Phylogenetic relationships of *Cylindrocladium pseudogracile* and *Cylindrocladium rumohrae* with morphologically similar taxa, based on morphology and DNA sequences of internal transcribed spacers and β-tubulin

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Abstract: Unidentified strains of *Cylindrocladium* that were baited from soil in the Amazonas state of Brazil or isolated from *Adiantum* in the Netherlands were examined morphologically and analysed phylogenetically in comparison with reference strains. Phylogenetic trees inferred from the 5.8S subunit and flanking internal transcribed spacers (ITS1 and ITS2) of rDNA, as well as the β-tubulin gene, separated species in accordance with their morphological features and characteristics. Although species differences based on ITS sequences were consistent, there were only a few informative sites available, making it difficult to clearly identify the unknown strains. Better resolution in separation of species was achieved from the β-tubulin data. The strains baited from soil in Brazil were found to represent two species, namely *Cylindrocladium gracile* (Bugnic.) Boesew. and *Cylindrocladium pseudogracile* Crous. Morphologically, these two species are similar, except that the latter has slightly narrower, 1(–3)-septate conidia, and produces a *Calonectria* teleomorph. Furthermore, the strain from the Netherlands represents *Cylindrocladium rumohrae* El-Gholl & Alfenas, a species previously known only from Panama. Results of this study also indicated that the β-tubulin gene is phylogenetically more informative than the ITS regions for distinguishing species of *Cylindrocladium*.

Key words: *Calonectria*, *Cylindrocladium*, phylogeny, sequence analysis, systematics.

Résumé : Par des études morphologiques et par analyse phylogénétique, les auteurs ont comparé des souches non-identifiées du *Cylindrocladium* trappées à partir du sol dans l'état Amazonas du Brésil, ou isolées à partir d'*Adianthum* en Hollande, et ils les ont comparées avec des souches de référence. Les dendrogrammes obtenus à partir de la sous-unité 5.8S et des espaceurs internes transcrits limitrophes (ITS1 et ITS2) du rADN, ainsi que du gène de la tubuline, permettent de séparer les espèces selon leurs particularités morphologiques et leurs caractéristiques. Bien que les différences spécifiques basées sur les séquences ITS soient congruentes, il n'y a que peu de sites révélateurs disponibles, ce qui rend difficile l'identification précise des espèces inconnues. On obtient une meilleure séparation des espèces à l'aide des données de la β-tubuline. On constate que les souches obtenues à partir du sol au Brésil représentent deux espèces, nommément le *Cylindrocladium gracile* (Bugnic.) Boesew. et le *Cylindrocladium pseudogracile* Crous. Morphologiquement, ces deux espèces se ressemblent, sauf que la dernière porte des conidies 1(–3)-septées légèrement plus étroites, et qu'elle produit un téléomorphe de type *Calonectria*. De plus, la souche provenant de la Hollande correspond au *Cylindrocladium rumorhae* El-Gholl & Alfenas, une espèce précédemment connue seulement au Panama. Les résultats de cette étude indiquent également que le gène de la β-tubuline est plus utile en phylogénie que les régions de l'ITS, pour distinguer les espèces du *Cylindrocladium*.

Mots clés : *Calonectria*, *Cylindrocladium*, phylogénie, analyse de séquences.

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Introduction

The genus *Cylindrocladium* Morgan, which has teleomorphs in *Calonectria* De Not. where known, includes several well-known pathogens of agricultural and forestry crops in tropical and subtropical regions of the world. Species are primarily distinguished based on their vesicle morphology, conidium dimensions, septation, and the morphology of their *Calonectria* teleomorphs. In many instances the *Calonectria* state is morphologically conserved, and vesicle and conidium morphology become the determining factors for species identification.

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In numerous cases, molecular techniques such as sequence analysis (Jeng et al. 1997; Schoch et al. 1999) and nuclear ribosomal DNA (nrDNA) Southern analysis (Crous et al. 1995, 1997*b*) have been employed to supplement morphology in determining the identity of new strains and the acceptable variation within species. In an effort to differentiate biological species in *Cylindrocladium* using the 5.8S subunit and flanking internal transcribed spacers (ITS) of the ribosomal RNA genes, Schoch et al. (1999) could only identify a low number of informative sites. In spite of this, these variable sites were shown to be consistent within a species and still supplied valuable phylogenetic information. DNA sequences of the β-tubulin gene have recently been used for the phylogenetic analysis of several different fungal taxa (Schardl et al. 1994; Tsai et al. 1994; O'Donnell et al. 1998). A study on the *Fusarium* Link species forming part of the *Gibberella fujikuroi* (Sawada) Wollenw. complex employed several unlinked DNA areas for phylogenetic analysis (O'Donnell et al. 1998). In comparison with the other DNA regions investigated in the latter study, however, more informative sites were found in the β-tubulin gene. This was useful in distinguishing species in *Fusarium*, including recently evolved ones (O'Donnell et al. 1998).

The aim of the present study was to employ these techniques to identify several unknown strains within two different *Cylindrocladium* species complexes. The first complex included several unidentified strains with clavate vesicles and one- to three-septate conidia that were isolated from soil. Morphologically these strains resembled *Cylindrocladium gracile* (Bugnic.) Boesew., *Cylindrocladium pseudogracile* Crous, as well as *Cylindrocladium theae* (Petch) Subram. The second complex included unidentified strains causing a disease of *Adiantum* in the Netherlands. These strains were characterized by clavate vesicles and multiseptate conidia and were most similar to *Cylindrocladium rumohrae* El-Gholl & Alfenas, *Cylindrocladium quinqueseptatum* Boedijn & Reitsma, *Cylindrocladium heptaseptatum* Sobers, Alfieri, & Knauss and *Cylindrocladium multiseptatum* Crous & M.J. Wingf.

Materials and methods

Strains

Cylindrocladium strains studied (Table 1), including reference strains obtained from various culture collections, are lodged in the culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa (STE-U). Strains of the unidentified *Cylindrocladium* species were received from The Hague in the Netherlands or were freshly baited from soil samples collected at Monte Dourado in the Amazonas state of Brazil, using methods explained in Crous et al. (1997*a*).

Morphological comparisons

Strains were cultured on 2% malt extract agar (MEA) (Oxoid), plated onto carnation-leaf agar (CLA) (Fisher et al. 1982; Crous et al. 1992), incubated at 25°C under near-ultraviolet light, and examined after 7 days. Only conidiophores occurring on carnation leaves were examined. Mounts were prepared in lactophenol, and measurements were made at 1000× magnification. Perithecia were rehydrated in 3% KOH and sectioned with a Leica CM 1100 cryostat freezing microtome. Sections (10 μm thick) were mounted in distilled water and examined microscopically. The 95% confidence intervals were derived from 30 observations, and the minimum and maximum values are given in parentheses. *Cylindrocladium* cultures were identified using the keys of Crous and Wingfield (1994) and Crous et al. (1997*a*).

DNA amplification and sequence determination

Single conidium strains were grown on MEA plates for approximately 7 days. Mycelia were collected from the plates, excess agar removed, and DNA extracted as described by Crous et al. (1993*a*). Both strands of the 5.8S subunit and ITS1 and ITS2 spacers of rDNA were sequenced and compared. Sequences were deposited at GenBank (accession No. AF231949–231973 for ITS; AF232849–232873 for β-tubulin). DNA was amplified using the primers ITS1 and ITS4 (White et al. 1990). In addition to this, both strands of the 5′ end of the β-tubulin gene, including several introns were sequenced. Primers based on those of Glass and Donaldson (1995) were used for initial amplifications. Additional primers were used as designed by O'Donnell and Cigelnik (1997). Finally, a 600 base pair (bp) fragment was amplified with the use of primers T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995). Polymerase chain reactions (PCR) were performed on a Rapidcycler (Idaho Technology, Idaho Falls, Idaho). Reaction conditions consisted of the following: an initial denaturation for 2 min at 96°C followed by 30 cycles of 15 s at 96°C, 30 s at 55°C, and 35 s at 75°C. A last elongation step of 2 min at 75°C was included. Reactions (total volume 25 μL) comprised 1.5 units Biotaq (Bioline, London) with the buffer as recommended by the manufacturer, 1 mM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 0.5 μM primer oligonucleotide, and approximately 10–30 ng of fungal genomic DNA as template. PCR products were purified using Magic PCR Preps (Promega Corporation, Madison, Wisc.). Both strands of the PCR product were sequenced using the ABI Prism 377 DNA sequencer (Perkin-Elmer, Norwalk, Conn.). 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 20–40 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, Princeton, N.J.) and loaded onto the sequencing gel.

Phylogenetic analysis

Alignments of sequences were done with computer package Malign version 2.7 (Wheeler and Gladstein 1991) and manually assessed for improvement. Phylogenetic analysis of the aligned DNA sequences was performed using PAUP* Version 4.0b2a (Swofford 1999). Sequences of *Fusarium subglutinans* (Wolknw. & Reinking) Nelson et al. and *Fusarium proliferatum* (Matsushima) Nirenburg (O'Donnell et al. 1998; Genbank β-tubulin: U34417, U34416; ITS: U34559, U34558) were used as outgroups. Gaps were treated as a fifth character to maximize the number of in-formative characters in all cases. Unweighted parsimony analyses were performed using only a simple heuristic search option for the ITS sequence based tree because of the high number of possible most parsimonious trees. The β-tubulin data set was analysed heuristically with 1000 random additions. Bootstrap analyses in both cases were done with 1000 replications to test clade stability (Felsenstein 1985). A partition-homogeneity test was performed on the ITS and β-tubulin data sets. Subsequently, a maximum-parsimony analysis was performed on the combined data using the branch and bound option. In addition to this, a heuristic analysis with 1000 random additions and 2000 bootstrap replications was performed. The decay indices were also calculated using AutoDecay (Eriksson 1998) to further test the robustness of the branches of the tree. Other measures, including tree length, consistency index, retention index, and rescaled consistency index (CI, RI, and RC), were also calculated. In addition to

Table 1. Strains of *Cylindrocladium* spp. studied.

Species	Accession No.	Substrate	Origin
	Complex 1		
Cylindrocladium clavatum	ATCC 22833	*Pinus*	Brazil[a,b]
	IMI 167580	*Camellia*	Mauritius
Cylindrocladium colhounii var. *colhounii*	STE-U 1339	Soil	Indonesia
	STE-U 681	Soil	Thailand
	STE-U 1237	*Eucalyptus*	KwaZulu-Natal, R.S.A.[c]
	STE-U 705	Soil	KwaZulu-Natal, R.S.A.
Cylindrocladium colhounii var. *macroconidiale*	STE-U 307	*Eucalyptus*	Mpumalanga, R.S.A.[a,d]
	STE-U 413	*Eucalyptus*	Mpumalanga, R.S.A.
Cylindrocladium gracile	PC 551197	*Argyreia*	Southeast Asia[a]
Cylindrocladium pseudogracile	AR 2677	*Manilkara*	Amazonas, Brazil[a]
Cylindrocladium pteridis	UFV 43	Unknown	Brazil
	STE-U 2190	*Eucalyptus*	Amazonas, Brazil
Cylindrocladium theae	ATCC 48895	*Rhododendron*	Florida, U.S.A.
	UFV 16	*Rhododendron*	Florida, U.S.A.
Cylindrocladium sp.	STE-U 1586	Soil	Amazonas, Brazil
	STE-U 1588	Soil	Amazonas, Brazil
	STE-U 623	Soil	Amazonas, Brazil
	Complex 2		
Cylindrocladium heptaseptatum	FTCC 1002	*Rumohrae*	Florida, U.S.A.
	FTCC 1003	*Rumohrae*	Florida, U.S.A.
Cylindrocladium multiseptatum	STE-U 1589	*Eucalyptus*	Indonesia[a]
	STE-U 1602	*Eucalyptus*	Indonesia
Cylindrocladium quinqueseptatum	ATCC 16550	*Scolopendrium*	United States
	STE-U 759	*Eucalyptus*	Madagascar
	STE-U 516	*Eucalyptus*	Thailand
Cylindrocladium rumohrae	UFV 218	*Rumohrae*	Panama[a]
	UFV 215	*Rumohrae*	Panama
Cylindrocladium sp.	STE-U 1603	*Adiantum*	The Netherlands

[a]Ex-type culture.
[b]*Cylindrocladium clavatum* is a synonym of *Cylindrocladium gracile*.
[c]Newly recorded from South Africa.
[d]A new species, *Cylindrocladium macroconidiale*.

the cladistic, parsimony methods, the phenetic, neighbor-joining method (Saitou and Nei 1987) was also used for comparative analyses.

Results

Morphology

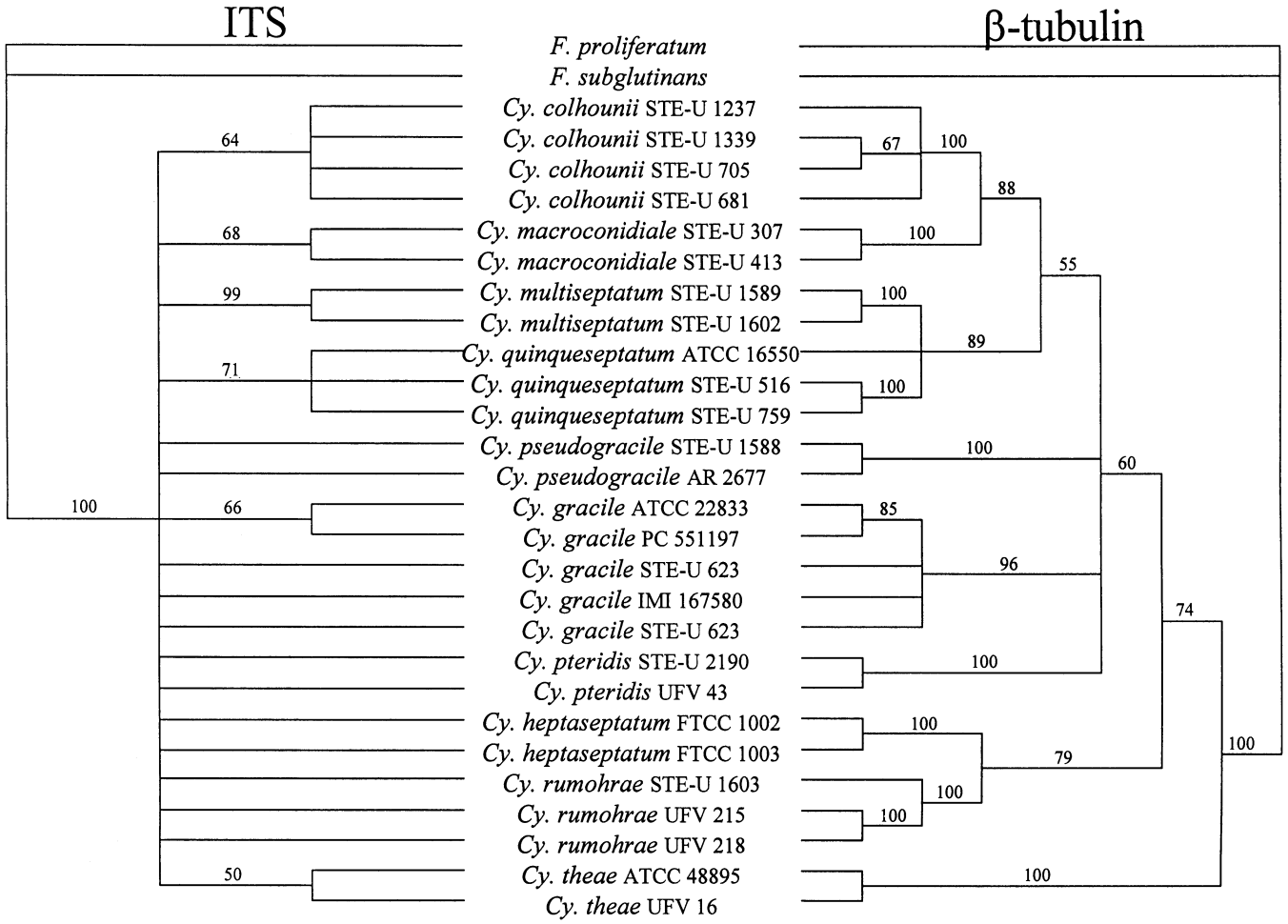
Cylindrocladium *complex 1*

The unidentified strains baited from soil collected in the Amazon were characterized by having clavate vesicles and conidia (42–)50–60(–65) µm in length, 4(–5) µm (STE-U 1588) or (4–)5–6 µm (STE-U 623, 1586) in width. Furthermore, although the latter two strains produced 1-septate conidia, STE-U 1588 had 1(–3)-septate conidia and produced a *Calonectria* teleomorph with red-brown perithecia on CLA, and one-septate ascospores, (33–)36–40(–45) × 5–7(–8) µm. These morphological features placed the strains into a complex characterized by clavate vesicles and one- to three-septate conidia. This suggested that species with one-septate conidia such as *Cylindrocladium gracile* (38–52 × 4–6 µm) (= *Cylindrocladium clavatum* Hodges & L.C. May), *Cylindrocladium pseudogracile* (40–65 × 4–5 µm) (teleomorph *Calonectria gracilis* Crous, M.J. Wingf., & Alfenas), and 1(–3)-septate conidia such as in *Cylindrocladium pteridis* (50–130 × 4–7 µm; Crous et al. 1997*b*) (teleomorph *Calonectria pteridis* Crous, M.J. Wingf., & Alfenas), and three-septate conidia as in *Cylindrocladium theae* (65–96 × 5–7 µm; Crous and Wingfield 1994) (teleomorph *Calonectria indusiata* Seaver) be included in molecular analyses for comparison. One species that also has clavate vesicles and three-septate conidia, but yellow perithecia, was also included, namely *Cylindrocladium colhounii* Peerally var. *colhounii* (45–70 × 4–6 µm) (teleomorph *Calonectria colhounii* Peerally var. *colhounii*) and *Cylindrocladium colhounii* var. *macroconidiale* Crous, M.J. Wingf., & Alfenas (86–112 × 5–8 µm; Crous and Wingfield 1994) (teleomorph *Calonectria colhounii* var. *macroconidialis* Crous, M.J. Wingf., & Alfenas). The unknown *Cylindrocladium* strains were morphologically most similar to *Cylindrocladium gracile* and *Cylindrocladium pseudogracile*. Based on ascospore dimensions, the *Calonectria* teleomorph of STE-U 1588 resembled *Calonectria gracilis*, which also has one-septate ascospores of similar dimensions (27–50 × 4–6 µm; Crous and Wingfield 1994).

Cylindrocladium complex 2

The unidentified strain obtained from *Adiantum* in the Netherlands (STE-U 1603) was characterized by having five-

Fig. 1. Phylogenetic trees of 29 taxa using the heuristic search option with 1000 random addition sequences and 1000 bootstrap replicates. Trees are rooted with the outgroups *F. subglutinans* (U34559) and *F. proliferatum* (U34558). The majority-role consensus tree (left) is derived from the alignment of the 5.8S rDNA, ITS1, and ITS2 spacers, while the phylogenetic tree (right) is derived from the alignment of β-tubulin sequences. The bootstrap values are indicated above the branches of both trees.

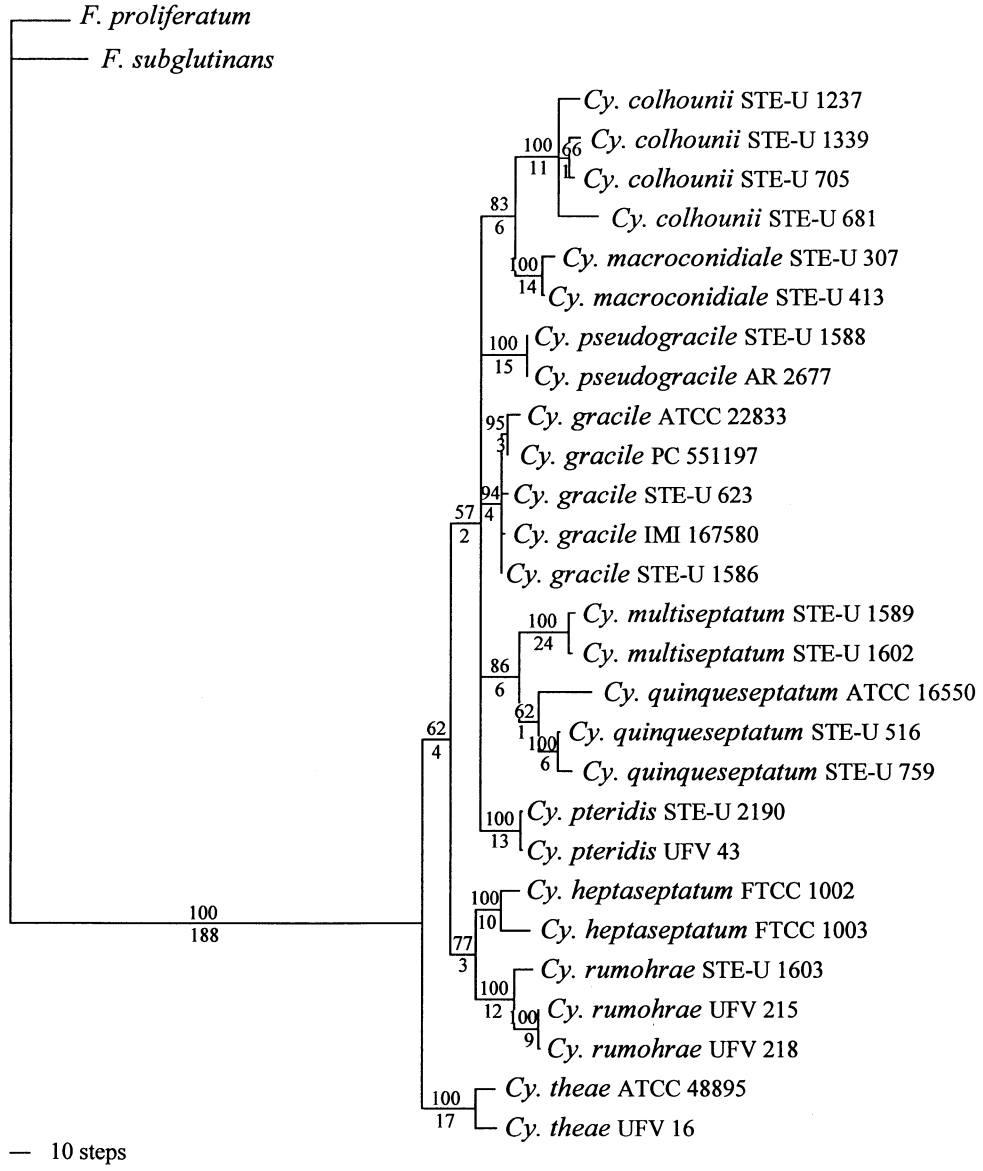


septate conidia, (95–)100–120(–130) × (8–)10 μm. This strain also formed a *Calonectria* teleomorph when cultured on CLA. Perithecia were brown to brown-red, and had 3–4 (–6)-septate ascospores, (60–)80–110(–140) × 6–7 μm. Morphologically, it was most similar to *Cylindrocladium rumohrae* (teleomorph *Calonectria rumohrae* El-Gholl & Alfenas), which has five-septate conidia (70–115 × 8–12 μm; Crous and Seifert 1998), and *Cylindrocladium quinqueseptatum* (teleomorph *Calonectria quinqueseptata* Figueiredo & Namekata), which also has five-septate conidia, (61–101 × 5–7 μm; Crous and Wingfield 1994). Other strains included in this complex were *Cylindrocladium heptaseptatum*, which has seven-septate conidia (96–144 × 6–9 μm; Crous and Wingfield 1994), and *Cylindrocladium multiseptatum* (teleomorph *Calonectria multiseptata* Crous & M.J. Wingf.), of which only the megaconidial form is known (120–200 × 8–10 μm; Crous and Seifert 1998).

Phylogenetic analysis

The ITS-based data set consisted of 527 characters, with 82 being informative. Only 10 of these characters occurred in the ingroup. Unweighted parsimony analyses were performed using a simple heuristic search option for the ITS sequence based tree and yielded 9958 equally parsimonious trees of 141 steps (CI = 0.95, RI = 0.838, RC = 0.609). Although the informative characters in the alignment of the ITS data set (data not shown) separate all the species in this study, their limited number limited the resolution of the ITS tree (Fig. 1). This could only differentiate a small number of clades, including those containing strains of *Cylindrocladium macroconidiale*, *Cylindrocladium colhounii*, *Cylindrocladium multiseptatum*, and *Cylindrocladium quinqueseptatum*. The β-tubulin-based data set consisted of 544 unordered characters, of which 207 were parsimony informative (150 being in the ingroup). The phylogenetic tree based on DNA sequences of part of the β-tubulin gene (Fig. 1) was obtained using the heuristic search option with 1000 random addition sequences and 1000 bootstrap replications. The tree had a length of 538 steps (CI = 0.699, RI = 0.803, RC = 0.561) and clearly separated all species. The result of the partition-homogeneity test ($P = 0.85$) suggested that ITS and β-tubulin data sets are highly homologous and support the same phylogeny. The maximum parsimony analysis on 311 parsimony informative characters of the combined data sets with the branch and bound option resulted in 4 most parsimonious trees (data not shown), which are almost identical except

Fig. 2. The most parsimonious tree derived from the combined ITS and β-tubulin data sets of 29 taxa using heuristic search option with 1000 random addition sequences and 2000 bootstrap replicates. The tree is rooted with the outgroups *F. subglutinans* (U34559) and *F. proliferatum* (U34558). The bootstrap values and the decay indices are indicated above and below the branches.



for minor changes in the positions of different isolates in the clades of *Cylindrocladium colhounii* and *Cylindrocladium gracile*. The maximum parsimonious phylogenetic tree (Fig. 2) based on the combined data set was generated using the heuristic search option with 1000 random input orders and 2000 bootstrap replications and is identical to one of the four most parsimonious trees obtained through the branch and bound option. The clades of *Cylindrocladium colhounii*, *Cylindrocladium macroconidiale*, *Cylindrocladium multiseptatum*, *Cylindrocladium quinqueseptatum*, *Cylindrocladium theae*, *Cylindrocladium heptaseptatum*, *Cylindrocladium rumohrae*, *Cylindrocladium gracile*, *Cylindrocladium pseudogracile*, and *Cylindrocladium pteridis* were strongly supported by the bootstrap values (94–100%). The basal clustering between *Cylindrocladium multiseptatum* and *Cylindrocladium quinqueseptatum* and that between *Cylindrocladium heptaseptatum* and *Cylindrocladium rumohrae* received relatively high bootstrap support (86 and 77%) suggesting close phylogenetic relationships among these species. Isolates of *Cylindrocladium colhounii* and *Cylindrocladium macroconidiale* clustered together, confirming the close relationship reflected by morphological characters. Neither data set supported the two species complexes hypothesized from morphology or any other interspecific relationships between them.

Discussion

The unidentified strains studied here represent two different *Cylindrocladium* species complexes, respectively characterised by *Cylindrocladium pseudogracile* and *Cylindrocladium rumohrae*. Both the latter species are poorly known, each having been collected only once (Crous et al. 1997*b*; El-Gholl et al. 1997). Other than morphology, sequencing of

the ITS1, ITS2, and β-tubulin genes were employed in the present study to try and circumscribe species among these strains. The ITS sequence data provided support that several previously delineated biological species are also phylogenetic species. The allocation of the strains STE-U 623, 1586, and 1588 to either *Cylindrocladium gracile* or *Cylindrocladium pseudogracile*, however, could not be resolved by the ITS DNA sequences. The data sets of the β-tubulin gene and morphological characteristics not only confirmed the results of the ITS data set but also clearly separated all the species investigated.

Cylindrocladium complex 1

The three unidentified strains from this complex had conidia that were similar in length (42–)50–60(–65) μm but were slightly wider (4–)5–6 μm in STE-U 623 and STE-U 1586, than in STE-U 1588 (4–5 μm). The latter strain also produced a *Calonectria* teleomorph resembling *C. gracilis*. Based on results obtained from the β-tubulin sequence data, strains STE-U 623 and 1586 are representative of *Cylindrocladium gracile*, while STE-U 1588 is representative of *Cylindrocladium pseudogracile*. These data suggest that, although the conidial lengths of these two *Cylindrocladium* species are similar, the minute differences in conidium width can be used to separate these taxa. It is possible that conidium width can also be used to separate other morphologically similar species of *Cylindrocladium*. The value of this feature to separate species has also been noted for cercosporoid fungi in vivo (Braun 1993), as well as in vitro (Crous 1998).

The β-tubulin data obtained here also support the recently proposed synonymy of *Cylindrocladium clavatum* under *Cylindrocladium gracile* (Crous et al. 1995). Furthermore, both strains of *Cylindrocladium pseudogracile* that are presently available readily produce a *Calonectria* teleomorph, which is absent in strains of *Cylindrocladium gracile*. Mating studies with *Cylindrocladium gracile*, using a wide variety of strains (data not shown), have thus far also proven unsuccessful in inducing the *Calonectria* teleomorph. A further complication is that some conidia of STE-U 1588 were found to develop up to three conidial septa. This was never observed in the type strain, AR 2677. The strongly supported cluster in the phylogenetic tree (Fig. 2) and very similar β-tubulin sequences (data not shown) observed between these two strains indicate that the species concept of *Cylindrocladium pseudogracile* as proposed by Crous et al. (1997*b*) should be expanded to also include strains with 1(–3)-septate conidia.

When *Cylindrocladium colhounii* var. *macroconidiale* (as *macroconidialis*) was originally described by Crous et al. (1993*b*), strains were compared with several collections of *Cylindrocladium colhounii* var. *colhounii* using isozymes and found to be distinct (A.C. Alfenas, unpublished data). However, because the teleomorphs were similar and so characteristic and the anamorph states found to differ only in conidial size and cultural characteristics, the South African material was described as a new variety of *Cylindrocladium colhounii* and not as a distinct species. Based on the topology of the phylogenetic tree of the combined data set (Fig. 2), however, it is clear that Crous et al. (1993*b*) incorrectly interpreted the variation observed between the South African material and that of *Cylindrocladium colhounii*. These two varieties are in fact two different species, as shown in the present study. The South African variety is therefore elevated to species status below.

Calonectria macroconidialis (Crous, M.J. Wingf., & Alfenas) Crous comb. & stat. nov. Figs. 3–5.

Calonectria colhounii var. *macroconidialis* Crous, M.J. Wingf., & Alfenas, Mycotaxon, **46**: 222 (1993).

ANAMORPH: *Cylindrocladium macroconidiale* (Crous, M.J. Wingf., & Alfenas) Crous comb. & stat. nov.

Cylindrocladium colhounii var. *macorconidiale* Crous, M.J. Wingf., & Alfenas, Mycotaxon, **46**: 222 (1993).

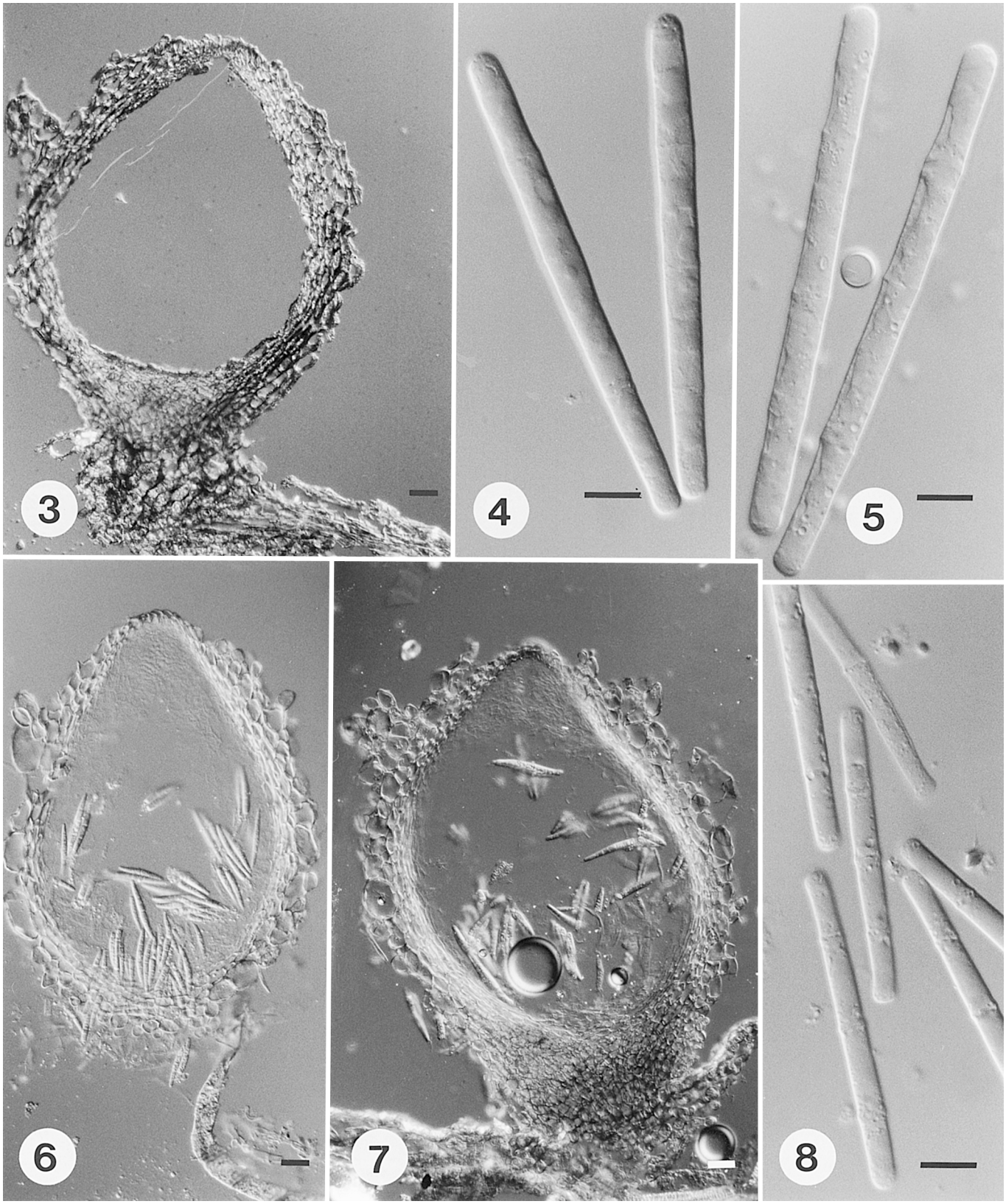
Cylindrocladium macroconidiale is easily distinguished from *Cylindrocladium colhounii* by its conidia, which are much larger, (68–)85–95(–112) × (5–)6–7(–8) μm, than in the latter species, (45–)55–65(–70) × (4–)5–6 μm. Although ascospores of both species are similar in size (30–)50–65(–70) × (4–)5–7(–8) μm, perithecia differ in colour (Figs. 3–8). Perithecia of *C. macroconidialis* are dirty yellow, and the red-brown pigmentation extends from the base upwards for most of the lower half of the perithecium. The latter part also colours dark red to red in 3% KOH (Fig. 3). Perithecia of *C. colounii* are bright yellow, and have a red-brown base, which colours dark red in 3% KOH (Figs. 6 and 7). *Cylindrocladium macroconidiale* has thus far only been collected from forestry nurseries in the Mpumalanga province of South Africa, where it is a serious pathogen of eucalypt cuttings in forestry nurseries. Single conidial and ascospore cultures have thus far never produced perithecia in culture. In contrast, several homothallic strains were obtained from forestry nurseries in the KwaZulu-Natal province of South Africa. Morphological and sequence data suggest, however, that these strains (STE-U 705, 1237) are representative of *Cylindrocladium colhounii*, and represent the first confirmed report of this pathogen from South Africa.

Cylindrocladium complex 2

Based on macroconidium, (95–)100–120(–130) × (8–)10 μm, and ascospore dimensions, (60–)80–110(–140) × 6–7 μm, the unidentified strain from *Adiantum* was similar to *Cylindrocladium rumohrae* (conidia 70–115 × 8–12 μm, ascospores 45–120 × 5–7 μm) and *Cylindrocladium quinqueseptatum* (conidia 61–101 × 5–7 μm, ascospores 54–100 × 4–8 μm). However, conidia of *Cylindrocladium quinqueseptatum* are much narrower, and the ascospores are smaller. The micro- and mega-conidial states of *Cylindrocladium rumohrae* and the strain from *Adiantum* are also similar and distinct from *Cylindrocladium quinqueseptatum* (Crous and Seifert 1998). In the phylogenetic tree of combined ITS and β-tubulin sequence data (Fig. 2), strain STE-U 1603 clustered close to other strains of *Cylindrocladium rumohrae* (UFV 215 and 218). These results confirm the occurrence of *Cylindrocladium ruhmorae* on *Adiantum* in the Netherlands. It has thus far only been known from Panama, where it causes petiole lesions on *Rumohrae adiantiformis* (El-Gholl et al. 1997).

The problems previously experienced in the use of the ITS regions for phylogenetic analysis (Jeng et al. 1997; Schoch et al. 1999) are, once again, underlined in this study.

Figs. 3–8. Vertical sections through perithecia and macroconidia. Figs. 3–5. *Calonectria macrocondialis* and its anamorph *Cylindrocladium macroconidiale* (holotype specimen). Figs. 6–8. *Calonectria colhounii* and its anamorph *Cylindrocladium colhounii* (STE-U 1339). Scale bars = 10 µm in Figs. 4, 5, and 8 and 35 µm in Figs. 3, 6, and 7.



By comparing several biological species in the *Cylindrocladium candelabrum* species complex, Schoch et al. (1999) found very few informative characters in the ITS region. More recently, the use of DNA sequences from part of the β-tubulin gene and the HMG box of the *MAT-2* mating type gene showed more conclusive evidence for characterizing these different species (unpublished results). In this study, the β-tubulin areas sequenced could provide clear differentiation of all the morphological species studied. However, several questions remain unanswered. The existence of several copies of the β-tubulin gene due to interspecies hybridization (Tsai et al. 1994; Schardl et al. 1994) have been reported before in fungi. In spite of these potential problems, the different morphological species dealt with in this study could be distinguished. This also proved to be the case for strains of one species collected from wide-ranging geographical locations, such as *Cylindrocladium quinqueseptatum* (United States, Madagascar, and Thailand). On the other hand, *Cylindrocladium multiseptatum* clustered together with the morphologically similar *Cylindrocladium quinqueseptatum* (Crous et al. 1998), suggesting that they may represent the same phylogenetic and morphological species. It is possible that *Cylindrocladium multiseptatum* represents strains of *Cylindrocladium quinqueseptatum* that have lost the ability to form micro- and macro-conidia. Based on these findings, a study aimed at re-evaluating the *Cylindrocladium quinqueseptatum* complex has been initiated and will be reported on elsewhere. As more strains are compared, and additional genes are sequenced, it is bound to cast more light on the general phylogeny of species within this genus.

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