compatibility.—Isolates of *Cy. naviculatum* collected and identified in this study were mated on CLA with their ex-type strains (STE-U 627–629) in all possible combinations using the technique previously described by Crous et al. (1993a). Petri dishes were inspected weekly for perithecial development. Single conidial isolates of the Colombian species (STE-U 1153, 1213) readily formed perithecia in culture, and were, therefore, accepted as being homothallic.

Molecular comparisons.—Morphologically the *Cylindrocladium* sp. isolated from Colombian soil is characterized by having 1-septate conidia and clavate vesicles. Based on these observations, it was further compared with *Cylindrocladium* spp. known to have clavate vesicles, and primarily 1-septate conidia. The nuclear ribosomal DNA (nrDNA) of the Colombian *Cylindrocladium* sp. (STE-U 1153) was compared with ex-type strains of *Cy. gracile* (PC 551197, = *Cy. clavatum*, ATCC 22833), *Ca. pteridis* Crous et al. (anam. *Cy. pteridis* Wolf, PPRI 4157) and *Ca. gracilis* Crous et al. (anam. *Cy. pseudogracile* Crous, PPRI 4176). The nrDNA of these isolates was digested with the restriction enzymes *Eco*RI, *Hin*dIII and *Xho*I, and a Southern blot analysis was performed using the 6.3-kb ribosomal DNA repeat unit of *Neurospora crassa* as probe (Crous et al., 1995).

TAXONOMY

Calonectria gracilipes Crous et G.R.A. Mchau, sp. nov. FIGS. 1–4, 9–11

Anamorph. **Cylindrocladium graciloideum** Crous et G.R.A. Mchau, sp. nov.

Perithecia globosa ad ovoidea, 350–400 µm alta, 300–380 µm lata, crocea ad rubra, pariete exteriore verrucosa, ostiolo papillato. Asci clavati, in stipitem longum tenuem gradatim angustatae, 80–120 × 12–18 µm, 8-spori. Ascosporae hyalinae, fusiformes, 1-septatae, ad septum constrictae, (28–)33–40(–45) × (5–)6–7(–7.5) µm. Filum septatum, hyalinum (150–)200(–260) µm, in vesiculam clavatam 3(–4) µm diam terminans. Rami primarii non septati vel raro 1-septati, 15–25 × 4–5 µm; rami secundarii non septati, 10–15 × 4–6 µm. Phialides doliiformes ad reniformes, hyalinae, non septatae, 10–15 × 2.5–4 µm. Conidia cylindrica, hyalina, 1-septata, apicibus obtusis, (35–)40–48(–60) × 4–5(–6) µm. Microconidiophora ignota.

HOLOTYPE. COLOMBIA. La Selva, soil, Jun. 1995, *M.J. Wingfield* (PREM 54417 teleomorph, PREM 55299 anamorph, ex-type culture STE-U 1153).

Perithecia orange, globose to ovoid, 350–400 µm high, 300–380 µm wide, turning dark red in 3% KOH; ostiole papillate, orange to red, generally darker than the perithecium body. Perithecial wall consisting of two layers: outside layer of *textura globulosa*, 40–50 µm wide; inner layer of *textura angularis*, 10–30 µm wide; 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, 80–120 × 12–18 µm, tapering to a long thin stalk. *Ascospores* hyaline, fusoid with rounded ends, straight to slightly curved, 1-septate, constricted at the septum, (28–)33–40(–45) × (5–)6–7(–7.5) µm. *Macroconidiophores* comprising a stipe, a sterile elongation and a penicillate arrangement of fertile branches. Stipe septate, (150–)200(–260) µm long, terminating in a narrowly clavate vesicle, 3(–4) µm diam; primary branches aseptate or rarely 1-septate, 15–25 × 4–5 µm; secondary and tertiary branches aseptate, 10–15 × 4–6 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, non-septate, 10–15 × 2.5–4 µm, apex with minute periclinal thickening. *Conidia* cylindrical, rounded at both ends, straight, (35–)40–48(–60) × 4–5(–6) µm, 1-septate, lacking a visible abscission scar, held in cylindrical packets by colorless slime. *Microconidiophores* not observed. *Chlamydospores* dark brown, walls thickened, formed in moderate numbers throughout the medium, and aggregated to form microsclerotia.

Cultures. Colony color (reverse) 13i sienna (Rayner, 1970). Colonies attaining a radius of 15–18 mm diam on MEA after 6 d in the dark at 25 C.

Cardinal temperature requirements for growth. Min. above 10 C, max. below 35 C, opt. 25 C. This is a high temperature species, with medium sporulation on aerial mycelium.

Substrate. Soil.

Distribution. Colombia.

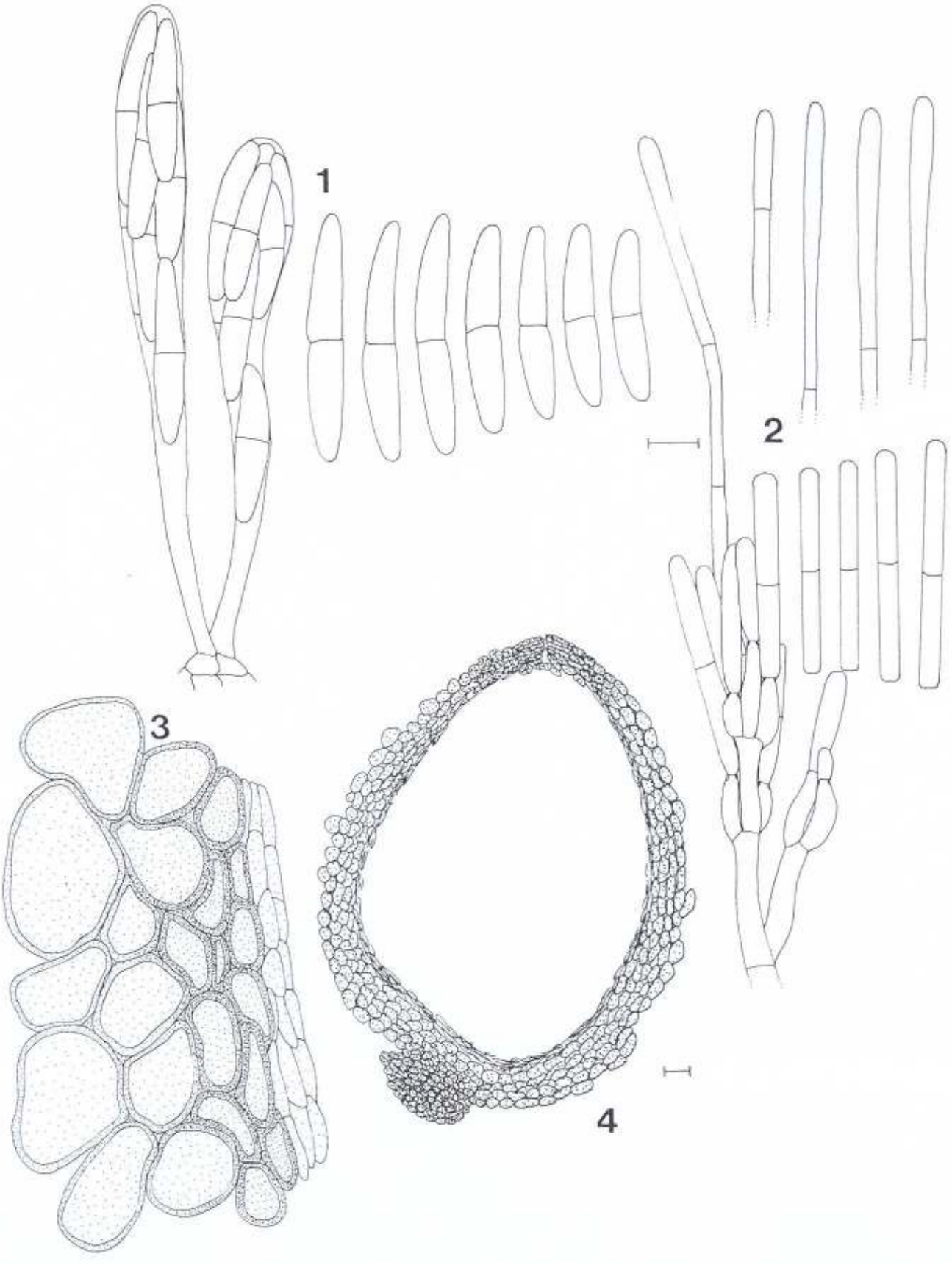
Calonectria naviculata Crous et M.J. Wingf., sp. nov. FIGS. 5–8, 12–14

Anamorph. Cylindrocladium naviculatum Crous & M.J. Wingf., Mycotaxon 50: 443. 1994.

Perithecia rubri-brunnea, globosa ad ovoidea, 350–450 µm alta, 350–400 µm lata, pariete exteriore verrucosa, ostiolo papillato. Asci clavati, in stipitem longum tenuem gradatim angustatae, 70–100 × 8–12 µm, 2–8-spori. Ascosporae hyalinae, fusiformes, falcatae, 1(–3)-septatae, ad septa constrictae, guttulatae, (20–)40–48(–52) × (3–)5–6(–6.5) µm.

→

FIGS. 1–4. *Calonectria gracilipes* and its anamorph *Cylindrocladium graciloideum*. 1. Asci and ascospores. 2. Conidiophore, clavate vesicles and 1-septate conidia. 3. Section through a perithecium wall showing the various wall layers. Bar = 10 µm. 4. Vertical section through a perithecium. Bar = 20 µm.


1
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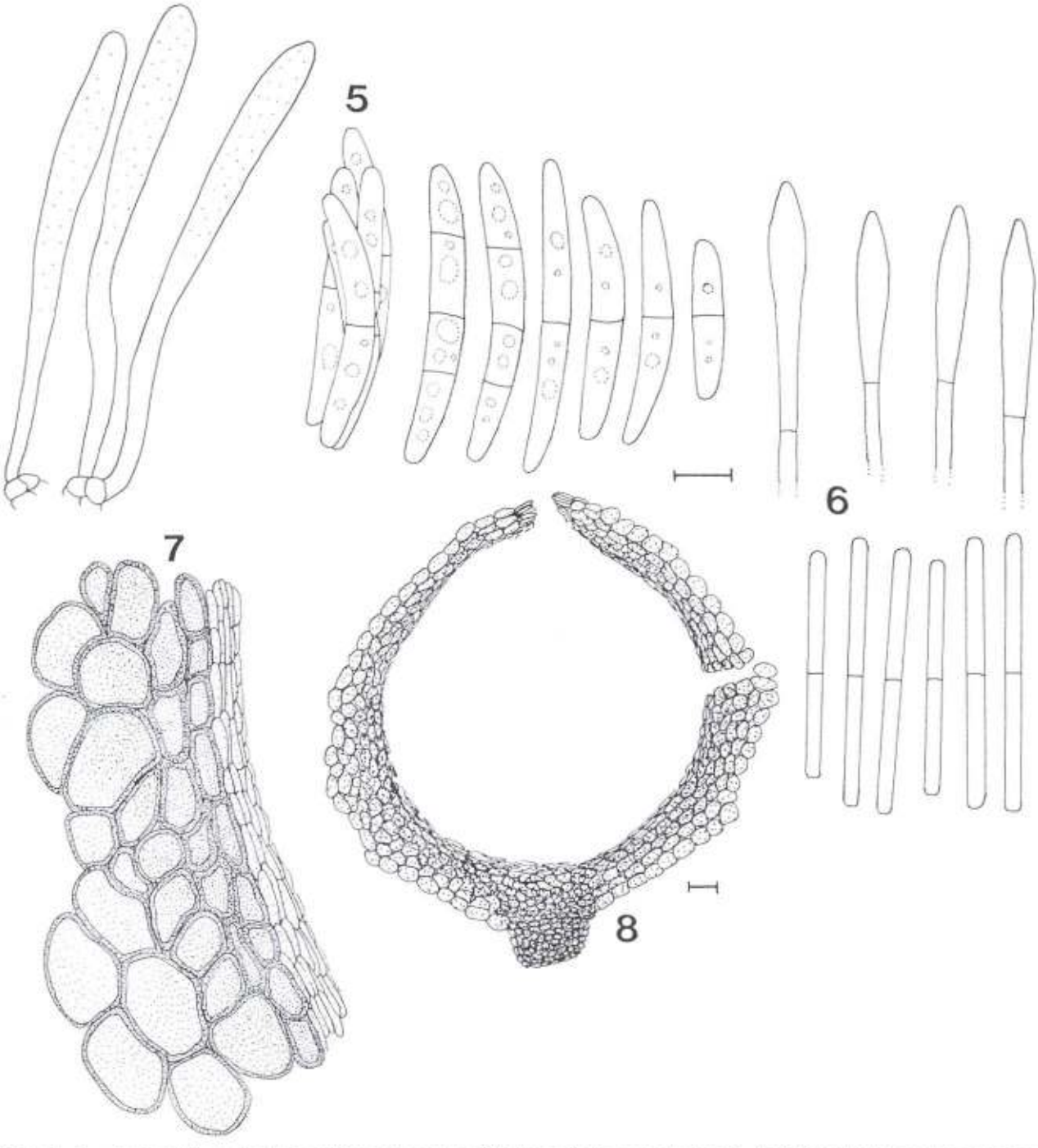
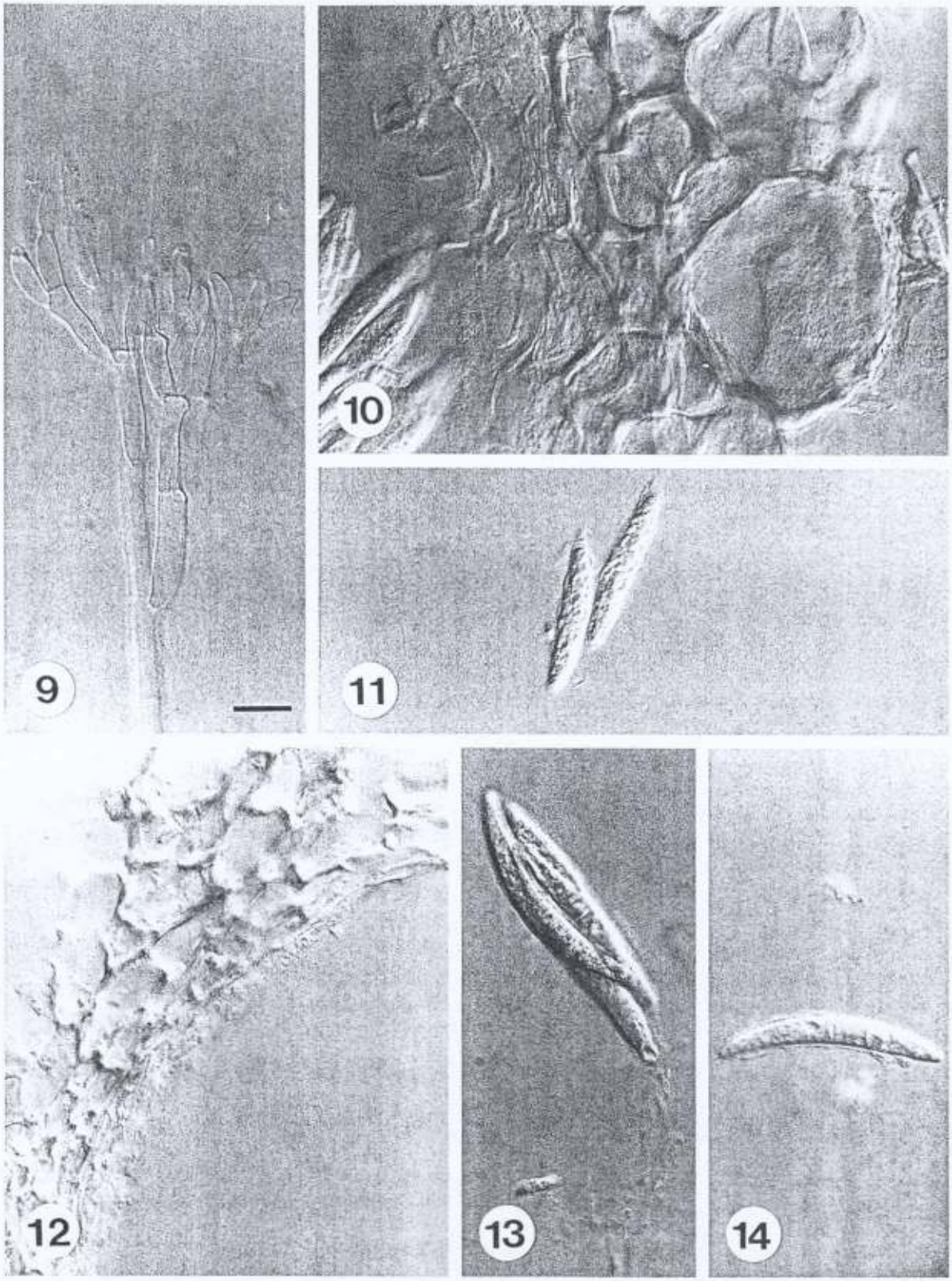


FIGS. 5–8. *Calonectria naviculata* and its anamorph *Cylindrocladium naviculatum*. 5. Asci (immature) and ascospores. 6. Naviculate vesicles (above) and 1-septate conidia. 7. Section through a perithecium wall showing the various wall layers. Bar = 10 μm. 8. Vertical section through a perithecium. Bar = 20 μm.

HOLOTYPE. BRAZIL × VENEZUELA. AMAZONAS: soil collected from Manaus (Brazil) and Río Orinoco (Venezuela), respectively, heterothallic mating of STE-U 627 × STE-U 962, Feb. 1995, *M.J. Wingfield* (PREM 54418).

Perithecia red-brown, globose to ovoid, 350–450 μm high, 350–400 μm wide; ostiole papillate, red, perithecium turning blood red in 3% KOH. Perithecial wall consisting of two layers: outside layer of *textura globulosa*, 20–40 μm wide; inner layer of *textura angularis*, 20–30 μm wide; hymenium layer of *textura prismatica*, hyaline, 5–10 μm wide; perithecial base up to 80 μm wide, consisting of dark red, angular cells. Asci 2- to 8-spored, clavate, 70–100 × 8–12 μm, tapering to a long thin stalk. Ascospores hyaline, smooth, fusoid with rounded ends, mostly slightly curved, 1(–3)-septate, becoming constricted at septa when 3-septate, guttulate, (20–)40–48(–52) × (3–)5–6(–6.5) μm.



FIGS. 9–14. *Calonectria gracilipes* and *Ca. naviculata*. 9–11. *Calonectria gracilipes*. 9. Penicillate conidiophore of *Cy. graciloideum*. 10. Section through a perithecium wall. 11. Ascospores. 12–14. *Calonectria naviculata*. 12. Section through a perithecium wall. 13. Ascus and ascospores. 14. Ascospore. Bar = 10 μm.

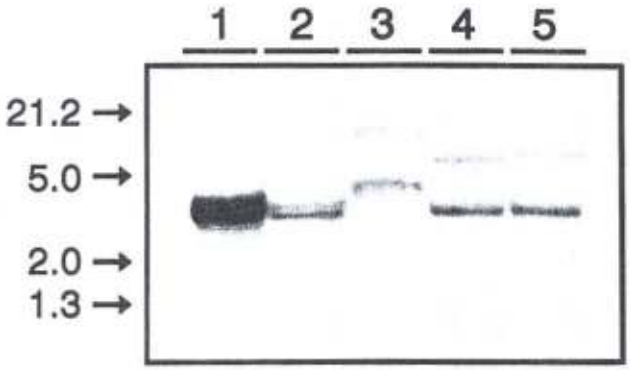


FIG. 15. Ribosomal DNA hybridization patterns for *Eco*RI digested nDNA of strains of *Cylindrocladium* and *Calonectria* species. Lanes 1, 2. *Cy. gracile* PC 551197 and ATCC 22833. Lane 3. *Ca. gracilipes* STE-U 1153. Lane 4. *Ca. gracilis* PPRI 4176. Lane 5. *Ca. pteridis* PPRI 4157. Size markers are lambda DNA digested with *Eco*RI and *Hin*dIII.

Cultures and cardinal temperature requirements for growth. As reported by Crous et al. (1994).

Substrate. Soil.

Distribution. Brazil, Amazonas state; Venezuela, Río Orinoco, Puerto Ayacucho, Amazonas.

Additional cultures examined. BRAZIL. AMAZONAS: Manaus, soil, Apr. 1993, *M.J. Wingfield*, STE-U 627–629 (ex-type of anamorph). VENEZUELA. AMAZONAS: Río Orinoco, Puerto Ayacucho, soil, 1994, *M.J. Wingfield*, STE-U 947, 955, 961, 962.

DISCUSSION

Species differences in *Cylindrocladium* are primarily manifested in conidial size, septation and in the form of the terminal vesicle of the conidiophore. Among the species having clavate vesicles and conidia that are primarily 1-septate, *Cy. gracile* has been recorded to have a wide range of conidial measurements. Crous and Wingfield (1994) retained *Cy. clavatum* (= *Cy. brassicae*) (conidia 38–52 × 4–6 µm) and *Cy. gracile* (conidia 40–56 × 3.5–5 µm) as separate species despite strong morphological similarity. El-Gholl et al. (1993) identified *Cy. clavatum* as the anamorph of *Ca. clavata* Alfieri et al. Crous et al. (1993b) linked *Cy. gracile* to *Ca. gracilis* Crous et al. However, in using nrDNA RFLP's, Crous et al. (1995) showed the ex-type strains of *Cy. clavatum* and *Cy. gracile* to be identical. The anamorph of *Ca. clavata* was furthermore found to be misidentified by El-Gholl et al. (1993) as *Cy. clavatum* (= *Cy. gracile*), and it was subsequently described as *Cy. flexuosum* Crous (Crous et al., 1995). To further confuse this issue, the strain described as *Ca. gracilis* was incorrectly linked to *Cy. gracile* (Crous et al., 1993b), and was thus described as *Cy. pseudogracile* (Crous et al., 1997). *Cylindrocladium* spp. in this group are difficult to identify, and it has now become obvious that with several species both morphs are required to accurately identify new strains, and that in problematic cases more specific molecular techniques would have to be employed to confirm identifications.

Cylindrocladium graciloideum (STE-U 1153, 1213) had shorter and wider conidia (35–)40–48(–60) × (4–)5(–6) µm than those of *Cy. pseudogracile* (40–)50–58(–65) × 4(–5) µm, but was consistent with the variation accepted in *Cy. gracile* (38–)40–50(–52) × 4–5(–6) (ATCC 22833), (40–)45–55(–60) × (3.5–)4–5 µm (PC 551197) (Crous et al., 1995). In this species complex, however, the nDNA profiles of *Cy. graciloideum* digested with the restriction enzymes *Eco*RI (FIG. 15; TABLE I) and *Xho*I, proved to be distinct from those of the other two species. Of the *Calonectria* states described for *Cylindrocladium* spp. with clavate vesicles and 1-septate conidia, *Ca.*

TABLE I. Estimated restriction fragment sizes (bp) of nuclear rDNA probed with the 6.3-kb ribosomal repeat unit of *Neurospora crassa*

Species	Accession no.	DNA fragment sizes		
		*Eco*RI	*Hin*dIII	*Xho*I
Cylindrocladium graciloideum (teleo. *Ca. gracilipes*)	STE-U 1153	6700 4300	6400	8100
Cylindrocladium pseudogracile (teleo. *Ca. gracilis*)	PPRI 4176	5700 3900	6300	>20 000
Cylindrocladium pteridis (teleo. *Ca. pteridis*)	PPRI 4157	5900 3900	6000	8300 800
Cylindrocladium gracile (teleomorph unknown)	PC 551197	4000 1800	13 000	7500
	ATCC 22833[a]	4000 1800	13 000	7500

[a] Ex-type culture of *Cy. clavatum*.

gracilipes can be distinguished by forming orange perithecia with 1-septate ascospores (28–)33–40(–45) × (5–)6–7(–7.5) µm, being most similar to, but wider than those of *Ca. gracilis* (27–)33–45(–50) × (4–)4.5–5(–6) µm. Although the nrDNA RFLP profiles of *Ca. pteridis* and *Ca. gracilis* were similar except for one distinct band (> 20 000), spores of *Ca. pteridis* [ascospores (30–)52(–75) × (4–)6(–7) µm, conidia 50–130 × 4–7 µm] were much larger than those of *Ca. gracilis* [ascospores (27–)33–45(–50) × (4–)4.5–5(–6) µm, conidia (40–)56(–65) × (4–)5 µm; Crous et al., 1997].

Cylindrocladium naviculatum can be easily distinguished from other species of *Cylindrocladium* by its characteristically naviculate vesicles, wide stipe extensions, and narrowly 1-septate conidia (40–55 × 3–4 µm; Crous et al., 1994). Although several single-conidial strains derived from the type collection from Amazonian Brazil produced protoperithecia on CLA, no fertile perithecia were observed. In mating studies with strains obtained in the present study from soil from Amazonian Venezuela, several combinations among six strains [STE-U 627 (+) with strains (−) STE-U 955, 961, 962, and STE-U 628 (+) with strains (−) STE-U 947, 961 and 962] produced perithecia with fertile progeny. The fact that both STE-U 627 and 628 mated with STE-U 961 and 962, suggests that isolates STE-U 627 and 628 are of the same mating type. This would explain the absence of fertile perithecia in the study where this species was described (Crous et al., 1994). Although strains were mated in all possible combinations, STE-U 627 (+) did not mate with STE-U 947 (−), nor did STE-U 628 (+) mate with STE-U 955 (−), suggesting that other factors could also have played a role in these particular matings.

Several media and techniques have been successfully employed in the past to bait *Cylindrocladium* strains from soil (Crous et al., 1991), and whether the alfalfa technique used in the present study is the most effective, has yet to be determined. Using this technique, however, a number of *Cylindrocladium* spp. other than those described here were also obtained from the soil samples collected. Samples from Venezuela rendered a few isolates of *Cy. pteridis* (STE-U 1181, 1182), *Cy. candelabrum* Viégas (STE-U 1183) and *Cy. gracile* (STE-U 920–922, 938–940). Colombian samples were more rewarding, and were extensively colonized by *Cy. reteaudii* (Bugnic.) Boesew. (STE-U 1069–1072, 1145–1152, 1165, 1166), and to a lesser extent by *Cy. parasiticum* Crous et al. (STE-U 723–725), *Cy. gracile* (STE-U 726, 1159, 1164) and *Cy. candelabrum* (STE-U 1160–1163).

Several *Cylindrocladium* spp. with 1-septate conidia have been linked to *Calonectria* states similar to *Ca. gracilipes* and *Ca. naviculata*. Although these species can be distinguished from each other based primarily on their anamorphs, identifications are greatly simplified if both morphs are formed in culture. Since publication of the initial key to *Calonectria* spp. with *Cylindrocladium* anamorphs (Crous and Wingfield, 1994), several new species have been added to the complex discussed in the present study, and thus an emended key is provided below.

KEY TO *CALONECTRIA* SPP. WITH *CYLINDROCLADIUM* ANAMORPHS HAVING 1-SEPTATE CONIDIA

1. Vesicles clavate or clavate to avesiculate 2
1. Vesicles not as above 5
   2. Stipe thick-walled, frequently avesiculate, conidia 57–77 × 4–7 µm, perithecia red-brown, ascospores 1(–3)-septate in ascus, (20–)40–48(–52) × (3–)5–6(–6.5) µm ..... *Cy. avesiculatum* (teleo. *Ca. avesiculata*)
   2. Stipe not thick-walled, never avesiculate, vesicles clavate 3
3. Teleomorph unknown; conidia (38–)40–55(–60) × (3.5–)4–5(–6) µm ..... *Cy. gracile*
3. Teleomorph readily formed in culture 4
   4. Perithecia orange, conidia (35–)40–48(–60) × 4–5(–6) µm, ascospores 1-septate, mostly constricted at septum, (28–)33–40(–45) × (5–)6–7(–7.5) µm ..... *Cy. graciloideum* (teleo. *Ca. gracilipes*)
   4. Perithecia red-brown, conidia (40–)50–58(–65) × 4(–5) µm, ascospores 1-septate, mostly not constricted at septum, (27–)33–45(–50) × (4–)4.5–5(–6) µm .... *Cy. pseudogracile* (teleo. *Ca. gracilis*)
5. Vesicles sphaeropedunculate, conidia 35–66 × 3–5 µm; perithecia orange-red, ascospores 1-septate in ascus, (18–)27–36(–48) × (4–)4.5–5(–7) µm ..... *Cy. floridanum* (teleo. *Ca. kyotensis*)
5. Vesicles not as above, perithecia red-brown 6
   6. Vesicles naviculate, conidia 40–55 × 3–4 µm, ascospores 1(–3)-septate in ascus, (20–)40–48(–52) × (3–)5–6(–6.5) µm ..... *Cy. naviculatum* (teleo. *Ca. naviculata*)
   6. Vesicles not as above, conidia 33–66 × 3–5 µm, ascospores 1-septate in ascus 7
7. Vesicles obovoid to pyriform or ellipsoidal, ascospores (24–)30–40(–49) × (4–)6–7(–8) µm ..... *Cy. scoparium* (teleo. *Ca. morganii*)
7. Vesicles obpyriform, rarely ellipsoidal, ascospores (28–)33–47(–68) × (4–)5–6(–7) µm ..... *Cy. candelabrum* (teleo. *Ca. scoparia*)

Results of the present and other recent studies (Crous et al., 1995; Victor et al., 1997) provide further evidence that the morphological variation observed in some species in *Cylindrocladium* and *Calonectria* can, in many instances be attributed to the lumping of distinct biological species. Of the approximately 20 known species of *Cylindrocladium* (Crous and Wingfield, 1994; Crous et al., 1995; Victor et al., 1997), only six have not yet been linked to *Calonec-*

tria states. This genus has been shown to include both homothallic and heterothallic species. All indications are, therefore, that additional collections will lead to the description of teleomorphs for those species known only in the anamorph state. The soil baiting technique provides a means for collecting isolates from diverse areas and ecological habitats. Utilization of this approach will eventually increase our knowledge of the diversity and mating systems within this group, and facilitate more detailed population studies on species complexes such as *Cy. candelabrum* (Crous et al., 1993a) and *Cy. floridanum* (Victor et al., 1997).

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