Delineation of *Cylindrocladium* species with 1-3-septate conidia and clavate vesicles based on morphology and rDNA RFLPs

P. W. Crous, L. Theron and W. H. van Zyl

Departments of Plant Pathology and Microbiology, University of Stellenbosch, P. Bag XI, Matieland 7602, South Africa

Unidentified isolates of *Cylindrocladium* with 1–3-septate conidia and clavate vesicles were compared with *C. theae*, *C. colhounii var. colhounii*, *C. colhounii var. macrocondia*, *C. gracie*, *C. pteridis* and *Calonectria graecis*. Ribosomal DNA (rDNA) of these isolates were digested with the restriction enzymes EcoR I, Hind III and Xho I, and Southern analysis performed with the 0.3-kb rDNA repeat unit of *Neurospora crassa* as DNA probe. Based on differences in general morphology, supported by their rDNA restriction fragment length polymorphisms, isolates of all species and varieties could be distinguished. Furthermore, the *Cylindrocladium* anamorph of *Calonectria graecis* was shown to be distinct from *Cylindrocladium pteridis* and *C. theae*. The name *Cylindrocladium pseudograecis* nov. is, therefore, proposed for the undescribed anamorph of *Calonectria graecis*.

Several species with 1–3-septate conidia and clavate vesicles were recently treated in a monograph of *Cylindrocladium* Morgan by Crous & Wingfield (1994). Within this complex, however, several distinct morphological groups can be recognized. *Cylindrocladium avescidatum* D. L. Gill, Alfieri & Sober is easily separated by its clavate to avesicate vesicles and thick-walled stipe extensions. *C. colhounii* Peerally var. **colhounii** and var. **macrocondia** Crous, M. J. Wingf. & Alfenas are distinguished by producing 3-septate conidia, being homothallic, and forming bright yellow perithecia with asci containing four 3-septate ascospores. *C. theae* (Petch) Subram. is also homothallic with 3-septate conidia, but forms reddish-brown perithecia with asci containing eight ascospores (Crous & Wingfield, 1994).

The remaining and more difficult group consists of a complex of three species. Crous & Wingfield (1994) recognized *C. clavatum* Hodges & L. C. May, *C. gracie* (Bagnic.) Boesew. and *C. pteridis* F. A. Wolf. In a subsequent study comparing the nuclear DNA polymorphisms of these species (Crous, Korf & Van Zyl, 1995), *C. clavatum* was reduced to synonymy under *C. gracie*, while their respective teleomorphs, *Calonectria clavata* Alfieri, El-Gholl & E. L. Barnard and *Calonectria gracie* Crous, M. J. Wingf. & Alfenas were shown to be distinct biological species. Uncertainty remained, however, concerning the status of *C. graecis*, as its banding pattern appeared similar to the only isolate of *Cylindrocladium pteridis* included in that study. Recently, another isolate with smaller conidia and slightly different vesicles than that ascribed to *C. pteridis* by Crous & Wingfield (1994) was isolated from soil. The aim of the present study, therefore, was to compare this isolate with isolates of *Calonectria gracie* and *Cylindrocladium pteridis*, and to delineate the biological species present in this complex.

**MATERIALS AND METHODS**

*Isolates of Cylindrocladium and Calonectria spp. examined:*

*Cylindrocladium gracie* (= *C. clavatum*), Brazil, Minas Gerais, Horta Correia, near Iabira, Pinus caribaea roots, Hodges & May, 16 Mar. 1971, ATCC 22833; *Calonectria graecis*, Brazil, Para, Belém, Mangueira Tapete, F. Albuquerque, PPRI 4176, IMI 354519, AR 2677; *Cylindrocladium pteridis*, Brazil, unknown host, J. C. Dinane, (Viçosa, Brazil, No. UVF 43), PPRI 4157; *C. pteridis*, needles of *Pinus* sp., T. L. Krügner, (Viçosa, Brazil, No. 10), PPRI 4177, IMI 354524; *C. pteridis* of *Pinus caribaea*, T. L. Krügner, (Viçosa, Brazil, No. UVF 37), PPRI 4178; *C. pteridis*, Eucalyptus grandis leaves, A. C. Alfenas, (Brazil, Viçosa, No. UVF 105), PPRI 4180, IMI 354530; *C. pteridis*, U.S.A., Arachnoides adiantiformis, F. Schickedanz, 1974, ATCC 34395; *C. pteridis*, Florida, Ramulina adiantiformis N. E. El-Gholl, (Brazil, Viçosa, No. UVF 50), PPRI 4179; *Cylindrocladium sp.*, South Africa, Natal Province, soil, P. W. Crous, 18 Nov. 1993, STE-U 675; *C. theae*, U.S.A., Florida, Rhododendron sp., N. E. El-Gholl, (Brazil, Viçosa, No. UVF 160), PPRI 4188; *C. colhounii* var. **colhounii**, U.S.A., unknown host, A. Alfenas, (Viçosa, Brazil, UVF 22A), PPRI 4183; *C. colhounii* var. **macrocondia**, E. Transvaal, Sabie, Frankfort, E. grandis cuttings, P. W. Crous, Mar. 1990, PPRI 4000.

**Morphology**

Single-conidial isolates were cultured on 2% malt extract agar (MEA) (Oxoid), plated onto cornation-leaf agar (CLA) (Crous, Phillips & Wingfield, 1992), incubated at 25 °C under light and examined after 7 d. Only material occurring on cornation leaves was examined. Mounts were prepared in lactophenol, and measurements made at × 1000 magnification.

**Total DNA isolation**

Single-conidial isolates were grown on MEA, and plugs of 7-d-old cultures transferred into 500 ml Erlemeyer flasks containing 100 ml glucose-yeast extract broth (Biolab)
Cultures were incubated for 7–14 d in the dark at 30°C until sufficient growth occurred. Mycelia were harvested by filtration (Whatman No. 1 filter paper) and freeze dried for 2 d. Total DNA was isolated according to Crous et al. (1993b), and subsequently redissolved in 200 μl TE buffer (pH 8.0) (Sambrook, Fritsch & Maniatis, 1989).

**Restriction enzyme analysis and Southern hybridization**

Total DNA (ca 5 μg) of each isolate was subjected to restriction digestion with EcoR I, Hind III and Xho I for 3 h respectively, according to the recommendation of the suppliers (Boehringer Mannheim). The DNA was separated on horizontal 1% agarose gels and transferred to Hybond-N nylon membranes (Amersham) according to standard procedures (Sambrook, et al., 1989). The *Neurospora crassa* ribosomal DNA (rDNA) was purified from plasmid pMF2 (Russell et al., 1984) as a 0.3-kb Pst I fragment and labelled with [α-32P]dATP (Amersham) as described by Feinberg & Vogelstein (1983). The Southern hybridizations and stringency washes were performed according to the method of Sambrook et al. (1989).

**RESULTS**

**Morphology**

*Calonectria gracilis* (PPRI 4176) formed a *Cylindrocladium* anamorph with 1-septate, 40–65 × 4–5 μm conidia, which were longer than those of *C. gracile* (ATCC 22833) at 38–52 × 4–6 μm. Among the species with 1–3-septate conidia, *C. collonii* var. *collonii* (PPRI 4183) had the shortest conidia at 45–65 × 4.5–5 μm, followed by *C. theae* (PPRI 4188) at 65–90 × 5–6 μm and *C. collonii* var. macroconidiale (PPRI 4000) at 86–112 × 5–8 μm. Brazilian isolates of *C. pteridis* (PPRI 4157, 4177–4180) had conidia within the range of 60–120 × 5–6 μm, while the Florida isolate (ATCC 34395) had slightly larger conidia at 65–130 × 4–7 μm. The *Cylindrocladium* species from South Africa (STE-U 675) had conidia similar in size to *C. theae*, *C. pteridis* and *Calonectria gracilis*, being 50–75 (100) × 4–5 μm, but were 1 (3)-septate, as found in *Cylindrocladium pteridis*. However, vesicles of STE-U 675 varied from narrowly ellipsoidal to clavate, and were not therefore strictly typical of *C. pteridis*.

**Restriction enzyme analysis and Southern hybridization**

Based on the rDNA restriction fragment length polymorphism (RFLP) profiles obtained with the restriction enzyme EcoR I, Hind III and Xho I, the South African isolate STE-U 675 fell into the variation accepted for *C. pteridis* (Table 1). Using the restriction enzymes EcoR I and Xho I (Fig. 1), isolate STE-U
to distinguish the species treated, and to allocate STE-U 675 to C. pteridis. When DNA was digested with additional restriction enzymes such as Sfi I, Xho I, Stu I, Msp I, Bgl II and Pst I, only the latter restriction enzyme showed a difference in the position of the two bands occurring in STE-U 675 and PPRI 4157. Banding patterns generated by the restriction enzyme Xho I (Fig. 4) showed the type strain of Calonectria gracilis (PPRI 4176) to be distinct from strains of Cylindrocladium pteridis collected from Brazil, U.S.A. and South Africa in having a band larger than 20000 bp.

**DISCUSSION**

In a previous study of Cylindrocladium species with 1-septate conidia and clavate vesicles four species, namely C. clavatum, C. pteridis, C. gracile and C. haucksworthii Peerally, were treated (Crous et al., 1995). Based on general morphology, C. haucksworthii could easily be distinguished from this group in having curved, 1-septate conidia and ellipsoidal to clavate vesicles. Results obtained using rDNA RFLP's firstly confirmed that the teleomorph Calonectria clavata was not conspecific with its purported anamorph, Cylindrocladium clavatum, and a new epithet C. flexuosum Crous was subsequently proposed. Furthermore, C. clavatum was shown to be synonymous with C. gracile, while once again, conspecificity could not be shown between C. gracile and its purported teleomorph Calonectria gracilis. The rDNA restriction patterns of EcoR I and Hind III showed only minor differences between C. gracilis and the strain of Cylindrocladium pteridis studied. It was only with a third enzyme, Xho I, that differences were detected between Calonectria gracilis and Cylindrocladium pteridis.

Conidia of Calonectria gracilis are 1-septate and 40–65 × 4–5 µm, while those of C. pteridis are 1(–3)-septate and 60–130 × 4–7 µm. In a previous study characterizing Cylindrocladium gracile, Crous et al. (1995) remained uncertain as to what variation was acceptable within C. pteridis, and accepted conidia to be 62–121 × 5–6 µm. Sobers (1968) stated that conidia of the latter are primarily 1-septate and 61–118 × 5–7 µm. Several isolates of C. pteridis have been studied that fail to develop 3-septate conidia. Although Sobers (1968) stated that microconidia of C. pteridis were curved, Crous & Wingfield (1994) reported that they could also be straight in some strains. Several strains seemed unable to produce a microconidial state, thereby lessening its value as a taxonomic feature in C. pteridis.

In Cylindrocladium, species identification relies on several features, of which conidial septation, dimensions, and vesicle shape are regarded to be of primary importance. Of those species with 1-septate conidia and more or less ovoid vesicles, small differences in vesicle morphology separate species such as C. candidum Viegas (obpyriform vesicles), C. scoparium Morgan (pyriform vesicles), C. ovatum El-Gholl, Alfenas & Crous, and T. S. Schub. (ovoid vesicles) and C. floridanum Sobers & C. P. Seym. (sphaeropediculate vesicles). However, as more data have become available, the separation of these species is further supported by differences in their protein (Alfenas & Wingfield, 1993a; El-Gholl et al., 1993) and DNA banding patterns, as well as distinct teleomorphs (Victor et al., 1996). Crous et al. (1993b) stated
that DNA RFLPs could distinguish variation between and among species, therefore enhancing attempts to isolate identities of *Cylindrocladium*. Using rDNA RFLPs, Lodolo, Van Zyl & Rabie (1992) and Crous et al. (1995) showed that this technique supported morphological differences in *Fusarium* and *Cylindrocladium*, respectively. Magee, D'Souza & Magee (1987) used rDNA RFLPs to distinguish several species of *Candida*, while Vidalys & Hester (1990) successfully employed this technique to distinguish species of *Cryptococcus*. Furthermore, intra- and interspecific variation could also be indicated in genera such as *Verticillium*, *Tapesia* and *Fusarium* (Cader & Barbara, 1991; Nicholson Razanoor & Hollins, 1993; Appel & Gordon, 1995). Using the same probe as in the present study, Kohn et al. (1988) combined this technique with morphological and epidemiological criteria to distinguish species of *Sclerotinia*. Haussner et al. (1993) reported RFLPs to be ideal for studying relatedness among both distantly and closely related species. The findings of the present study thus concur with that of others, namely that RFLP patterns support the recognition of individual species, as well as strains within a species.

The results we obtained in the present study showed the RFLP profiles of STE-U 675 with the restriction enzymes *EcoRI*, *HindIII* and *XhoI* to be similar to that of *C. pteridis* (Table 1), but distinct from all other species studied. Isolate STE-U 675 was initially accepted as distinct from *C. pteridis* based on its smaller conidia, at 50–75 × 4–5 μm, as well as narrowly ellipsoidal vesicles with acutely rounded apices. In older cultures, STE-U 675 produced a few conidia up to 100 μm long, thus suggesting that although conidia were usually 50–75 μm long, conidia more comparable with typical isolates of *C. pteridis* could be formed. These findings suggest that in contrast to the earlier concept accepted for *C. pteridis* as having clavate vesicles and conidia above 60 μm in length (Wolf, 1926; Sherbakof, 1928; Boedijn & Reitsma, 1950; Sober, 1968; Sober & Alferi, 1972; Peerally, 1991; Crous & Wingfield, 1994), vesicles can be narrowly ellipsoidal (widest in the middle or upper third of the vesicle with acutely rounded apices) or clavate, and conidia can be 1–(3-)septate and 50–130 × 4–7 μm.

The different banding pattern generated by the restriction enzyme *XhoI* (Fig. 4) for *Calonectria gracilis* (PPRI 4176) (presence of a band larger than 20000 bp), and strains of *Cylindrocladium pteridis* collected from Brazil, U.S.A. and South Africa, suggested the former species to be distinct. Conidia of *Calonectria gracilis* are 1-septate and 40–65 × 4–5 μm, overlapping to some degree with those of *C. pteridis*, which are 1–(3-)septate and 50–130 × 4–7 μm. However, identification based solely on the anamorph states can be problematic, especially as this study has shown that isolates at the lower end of the range of *C. pteridis*, such as STE-U 675, have conidia falling between what is typically associated with both *C. pteridis* and *Calonectria gracilis*. In the latter case, the teleomorph (when produced) will greatly assist in identification, as ascospores of the homothallic *C. gracilis* are 1-septate, (27–)30–(50) × (4–)5(–6) μm, while those of the heterothallic *Calonectria pteridis* Crous et al. are 1–(3-)septate, (30–)50–(75) × (4–)6(–7) μm (Crous, Wingfield & Alfenas, 1993c). Based on these differences between *Cylindrocladium* and *C. gracilis*, an epithet is provided for the *Cylindrocladium*

![Figure 5](image-url)

**Fig. 5.** Conidia, vesicles and ascospores of *Cylindrocladium* and *Calonectria* spp. A. One-septate conidia and elavate vesicles of *C. gracile* (ATCC 22833). B. One-septate conidia and clavate vesicles of *C. pseudogracile*, and ascospores of its teleomorph *Calonectria gracilis*. C. Range of 1–3-septate macroconidia (STE-U 675, PPRI 4157), microconidia, 1–3-septate ascospores (PREM 51033), and clavate to narrowly ellipsoidal vesicles (PPRI 4157, STE-U 675) of *C. pteridis* (bar. 10 μm).

**Cylindrocladium pseudogracile** Crous, sp. nov. (Fig. 5B) Teleomorph: *Calonectria gracilis* Crous, M. J. Wingf. & Alfenas, *Mycothax* 46: 224 (1993).

**Etym.:** morphologically intermediate between *C. gracile* and *C. pteridis*.


**Macroconidiosphaera.** Stipe septate, hyaline, terminating in a narrowly clavate vesicle, (2–)4(–5) μm diam.: stipes (160–)220–350 μm long. **Conidiophore branches:** primary


Cardinal temperature requirements for growth. Min. above 10° max. above 35°, opt. 30°. This is a high temperature species, with medium sporulation on aerial mycelium.

Holotype: Brazil, Para, near Belém, Manilkara zapota, 1990, F. Carneiro de Albuquerque, PREM 51031.

This study has demonstrated once again the value of integrating molecular techniques with traditional morphology to assess morphological variation accurately, and also to confirm anamorph/teleomorph relationships. Furthermore, results have suggested that in Cylindrocladium, where species are identified primarily by minor differences in conidium size, septation and vesicle shape, isolates falling within the range of accepted species complexes can only safely be identified if those complexes have been adequately characterized. In contrast to the acceptable variation found for C. pteridis in this study, other studies (Crouse et al., 1993; Victor et al., 1996) have shown that morphologically similar isolates could easily belong to distinct mating populations, and possible distinct biological species within a Cylindrocladium complex. Research involving the integration of studies on morphology, mating behaviour, vegetative compatibility groups and molecular techniques are therefore required to further attempts to elucidate other species complexes within Cylindrocladium and Colomnomyces.

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