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

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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 (rDNA) polymorphisms. The rDNA of *C. clavatum*, *C. hawksworthii*, *C. pteridis*, *C. gracile*, *Calonectria gracilis* and *Calonectria clavata* were digested with the restriction enzymes EcoRI, HindIII and Xhol, 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* Panero & Bobra (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

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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 Sober (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, 1993b). 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* (PPRI 55197, ex type strain); *Calonectria gracilis* (PPRI 4176 = AR 2677, ex type strain); *Cylindrocladium hawksworthii* (MUCL 30866, ex type strain); *Cylindrocladium pteridis* (PPRI 4157 = UYF 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 (Biolog) (Zumpeetta, 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 EcoRI, HindIII and XhoI 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 Psrl fragment and labelled with [32p]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 55197), 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–17k, pepper brown to Saccardo's umber, except *Cylindrocladium hawksworthii* and *Calonectria clavata*, which were 13k verona brown and 133–134, 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 EcoRI, XhoI and HindIII restriction patterns were obtained for the different strains (Figs. 1–3, Table 1). The
Fig. 1. rDNA hybridization patterns for EcoRI-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 baeckeavorithii* MUCL 30866. Lane 8: *C. clavatum* ATCC 22833. Size markers are lambda DNA digested with EcoRI and HindIII.

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

Fig. 3. rDNA hybridization patterns for XhoI-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

<table>
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a Nuclear rDNA fragments highlighted by the 6.3-kb ribosomal repeat unit of Neurospora crassa 32P-labelled probe.

The 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 (Peerrally, 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 Peerrally (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 EcoRI, Xhol and HindIII 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.


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. gracilis* 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*.

The rDNA restriction patterns obtained with EcoRI and HindIII showed only minor differences between *Calonectria gracilis* and *Calonectria pteridis*. These two isolates could, however, easily be distinguished with the restriction enzyme *XhoI* (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.

Macroconiophora. Filum septatum, hyalium (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, doliformes 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.

Microconiophora. 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, cylindraceae, 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.

**Macroconiophores. 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, doliform 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.

**Microconiophores. 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) 13.3'6e13.1', ochreous to umber (Rayner, 1970). Chlamydothorbes extensive, dense, forming numerous microsclerotia.

Etymology. Flexiocystis = in reference to its curved conidia.

Holotype. USA, Florida, Lake Placid, roots and stems of Callistemon vinifolius, 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 Ramalibuspora 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, Lodolet 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 Cylindrical. 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 Cylindrical (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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