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Nuclear DNA Polymorphisms of *Cylindrocladium* Species with 1-septate Conidia and Clavate Vesicles

P. W. Crous[1]*, A. Korf[2], and W. H. van Zyl[2]

[1,2] Departments of Plant Pathology and Microbiology, University of Stellenbosch, South Africa

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Summary

Four *Cylindrocladium* species characterized by clavate terminal vesicles and 1-septate conidia were compared on the basis of morphology, cultural characteristics, and nuclear DNA (nDNA) polymorphisms. The nDNA of *C. clavatum, C. hawksworthii, C. pteridis, C. gracile, Calonectria gracilis* and *Calonectria clavata* were digested with the restriction enzymes *Eco*RI, *Hin*dIII and *Xho*I, and Southern analysis performed with the 6.3-kb ribosomal DNA repeat unit of *Neurospora crassa* as probe. Based on general morphology and DNA banding patterns *Cylindrocladium gracile* was found to be the earlier name for *C. clavatum*. *Calonectria gracilis* was shown to be similar, but distinct from *Calonectria pteridis*, the teleomorph of *Cylindrocladium pteridis*. *Calonectria clavata* appeared to be a distinct species with an undescribed anamorph, for which the nomenclator *Cylindrocladium flexuosum* was provided.

Key words: *Cylindrocladium clavatum* – *Cylindrocladium flexuosum* – *Cylindrocladium gracile* – *Cylindrocladium hawksworthii* – *Cylindrocladium pteridis* – rDNA polymorphisms – Systematics

Introduction

In a recent monograph of *Cylindrocladium* Morgan, *Crous* and *Wingfield* (1994) recognized 22 species and 2 varieties. Four of these species, namely *C. clavatum Hodges & May, C. pteridis Wolf, C. gracile (Bugn). Boesewinkel* and *C. hawksworthii Peerally* are known to have 1-septate conidia and thin-walled stipe extensions terminating in clavate vesicles. These species are also regarded as serious pathogens, and are associated with symptoms such as damping-off, seedling blight, leaf spot, stem cankers, shoot blight, root disease, and tuber rot of numerous hosts worldwide (*Crous* and *Wingfield,* 1994; *Peerally,* 1991a).

Cylindrocladium hawksworthii can easily be distinguished in having curved conidia, whereas those of *C. clavatum, C. gracile* and *C. pteridis* are straight. The latter three species have similar temperature requirements for growth, and are primarily distinguished on cultural characteristics and dimensions of conidia and stipe extensions (*Crous* and *Wingfield,* 1994). These species represent a range in size of conidia and stipe extensions, varying from the smaller *C. clavatum* and *C. gracile* to the larger *C. pteridis* (*Crous* et al., 1994).

Hodges and *May* (1972) described a small-spored *Cylindrocladium* species from Brazil as *C. clavatum*. The latter was found to be an important root and leaf pathogen of several hosts worldwide (*Lopes* and *Reifschneider,* 1982; *Ooka* and *Uchida,* 1983; *Dianese* et al., 1986; *Blum* and *Dianese,* 1993; *Crous* et al., 1993b). Based on an examination of type specimens, *C. brassicae Panwar & Bohra* (1974) was later recognized as a synonym of *C. clavatum* (*Crous* and *Wingfield,* 1994). In a recent study, *El-Gholl* et al. (1993) described the heterothallic teleomorph of *C. clavatum* as *Calonectria clavata Alfieri, El-Gholl & Barnard*. An examination of these two heterothallic strains (078-1261, 078-1543) revealed, however, that the conidia were far larger than those produced by the type culture of *C. clavatum*. Furthermore, they were strongly curved, resembling those of *C. hawksworthii,* but also had curved microconidia resembling those of *C. pteridis*.

Cylindrocarpon gracile Bugnicourt (1939) was transferred to *Cylindrocladium* by *Boesewinkel* (1982). The latter

* Corresponding author

species was distinguished from *C. clavatum* and *C. pteridis* in having conidia and stipes of intermediate dimensions. A morphologically similar homothallic isolate was later obtained from Brazil, which led to the description of the teleomorph as *Calonectria gracile Crous, Wingfield & Alfenas* (1993c).

Cylindrocladium pteridis Wolf (1926) was described from leaf spots of *Rumohra adiantiforme* in Florida, and recognized as the earlier name for *C. macrosporum Sherb.* by *Sobers* (1968). The latter species is also distinct from all others presently known in *Cylindrocladium* by producing curved microconidia (*Crous* and *Wingfield,* 1994). Its heterothallic teleomorph, *Calonectria pteridis, Crous, Wingfield & Alfenas* (1993c) was recently induced by mating several single-conidial isolates obtained from Brazil.

Complementing alpha and beta taxonomy, several molecular techniques have recently been employed in *Cylindrocladium,* ranging from total protein and isozyme banding patterns to DNA restriction fragment length polymorphisms (RFLPs) (*Crous* et al., 1993a, 1993d). These techniques proved to be time consuming, and in the case of proteins, often influenced by host and geographical variation. *Crous* et al. (1993a) stated, however, that DNA RFLPs can clearly distinguish variation between and among species, and therefore substantially enhance attempts to allocate isolates in *Cylindrocladium. Lodolo* et al. (1992) proposed using ribosomal DNA (rDNA) restriction fragment length polymorphisms (RFLPs) as a rapid technique to demonstrate species-specific differences in *Fusarium Link.* The aim of the present study, therefore, was to use the technique proposed by *Lodolo* et al. (1992) to establish variation among the four *Cylindrocladium* species discussed above, and to determine the correct taxonomic position of the type strain of *Calonectria clavata.*

Materials and Methods

Morphological characterization. The following type and verified strains of *Cylindrocladium* and *Calonectria* spp. were studied: *Cylindrocladium clavatum* (PPRI 3994 = CPC 328), (ATCC 22833, ex type strain); *Calonectria clavata* (078-1261 = ATCC 66388 and 078-1543 = ATCC 66389, ex type strains); *Cylindrocladium gracile* (PC 551197, ex type strain); *Calonectria gracilis* (PPRI 4176 = AR 2677, ex type strain), *Cylindrocladium hawksworthii* (MUCL 30866, ex type strain); *Cylindrocladium pteridis* (PPRI 4157 = UFV 43).

Single-conidial 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 cotton blue, and measurements made at 1000 × magnification.

Culture characteristics. Colony color and chlamydospore formation were determined at 25 °C in the dark after 6 d. Color designations used were those of *Rayner* (1970).

Chromosomal DNA isolation. Single-conidial isolates were grown on MEA, and plugs of 7-d-old cultures transferred into 500 ml Erlenmeyer flasks containing 100 ml glucose-yeast extract broth (Biolab) (*Zumpetta,* 1976). Cultures were incubated for 7–14 d in the dark at 25 °C until sufficient growth occurred. Mycelia were harvested by filtration (Whatman No. 1 filter paper), the mycelial mat immersed into liquid nitrogen and chromosomal DNA isolated according to *Raeder* and *Brode* (1988). Chromosomal DNA was subsequently redissolved in 200 µl TE buffer (pH 8.0) (*Sambrook* et al., 1989).

Restriction enzyme analysis and Southern hybridization. Chromosomal DNA (ca. 5 µg) of each isolate was subjected to restriction digestion with *Eco*RI, *Hin*dIII and *Xho*I for 3 h respectively, according to the recommendation of the suppliers (Boehringer Mannheim). The DNA was separated on horizontal 0.8% agarose gels and transferred to Hybond-N nylon membranes (Amersham) according to standard procedures (*Sambrook* et al., 1989). The *Neurospora crassa* rDNA was purified from plasmid pMF2 (*Russell* et al., 1984) as a 6.3-kb *Pst*I fragment and labelled with [α-^{32}P]dATP (Amersham) as described by *Feinberg* and *Vogelstein* (1983). The Southern hybridizations and stringency washes were performed according to the method of *Sambrook* et al. (1989).

Results

Morphological characterization

All the species examined in the present study produced stipe extensions terminating in clavate vesicles. Based on conidium morphology on CLA, two groups could be distinguished. Conidia of *C. clavatum, C. gracile* and *C. pteridis* were straight, whereas those of *C. hawksworthii* and *Calonectria clavata* were curved. Conidia of *Cylindrocladium clavatum* were 38–52 × 4–6 µm, overlapping with the slightly larger conidia of *Cylindrocladium gracile* (PC 551197), which were 40–56 × 3.5–5 µm. Conidia of *Calonectria gracilis* were 40–65 × 4–5 µm, overlapping to some degree with the lower range of *Calonectria pteridis,* which were 62–121 × 5–6 µm. The same trend was also observed for their ascospores, those of *C. gracilis* being 1-septate, (27–)36.5(–50) × (4–)5(–6) µm, and those of *C. pteridis* 1(–3)-septate, (30)–51.5(–75) × (4.5)5.5(–7) µm. Of the two species with curved conidia, those of *C. hawksworthii* (42–76 × 4–4.5 µm) were similar in length, but slightly narrower than those of *Calonectria clavata* (50–80 × 5–6 µm).

Culture characteristics

Chlamydospore formation was found to be extensive in all species. Colony color (bottom) on MEA for all species ranged from 13k–17″k, umber brown to Saccardo's umber, except *Cylindrocladium hawksworthii* and *Calonectria clavata,* which were 13″k verona brown and 13′b–13′i, ochreous to umber, respectively (*Rayner,* 1970). The aerial mycelium of *Cylindrocladium clavatum* was yellowish, in contrast to the white-brown aerial mycelium of the other species. Optimum growth for all species occurred at 30 °C, except *Cylindrocladium clavatum* and *Calonectria clavata* which obtained optimum growth at 25 °C.

Restriction enzyme analysis and Southern hybridization

Using the *Neurospora crassa* rDNA probe five distinct *Eco*RI, *Xho*I and *Hin*dIII restriction patterns were obtained for the different strains (Figs. 1–3, Table 1). The

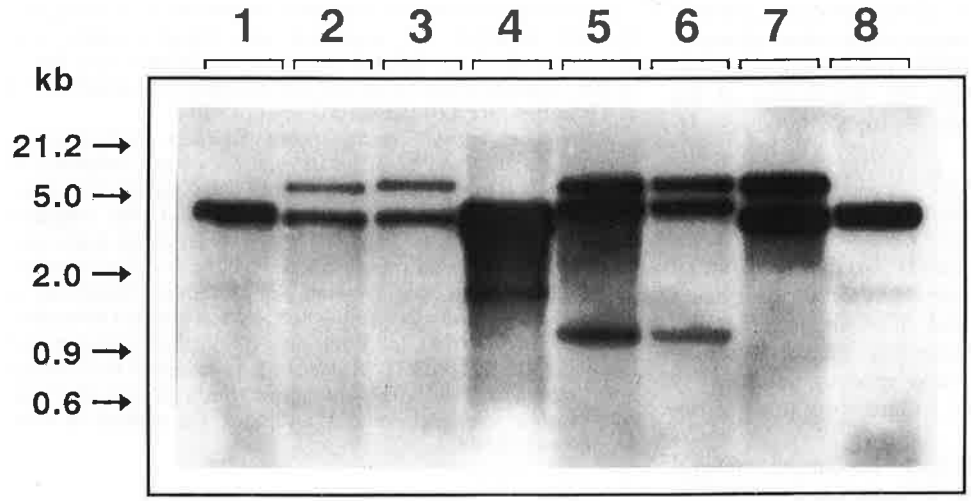

Fig. 1. rDNA hybridization patterns for *Eco*RI-digested nDNA of strains of *Cylindrocladium* and *Calonectria* species. Lane 1: *Cylindrocladium clavatum* PPRI 3994. Lane 2: *Calonectria gracilis* PPRI 4176. Lane 3: *C. pteridis* PPRI 4157. Lane 4: *Cylindrocladium gracile* PC 551197. Lanes 5 and 6: *Calonectria clavata* ATCC 66388 and 66289. Lane 7: *Cylindrocladium hawksworthii* MUCL 30866. Lane 8: *C. clavatum* ATCC 22833. Size markers are lambda DNA digested with *Eco*RI and *Hin*dIII.

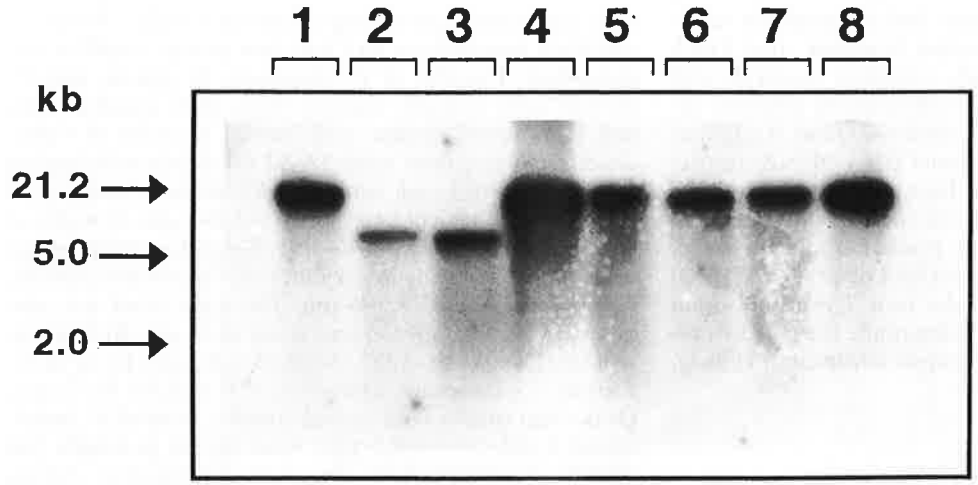

Fig. 2. rDNA hybridization patterns for *Hin*dIII-digested nDNA of strains of *Cylindrocladium* and *Calonectria* species. Details as in Fig. 1.

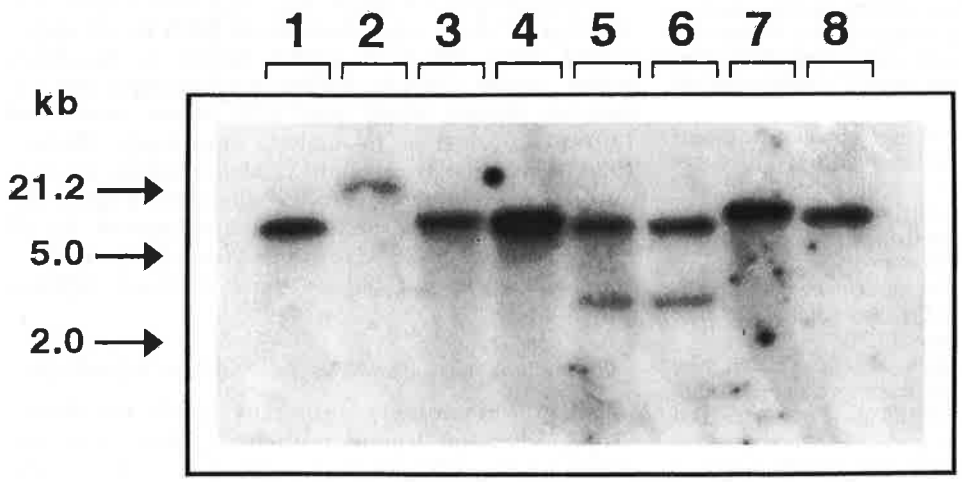

Fig. 3. rDNA hybridization patterns for *Xho*I-digested nDNA of strains of *Cylindrocladium* and *Calonectria* species. Details as in Fig. 1.

Table 1. Nuclear DNA polymorphisms observed for the *Cylindrocladium* and *Calonectria* species

Species	Accession No.	DNA fragment sizes[a]		
		*Eco*RI	*Hin*dIII	*Xho*I
Cylindrocladium clavatum	PPRI 2994	4000 1800	13000	7500
Calonectria gracilis	PPRI 4176	5700 3900	6300	>20000
Cylindrocladium pteridis	PPRI 4157	5900 3900	6000	8300
Cylindrocladium gracile	PC 551197	4000 1800	13000	7500
Calonectria clavata	ATCC 66388	5700 4300 1000	12000	6300 2600
Calonectria clavata	ATCC 66289	5700 4300 1000	12000	6300 2600
Cylindrocladium hawksworthii	MUCL 30866	5700 3900	12000	10500
Cylindrocladium clavatum	ATCC 22833	4000 1800	13000	7500

[a] Nuclear rDNA fragments highlighted by the 6.3-kb ribosomal repeat unit of *Neurospora crassa* ^{32}P-labelled probe.

restriction pattern of the South African collection of *Cylindrocladium clavatum* (PPRI 3994) was similar to that of the type culture of *C. clavatum* (ATCC 22833). However, the type culture of *C. gracile* (PC 551197) could not be distinguished from that of *C. clavatum*. The two heterothallic isolates of *Calonectria clavata* were similar to each other, but distinct from that of *Cylindrocladium clavatum*, and all other species studied. The profile of *Calonectria gracile* (AR 2677) was distinct from *Cylindrocladium gracile* (PC 551197), and closer to that of *Cylindrocladium pteridis*.

Discussion

Type and verified strains of four *Cylindrocladium* species were investigated in this study. Three of these have been associated with *Calonectria* teleomorphs, and type strains of the latter were also included. Comparisons were done on the basis of morphology, culture characteristics and nuclear DNA polymorphisms.

Cylindrocladium hawksworthii. This species is known from two collections made in Mauritius (*Peerally*, 1991b). It is distinguished from other species in *Cylindrocladium* in having primarily clavate vesicles, and curved 1-septate conidia. The phenomenon of curved macroconidia in *Cylindrocladium* is known from *C. curvatum Boedijn & Reitsma* (1950) with sphaeropedunculate vesicles, and *C. variabile Crous* et al. (1993a), with sphaeropedunculate to ellipsoidal or clavate vesicles and (1–)3(–4)-septate conidia. The present study also found the two heterothallic strains of *Calonectria clavata* to have prominently curved macroconidia. The latter strains could, however, be distinguished from *Cylindrocladium hawksworthii* by their larger conidium dimensions and septation, as well as distinct rDNA restriction patterns.

Cylindrocladium clavatum and Cylindrocladium gracile. When *Bugnicourt* (1939) described *Cylindrocarpon gracile*, he gave the conidial dimensions as being 24–48 × 2.5–4 µm. *Hodges* and *May* (1972) described *Cylindrocladium clavatum* with conidia being 37.5–48 × 3.5–5.5 µm. *Boesewinkel* (1982) transferred *Cylindrocarpon gracile* to *Cylindrocladium*, and retained it as a separate species because of its longer stipe extensions and narrowly clavate vesicles. This example was followed by *Peerally* (1991a). *Crous* and *Wingfield* (1994) characterized *Cylindrocladium gracile* (PC 551197) by having conidia slightly longer (40–56 × 3.5–5 µm) than that of *C. clavatum* (38–52 × 4–6 µm), and smaller than those of *C. pteridis* (62–121 × 5–6 µm).

Results obtained with rDNA restriction patterns for *Eco*RI, *Xho*I and *Hin*dIII in the present study (Table 1) clearly indicate, however, that the type culture of *Cylindrocladium gracile* (PC 551197) is indistinguishable from that of *C. clavatum* (ATCC 22833). The similarity in morphology and rDNA restriction patterns suggest, therefore, that the two species are synonymous. Because *gracile* is the older epithet, it is herewith proposed as the name for this species.

Cylindrocladium gracile (Bugn.) Boesewinkel, Trans. Br. mycol. Soc. *78*, 554 (1982)

Cylindrocarpon gracile Bugnicourt, Encycl. Mycol. *11*, 162 (1939)

Cylindrocladium clavatum Hodges & May, Phytopathology 62, 900 (1972)

Cylindrocladium brassicae Panwar & Bohra Indian Phytopathology 27, 425 (1974)

Calonectria pteridis (anam. Cylindrocladium pteridis) and Calonectria gracilis. Of the 1-septate species with clavate vesicles, *C. pteridis* has the largest macroconidia, and frequently also forms curved microconidia in culture. In the present study curved microconidia have also been observed for *Calonectria clavata.* Similar to *C. clavata, C. pteridis* has also been found to be heterothallic, with successful matings producing the teleomorph in culture (*Crous* et al., 1993c).

A homothallic strain with conidial dimensions of 40–65 × 4–5 µm was recently obtained from Brazil. Because these dimensions overlapped considerably more with that of *C. gracile* than *C. pteridis,* and *C. pteridis* was known to be heterothallic with larger, 1(–3)-septate ascospores, the isolate was described as the *Calonectria* teleomorph of *Cylindrocladium gracile* (*Crous* et al., 1993c). As stated above, the present study found *Cylindrocladium gracile* to be distinct from *Calonectria gracilis,* and synonymous with *Cylindrocladium clavatum.*

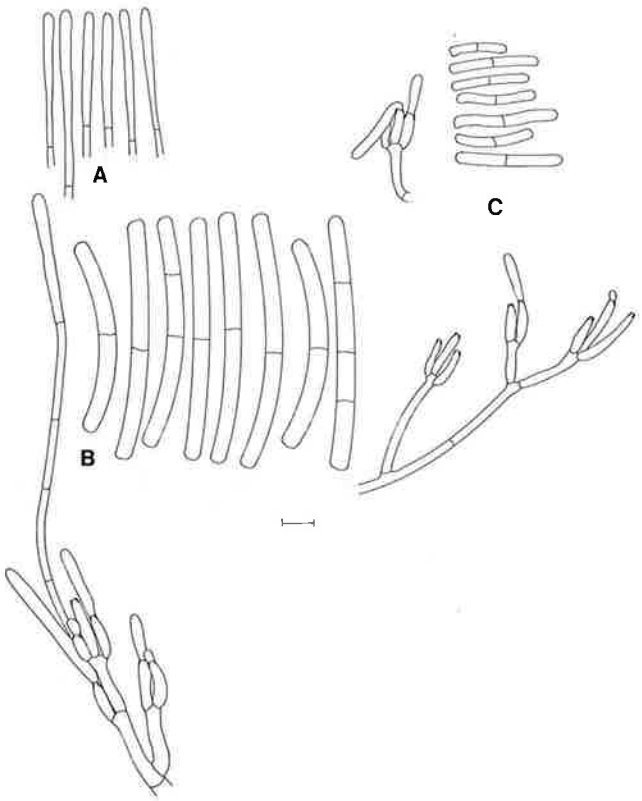

Fig. 4. Morphological structures of *Cylindrocladium flexuosum,* the anamorph of *Calonectria clavata.* A, clavate vesicles; B, penicillate conidiophore with curved, 1(–3)-septate macroconidia; C, conidiophores with irregularly curved, 1-septate microconidia (ATCC 66389, bar = 10 µm).

The rDNA restriction patterns obtained with *Eco*RI and *Hin*dIII showed only minor differences between *Calonectria gracile* and *Calonectria pteridis.* These two isolates could, however, easily be distinguished with the restriction enzyme *Xho*I (Table 1). Based on the differences in the banding patterns, as well as conidium and ascospore dimensions, it would appear that *Calonectria gracilis* represents a distinct species between *Cylindrocladium clavatum* and *Cylindrocladium pteridis.* We are however of the opinion that additional isolates of *Calonectria pteridis* will have to be studied to suitably resolve the validity of *Calonectria gracilis.*

Calonectria clavata. The heterothallic strains of *Calonectria clavata* are morphologically distinct from all presently described species of *Cylindrocladium.* Furthermore, their rDNA restriction patterns (Figs. 1–3) show these two isolates to be similar, but distinct from all other species investigated. We therefore propose the following nomenclator for this species.

Cylindrocladium flexuosum Crous sp. nov.

Teleomorph: *Calonectria clavata Alfieri, El-Gholl & Barnard,* Mycotaxon *48:* 206 (1993)

Macroconidiophora. Filum septatum, hyalinum (120–)180(–210) µm, in vesiculam clavatum (2–)3.5(–5) µm diam. terminans. Rami primarii non septati vel raro 1-septati, (20–)25(–33) × (3.5–)4(–5) µm; rami secundarii non septati, (15–)18(–22) × (3.5–)4(–5) µm. Phialides elongatae, doliiformes ad reniformes, hyalinae, non septatae, (10–)18(–30) × (3.5–)4(–4.5) µm. Conidia cylindrica, hyalina, curvata, 1(–3)-septata, apicibus obtusis, (50–)73(–80) × 5(–6) µm.

Microconidiophora. Filum nullum. Rami primarii non septati vel raro 1-septati, (15–)25(–35) × (2.5–)3(–3.5) µm; rami secundarii non septati, (10–)15(–20) × (2.5–)3(–3.5) µm. Phialides terminales, cylindricae, hyalinae, non septatae, (12–)18(–24) × 3(–3.5) µm. Conidia cylindrica, hyalina, irregulariter curvata, 1-septata, apicibus obtusis, (21–)32(–37) × 3(–3.5) µm.

Macroconidiophores. Stipe extension septate, hyaline, terminating in a clavate vesicle, 2–)3.5(–5) µm diam.; stipes (120–)180(–210) µm long. *Conidiophore branches:* primary branches non-septate or rarely 1-septate, (20–)25(–33) × (3.5–)4(–5) µm; secondary branches non-septate, (15–)18(–22) × (3.5–)4(–5) µm. *Phialides* elongate, doliiform to reniform, hyaline, non-septate, (10–)18(–30) × (3.5–)4(–4.5) µm. *Conidia* cylindrical, hyaline, curved, 1(–3)-septate, rounded at both ends, (50–)73(–80) × 5(–6) µm.

Microconidiophores. Stipe extension absent. *Conidiophore branches:* primary branches non-septate to rarely 1-septate, (15–)25(–35) × (2.5–)3(–3.5) µm; secondary branches non-septate, (10–)15(–20) × (2.5–)3(–3.5) µm. *Phialides* terminal, cylindrical, straight or slightly curved, hyaline, non-septate, (12–)18(–24) × 3(–3.5) µm; collarettes absent. *Conidia* cylindrical, irregularly curved, hyaline, 1-septate with obtuse ends, (21–)32(–37) × 3(–3.5) µm.

Culture characteristics. Minimum temperature above 10 °C; maximum temperature above 35 °C; optimum temperature 25 °C. This is a high temperature species

(*Crous* and *Wingfield,* 1994), with slight sporulation on aerial mycelium. *Colony colour* (bottom) 13'b–13'i, ochreous to umber (*Rayner,* 1970). *Chlamydospores* extensive, dense, forming numerous microsclerotia.

Etymology. *Flexuosum* = in reference to its curved conidia.

Holotype. USA, Florida, Lake Placid, roots and stems of *Callistemon viminalis,* 5 Apr. 1978, leg. *C. P. Seymour* & *E. L. Barnard,* 078-1543, ATCC 66389, PREM 51721.

Maclean et al. (1993) stated that DNA based techniques are essential to confirm the position of fungi exhibiting a pleomorphic nature in culture. The use of RFLP analysis and DNA probes has been demonstrated to distinguish groups within several fungal species, including *Ramulispora herpotrichoides* (Fron) von Arx [= *Pseudocercosporella herpotrichoides* (Fron) Deighton] (*Nicholson* et al., 1991; *Frei* and *Wenzel,* 1993), *Septoria tritici Roberge* ex *Desmaz* (*McDonald* and *Martinez,* 1990), and *Stagonospora nodorum* (Berk.) *Castellani* & *E. G. Germano* (*McDonald* et al., 1994). In the Hypocreales, *Lodolo* et al. (1992) found this technique a fast and effective means to distinguish among three closely related *Fusarium* species.

In conclusion, the present study has found rDNA RFLPs to be effective in distinguishing among morphologically similar species of *Cylindrocladium.* Our results further suggest that this is also an excellent technique for validating anamorph teleomorph relationships where the two states have been described from separate collections. With the recently completed monograph of *Cylindrocladium* (*Crous* and *Wingfield,* 1994), results obtained using this technique can now be integrated with alpha and beta taxonomic criteria. This approach would help to determine the morphological and genetic range of species occurring in morphologically similar complexes.

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Dr. *Pedro W. Crous*, Department of Plant Pathology, University of Stellenbosch, 7600 Stellenbosch, South Africa; e-mail PWC@MATIES.SUN.AC.ZA