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Cylindrocladium angustatum sp. nov., a new leaf spot pathogen of *Tillandsia capitata* from Florida, U.S.A.

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Cylindrocladium angustatum **is described as a new species from *Tillandsia* introduced with plant material into the U.S.A. from Central America. Koch's postulates are established to prove its pathogenicity to this host. The species is compared with and distinguished from *C. heptaseptatum* and *C. rumohrae* based on morphology, cultural characteristics and phylogenetic analysis of sequence data of the beta-tubulin gene.**

Key Words——*Cylindrocladium*; foliar fungal pathogen; *Hypocreales*; *Tillandsia*.

During March 1999, a new *Cylindrocladium* disease was encountered on *Tillandsia capitata* Griseb. plants at a large commercial containerized plant production greenhouse in Sarasota, FL in the U.S.A. The plants, which were showing symptoms of crown, basal rot and leaf spots, were collected by nursery inspectors of the Florida Department of Agriculture and Consumer Services—Division of Plant Industry, who submitted them to the Gainesville plant disease clinic for diagnosis (log number P99-0454). The *Cylindrocladium* isolate appeared unique from the first time it was observed in the clinic. The *Tillandsia* plants at this nursery were imported exclusively from Central America. The disease was detected a second time (log number P99-1321) in a subsequent visit to the same nursery on 11 June 1999. As part of a collaborative research programme on *Cylindrocladium* diseases, isolates were subsequently sent to the Department of Plant Pathology at the University of Stellenbosch, South Africa, for further characterisation.

The aim of the present study was thus to report this new disease of *Tillandsia* from the U.S.A., to identify and characterise the *Cylindrocladium* species involved, prove Koch's postulates, and discuss its morphological similarities to other species in the genus.

Materials and Methods

Morphology Leaf spots and blotches were surface-sterilised with 1% NaOCl for 3 min and plated on acidified potato dextrose agar (APDA, pH 4.3) to inhibit bacterial growth. APDA was prepared from the broth of 200 g of freshly peeled, diced, and boiled Irish potatoes (*Solanum tuberosum* L.) supplemented with 20 g dextrose, 1 g KH_2PO_4, and 18 g Difco Bacto agar and made up to 1 L with deionized water. After cooling to 50°C, 1.4 ml of 50% lactic acid was added per litre of autoclaved medium to obtain a pH of 4.3. Single conidi-

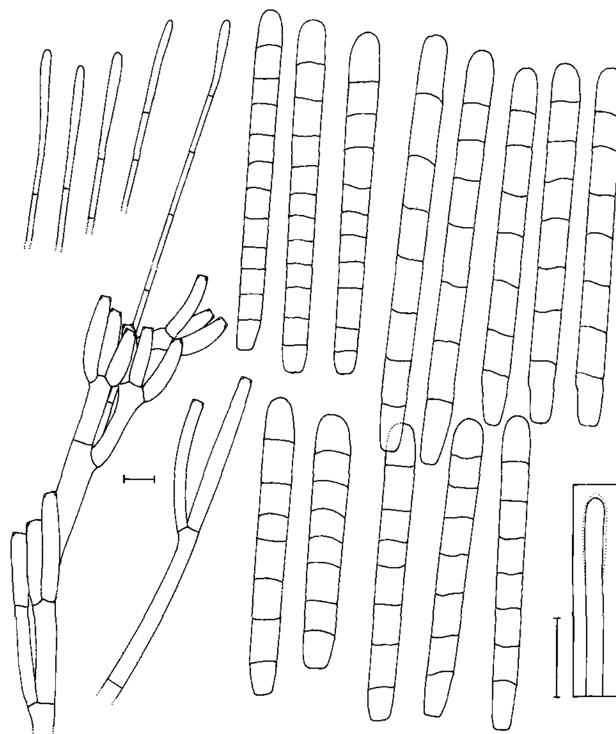

Fig. 1. *Cylindrocladium angustatum*. Clavate vesicles, macroconidia and conidiophores formed on carnation leaf agar. Subverticillate conidiophores were rarely observed. The variation in vesicle shape is given in the lower right corner, with the solid line indicating the dominant shape. Bars = 10 μm.

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um isolates were obtained, and subsequently transferred to divided plates containing 2% malt extract agar (MEA) (Biolab, Midrand, Johannesburg, South Africa), and carnation leaf agar (CLA) (Fisher et al., 1982; Crous et al., 1992). Plates were incubated at 25°C under continuous near-ultraviolet light. For microscopic examination fungal structures were mounted in lactophenol and measurements made at 1000× magnification. Thirty observations were made of each structure, the 95% confidence intervals determined, and the extremes given in parentheses. Reference cultures are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U) and the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia 20110–2209, U.S.A.

Sequencing Single conidial isolates selected for DNA comparison were grown on MEA plates. Mycelial mats were removed from the plates by means of a sterile scalpel and ground to a powder using liquid nitrogen and a mortar and pestle. Approximately 40 mg of ground mycelium was added to 2 ml microtubes containing 600 μl of extraction buffer. The extraction buffer consisted of 1% SDS, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 100 mM EDTA. Subsequently, the protocol was followed as suggested for the Wizard Genomic DNA Purification kit (Promega, Madison, U.S.A.). A 600 bp fragment of the β-tubulin gene was amplified with primers T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995). Additional amplification and sequencing conditions were the same as described by Schoch et al. (1999). Representative sequences used for comparison (*C. quinqueseptatum* Boedijn & Reitsma, *C. multiseptatum* Crous & M. J. Wing., *C. rumohrae* El-Gholl & Alfenas, *C. heptaseptatum* Sobers, Alfieri & Knauss and *C. theae* (Petch) Subram.) were taken from Crous et al. (1999).

Phylogenetic analysis Phylogenetic analysis of aligned DNA sequences was performed using PAUP* Version 4.0 bl (Swofford, 1999) and printed with the help of Treeview Version 1.5 (Page, 1996). Gaps were treated as missing. A single most parsimonious tree was obtained through branch and bound analysis in PAUP* and clade stability was assessed after 1000 bootstrap repetitions. The sequence of *Fusarium subglutinans* (Wollenw. & Reinking) Nelson et al. (NRRL 22016) deposited by O'Donnell et al. (1998), was obtained (GenBank accession number β-tubulin: U34417), and used as outgroup. New sequence data of the *Cylindrocladium* sp. were also deposited at GenBank (AF 207543, 207544).

Pathogenicity To confirm Koch's postulates, 30 ml of a conidial suspension (123,500 conidia/ml) of the *Cylindrocladium* sp. was used to spray inoculate three cultivars of *Tillandsia capitata* ('Guatemala'–10 cm tall; 'Hondurensis'–5 cm tall; and 'Red Mexico'–8 cm tall). Two plants per cultivar were inoculated. Two plants were used for control, and sprayed with sterile water. Plants were individually maintained in a moist chamber at room temperature (25°C, ±2°). Plants were removed from the moist chambers after 3 d. Lesions were removed from infected leaves by means of a sterile scalpel blade. Leaf disks were surface sterilised by immersion in 70% ethanol for 30 s, 1% NaOCl for 1 min and again in 70% ethanol for 30 s. Leaf disks were placed in Petri dishes containing 1.5% water agar and incubated for seven days at 25°C. Cultures were isolated and identified as discussed above.

Results

Morphology Isolates sporulated well on CLA, and produced penicillate macroconidiophores. Stipe extensions terminating in narrowly clavate vesicles were infrequently observed. Macroconidia were predominantly 7–10-septate, and (90–)100–120(–130) × 9–11(–12) μm (Figs. 1–6). No other conidial forms could be induced in culture. Morphologically this species was most similar to *C. heptaseptatum* and *C. rumohrae* (Figs. 7–10). It's cultural morphology was quite distinct, however, in that isolates rarely produced chlamydospores, and relatively few microsclerotia formed scattered throughout the medium.

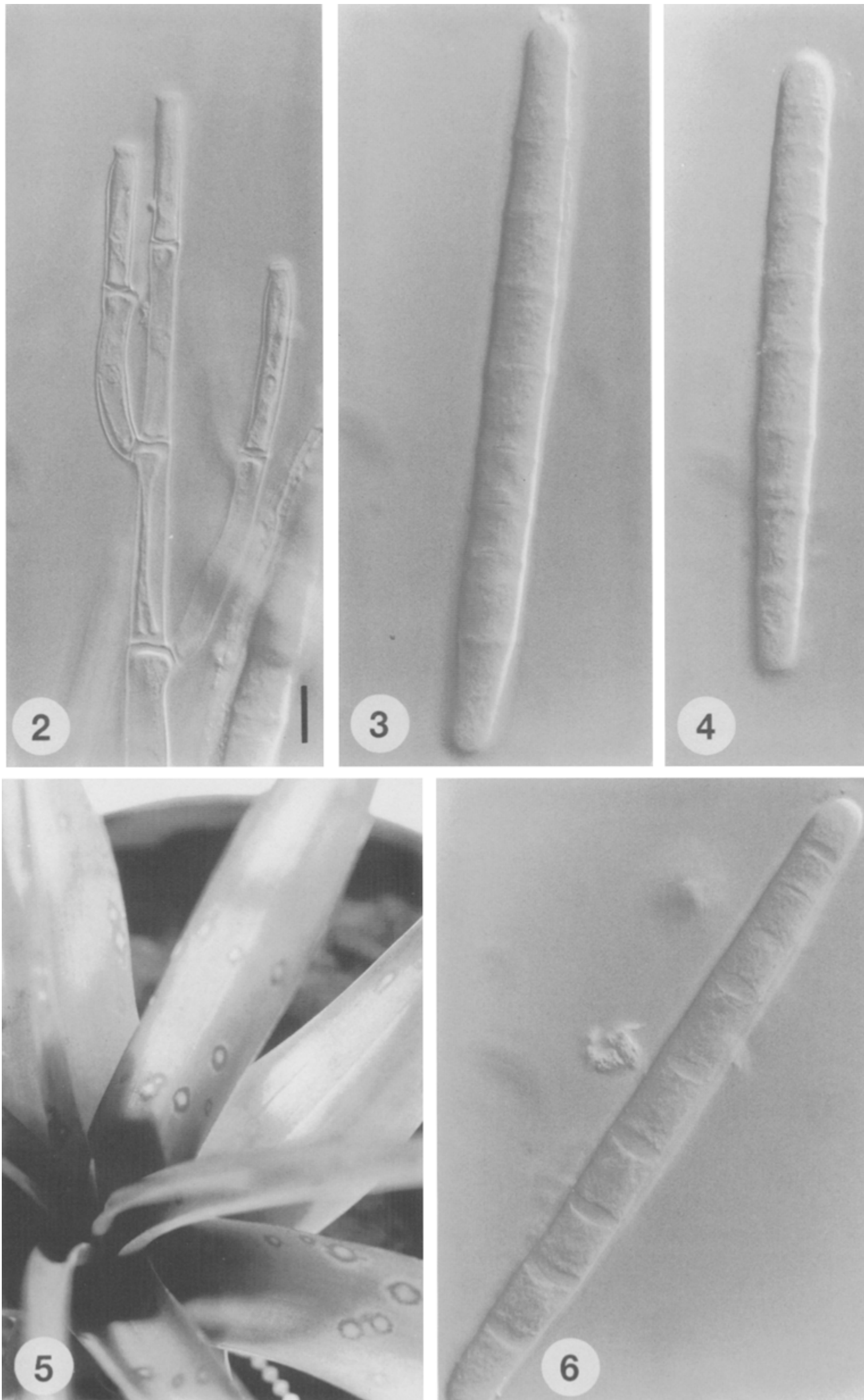
Sequencing DNA sequences amplified from the 5′ end of β-tubulin contained several introns and consisted of 543 characters with 127 being informative. The single most parsimonious tree obtained from this data set consisted of 311 steps (Fig. 11). The two isolates of the *Cylindrocladium* species from *Tillandsia* clustered together, and were most similar to the clade represented by isolates of *C. rumohrae* (56% bootstrap support).

Pathogenicity Symptoms appeared within 3 d on all three cultivars, and varied from leaf spots to foliar blight. No crown rot symptoms developed on inoculated plants. On *T. capitata* 'Guatemala', very few leaf spots formed. Spots had tan centers with reddish-brown borders. On the cv. 'Red Mexico', leaf spots appeared with tan centers surrounded by reddish-brown water-soaked borders (Fig. 5). Symptoms on cv. 'Hondurensis' were light reddish-brown leaf spots with tan centers, advancing to foliar blighting. Within 6 d of inoculation, the plants were dead and sporulation of the pathogen was evident on the necrotic tissues. The pronounced symptoms on 'Hondurensis' implied extreme susceptibility of this cultivar. The *Cylindrocladium* sp. was recovered from symptomatic leaf tissue of all inoculated hosts.

Taxonomy

Cylindrocladium angustatum Crous & El-Gholl, sp. nov. Figs. 1–4, 6

Filum septatum, hyalinum, (200–)250–300 μm, in vesiculam clavatam 2–3 μm diam terminans. Rami primarii non vel 1-septati, 33–70 × 4–6 μm. Rami secundarii aseptati, 20–30 × 4–5 μm. Rami tertiarii vel quarti raro visi, aseptati, 15–20 × 4–5 μm. Phialides ad extremum ramorum 1–4 exorientes, cylindricae, rectae vel leviter curvatae, hyalinae, aseptatae, 20–40 × 4–5 μm. Conidia cylindrica, hyalina, (1–)7–10(–12)-septata, apice utrinque obtusa, interdum basi truncata, recta, saepe in medio leviter inflata, (90–)100–120(–130) × 9–11(–12)



Figs. 2–6. *Cylindrocladium angustatum*. 2. Conidiophore with cylindrical phialides. 3, 4, 6. Multiseptate conidia that taper slightly at their ends. 5. Leaf spot symptoms on *Tillandsia capitata* cv. 'Red Mexico', confirming Koch's postulates. Bar = 10 μm.

μm.

HOLOTYPE: U.S.A. FLORIDA: Sarasota nursery, leaf spots of *Tillandsia capitata*, 1 Mar. 1999, R. M. Leahy, PREM 56546, culture ex-type P99-0454, STE-U 2347.

Etymology: Refers to the very thin stipe extensions.

Macroconidiophores comprised of a stipe, a sterile elongation and a penicillate arrangement of fertile branches. Stipe extension septate, (200–)250–300 μm long, terminating in a narrowly clavate vesicle, 2–3 μm diam; primary branches aseptate or 1-septate, 33–70 × 4–6 μm; secondary branches aseptate, 20–30 × 4–5 μm, tertiary and quaternary branches rarely observed, aseptate, 15–20 × 4–5 μm, each terminal branch producing 1–4 phialides; phialides cylindrical, straight to slightly curved, hyaline, aseptate, 20–40 × 4–5 μm, apex with minute periclinal thickening and inconspicuous collarette. Conidia cylindrical, bluntly rounded at both ends, or truncate at the base, straight, frequently slightly swollen in the middle, (90–)100–120(–130) × 9–11(–12) μm, (1–)7–10(–12)-septate, lacking a visible abscission scar, held in cylindrical clusters by colorless slime. Megaconidiophores and Microconidiophores not observed. Chalamydospores rarely observed, dark brown, thickened, sparse, occurring in aggregated clusters to form microsclerotia.

Cultures: Colony color (underneath and surface) buff 15′d, with moderate white aerial mycelia. Colony margin regular, with slight to no sporulation on aerial mycelia. Colonies reaching 33 mm diam on MEA after 6 d in the dark at 25°C.

Cardinal temperatures for growth: Min above 10°C, max below 35°C, opt 25°C. This is a moderate temperature species, not growing below 10°C, or above 30°C.

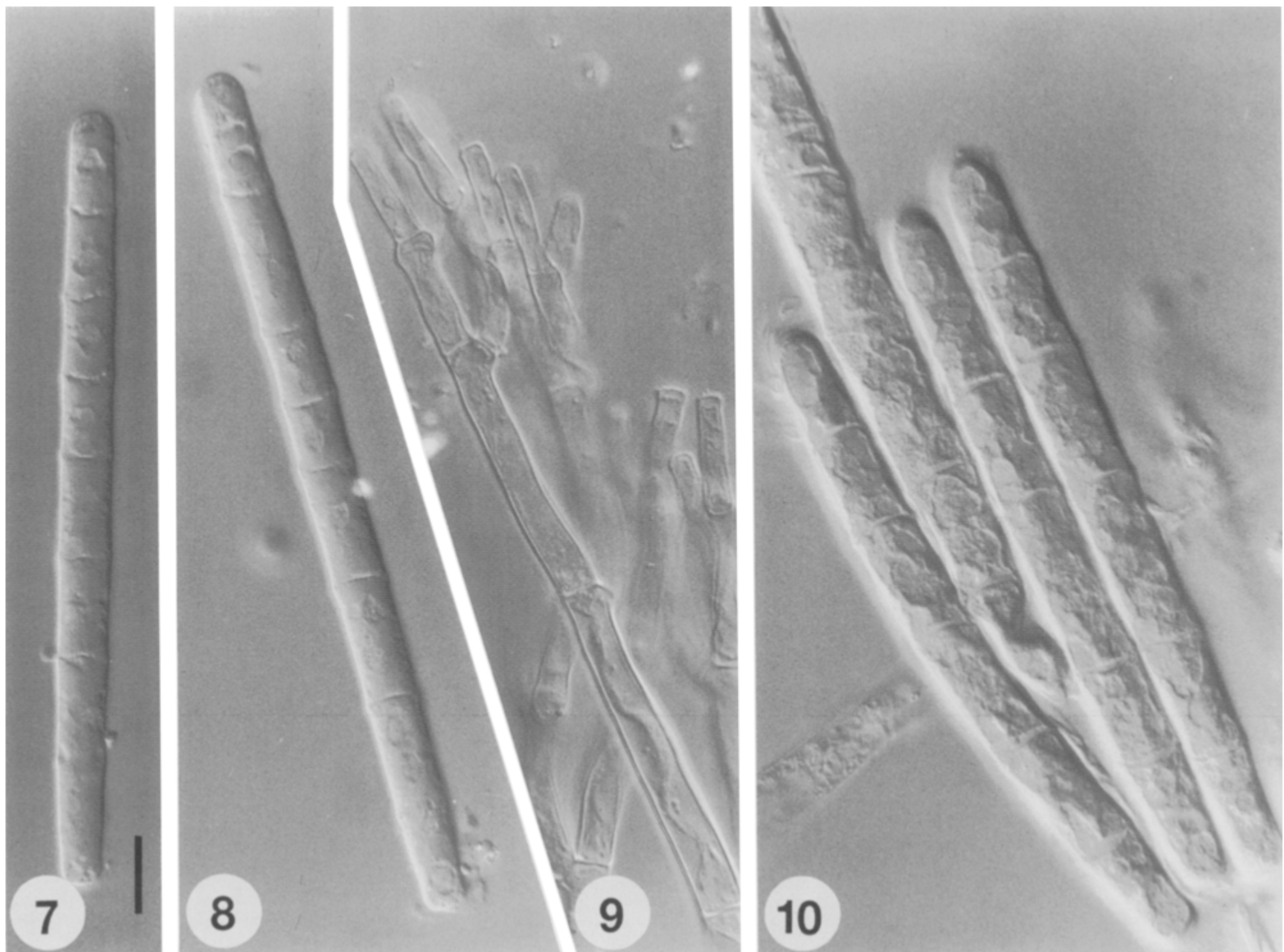
Substrate: *Tillandsia capitata* Griseb.

Distribution: Florida, U.S.A.

Additional culture examined: U.S.A. FLORIDA, Sarasota nursery, leaf spots of *Tillandsia capitata*. 11 Jun. 1999, R. M. Leahy, culture P99-1321, STE-U 3152.

Notes. *Cylindrocladium heptaseptatum* has (1–)7(–8)-septate macroconidia, (80–)110–130(–144) × (6–)7–8(–9) μm in size, and clavate vesicles that are (3–)4–5 μm wide. Macroconidia of *C. rumohrae* are (1–)5(–7)-septate, (70–)100–120(–130) × (8–)10(–12) μm, and its vesicles are clavate, (3–)4–5 μm wide (Figs. 7–10). Both species also form extensive chlamydospores in culture.

Macroconidia of *C. angustatum* are (1–)7–10(–12)-septate, (90–)100–120(–130) × 9–11(–12) μm, thus



Figs. 7–10. Conidiophores and macroconidia of *Cylindrocladium* spp. 7–9. *C. heptaseptatum*. 10. *C. rumohrae*. Bar = 10 μm.

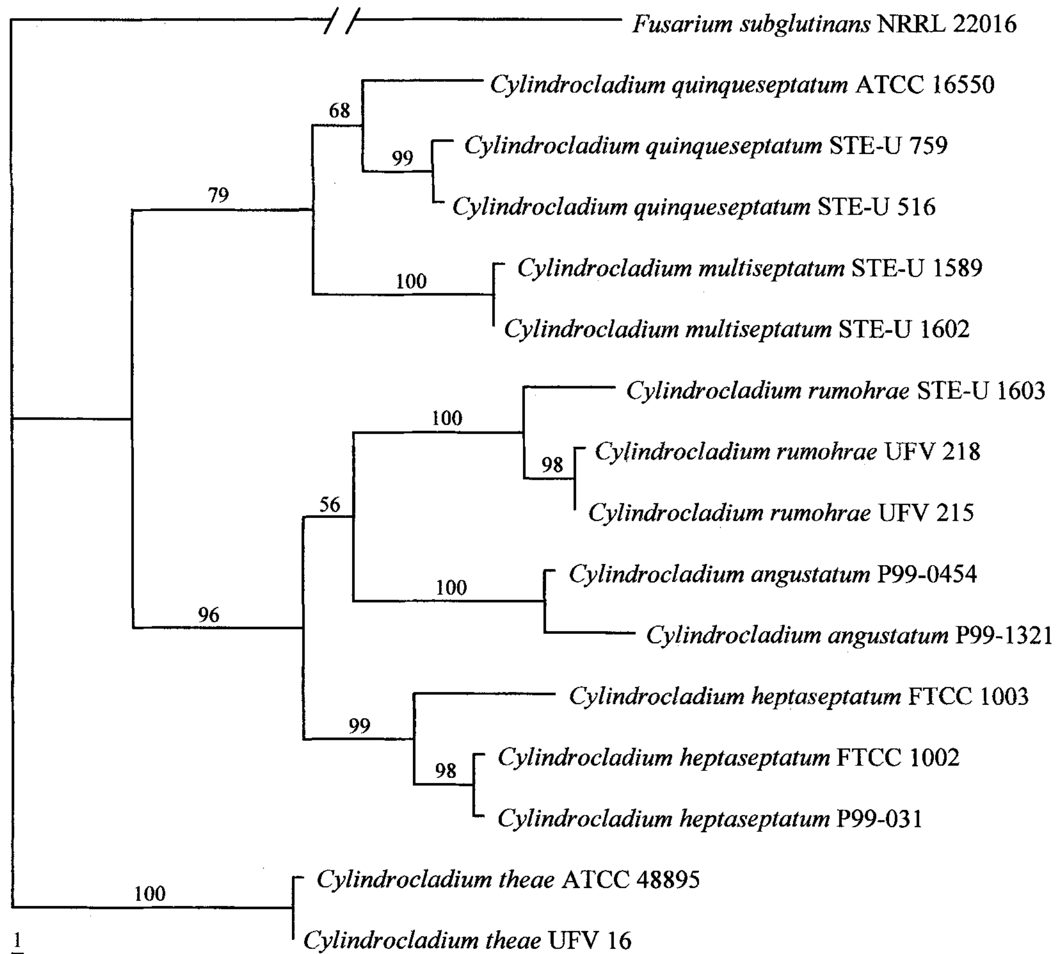


Fig. 11. Most parsimonious tree (307 steps, CI=0.827, RI=0.859, RC=0.711) generated by the branch and bound algorithm in PAUP* version 4.0 bl based on sequences of the 5′ end of the β-tubulin gene. Clade stability was assessed with 1000 bootstrap replications (values above 50% are shown) and *F. subglutinans* was used as outgroup.

being wider and developing more conidial septa than *C. heptaseptatum*. Although macroconidia of *C. angustatum* are similar in size to those of *C. rumohrae*, they tend to develop more septa. A further difference between these species can also be found in their terminal vesicles. Although those of *C. heptaseptatum* and *C. rumohrae* are clavate, (3–)4–5 μm wide, those of *C. angustatum* are narrowly clavate, 2–3 μm wide, and rarely develop in culture. A final distinguishing feature in this species complex can be found in their growth on MEA. Both *C. heptaseptatum* and *C. rumohrae* are observed to form extensive chlamydospores in this medium, while isolates of *C. angustatum* rarely form chlamydospores in culture, but rather tend to form a few scattered microsclerotia. These morphological differences are also supported by their β-tubulin sequence data (Fig. 11), which showed these three morphological species to also by phylogenetically distinct, with *C. angustatum* being more closely related to *C. rumohrae* than to *C. heptaseptatum*. No *Calonectria* teleomorphs are known for *C. angustatum* and *C. heptaseptatum*, and all mating studies have thus far proven unsuccessful.

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