Cylindrocladium pseudonaviculatum sp. nov. from New Zealand, and new *Cylindrocladium* records from Vietnam

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Cylindrocladium pseudonaviculatum is newly described from leaf spots of *Buxus sempervirens* collected in New Zealand. This species resembles *Cy. naviculatum*, but is distinct in having longer and wider conidia. Sequence data derived from the β-tubulin gene also support *Cy. pseudonaviculatum* as being distinct from the taxa presently acknowledged in *Cylindrocladium*. No *Calonectria* teleomorph was observed in culture, and mating studies proved unsuccessful. *Cylindrocladium insulare* and *Cy. pauciramosum* are also recorded for the first time from Vietnam, where they are associated with leaf spot symptoms on *Eucalyptus* spp.

Keywords: *Buxus*, *Calonectria*, *Cylindrocladium*, *Eucalyptus*, leaf spot.

Cylindrocladium spp. (teleomorph: *Calonectria*) are commonly associated with leaf spots and root rots of numerous hosts in tropical and subtropical countries. Species are distinguished primarily on the morphology of their anamorphs (vesicle and conidium morphology) on cultural characteristics, and characteristics of their teleomorphs (ascospore, ascus and perithecial morphology). In several species complexes, however, the taxa are morphologically conserved, and mating studies, as well as a variety of molecular techniques are required to elucidate the various biological and phylogenetic species.

In a recent study, Schoch & al. (2001b) employed sequence data from the 5' end of the β-tubulin gene to establish a phylogeny for 86 isolates, representing more than 30 species of *Cylindrocladium*. This study concluded that the various phylogenetic species correlated with the biological and morphological species accepted in the genus, and that vesicle shape was a highly informative morphological character. In the present study, several *Cylindrocladium* isolates morphologically similar to *Cy. candelabrum* and *Cy. naviculatum* were obtained from leaf spots of *Buxus* and *Eucalyptus*, respectively.

These strains could, however, be separated based on certain morphological characters, and appeared to be distinct species. The aim of the present study was, therefore, to determine whether these isolates were also distinguishable phylogenetically. To this end partial sequence data of the β-tubulin gene was derived for each, and compared with those of the species presently acknowledged in *Cylindrocladium* (Schoch & al., 2001b; Crous, 2002).

Materials and methods

Morphology

Isolates were cultured on 2% malt extract agar (MEA) (Oxoid), plated onto carnation-leaf agar (CLA) (Fisher & al., 1982; Crous & al., 1992), incubated at 25°C under near-ultraviolet light, and examined after 7 d. The 95% confidence intervals of conidial measurements were derived from 30 observations of structures formed on carnation leaves. Growth rates and cultural characteristics were determined after 6 d on MEA at 25°C in the dark, using procedures described by Crous & Wingfield (1994). Mating studies were conducted using the technique as outlined by Schoch & al. (1999). Colony colours were coded according to Rayner (1970). Isolates are maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa (STE-U), the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands and the International Collection of Micro-organisms from Plants (ICMP) in New Zealand.

PCR amplification and sequencing

The isolation protocol of Lee & Taylor (1990) was used to isolate genomic DNA from fungal mycelia grown on MEA plates. Template DNA (approximately 20 ng) was amplified in a 25 µl PCR reaction mixture consisting of 1 × PCR reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) and 200 µM each of dATP, dCTP, dGTP, and dTTP, with 15 pmols T1 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995) primers, and 1.25 units Taq DNA polymerase enzyme (Roche Diagnostics GmbH, Mannheim, Germany). The reaction was set up as follows: initial denaturation at 96°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 90 s, and final extension at 72°C for 7 min in a GeneAmp PCR System 2700 (Perkin-Elmer, Norwalk, Connecticut). A negative control, in which water was used instead of template DNA, was set up for each experiment. PCR products were separated by electrophoresis at 75 V for 1 h in a 0.8% (w/v) agarose gel in 0.5 × TAE buffer (0.4 M Tris, 0.05 M NaAc,

and 0.01 M EDTA, pH 7.85) and visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK) following ethidium bromide staining.

PCR products were purified by using a NucleoSpin Extract 2 in 1 Purification Kit (Macherey-Nagel GmbH, Germany). The cycle sequencing reaction with 20 to 40 ng of purified PCR products and 10 pmol primer in a total volume of 10 µl was carried out with an ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA, USA) containing AmpliTaq DNA Polymerase. The reaction was set up as denaturing at 94°C for 5 min., followed by 25 cycles of 96°C for 10 s, 55°C for 10 s, and 60°C for 4 min., with a final incubation of 30 s at 60°C. The resulting fragments were analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut).

Phylogenetic analysis

The nucleotide sequence of the β-tubulin gene of this study and those of the outgroup, *Fusarium proliferatum* (Matsush.) Nirenberg (GenBank accession numbers: X94171 and U34558) were assembled using the editor in Phylogenetic Analysis Using Parsimony version 4.0b8a (PAUP) (Swofford, 2000). The sequence files were aligned using the CLUSTAL W software (Thompson & al., 1994). Adjustments for improvement were made by eye where necessary. Phylogenetic analyses were undertaken using PAUP version 4.0b8 (Swofford, 2000). Alignment gaps were treated as a new state and all characters were unordered and of equal weight. Heuristic searches were conducted using stepwise simple addition and tree bisection and reconstruction (TBR) as the branch swapping algorithm to find maximum parsimony trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees was evaluated by 1000 bootstrap replications (Hillis & Bull, 1993). Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC) were also calculated. Resulting trees were printed with TreeView Version 1.6.5 (Page, 1996) and decay indices were calculated with AutoDecay Version 4.0.2 (Eriksson, 1998).

Results

Morphology

The *Cylindrocladium* isolates obtained from diseased *Eucalyptus* leaves in Vietnam have 1-septate conidia, 33–55 × 3–4 µm, and obpyriform to ellipsoid or clavate vesicles. Based on morphology, isolates resembled those placed in the *C. pauciramosum*-complex.

However, mating studies proved unsuccessful, and hence a definite identification to species level could not be obtained. The species collected from *Buxus* was morphologically similar to *C. naviculatum* Crous & M. J. Wingf. in having naviculate vesicles and 1(–3)-septate conidia. However, conidia were much longer (50–80 µm) and wider (4–6 µm), than those of *C. naviculatum*, which measure 40–50 × 3–4 µm.

Alignment of nucleotide sequences

For each isolate, approx 530 bases (spanning the first three introns and exons as well as part of the fourth exon) of the 5' end of the β-tubulin gene were determined. The manually adjusted alignments of the nucleotide sequences contained 560 sites for the data set (data not shown). Of the aligned nucleotide sites for the data set, 197 characters were parsimony-informative, 89 variable characters were parsimony-uninformative and 274 were constant. Sequences were deposited at GenBank (Tab. 1), and the alignment in TreeBase (SN1019-2816).

Phylogenetic relationships

The aligned sequences of the β-tubulin gene of 32 isolates and an outgroup were subjected to maximum parsimony analysis using the heuristic search option in PAUP (Swofford, 2000). The majority consensus tree of 5 equally most parsimonious trees obtained from the heuristic search was evaluated with 1000 bootstrap replications. The topology of one of the most parsimonious trees clearly segregated *Cy. naviculatum* and *Cy. pseudonaviculatum* with 99% bootstrap support. Isolates STE-U 3211, 3199 and 3219 clustered with isolates of *Cy. insulare* with 100% bootstrap support and STE-U 3207 with *Cy. pauciramosum* with 91% bootstrap support.

Taxonomy

Cylindrocladium pseudonaviculatum Crous, J. Z. Groenewald & C. F. Hill, **sp. nov.** – Figs. 1–7.

Teleomorph: unknown.

A *Cylindrocladium naviculatum* differt macroconidiis 50–80 × 4–6 µm.

Macroconidiophores consisting of a stipe, a penicillate arrangement of fertile branches, a stipe extension, and a terminal vesicle; stipe septate, hyaline, smooth, 50–150 × 5–6 µm; stipe extensions septate, straight to flexuous, 120–180 µm long, 3–4 µm wide at apical septum, terminating in a naviculate vesicle, 4–8 µm diam. – Conidiogenous apparatus 30–60 µm long, 30–45 µm wide; pri-

Tab. 1. – Isolates of *Cylindrocladium* spp. studied.

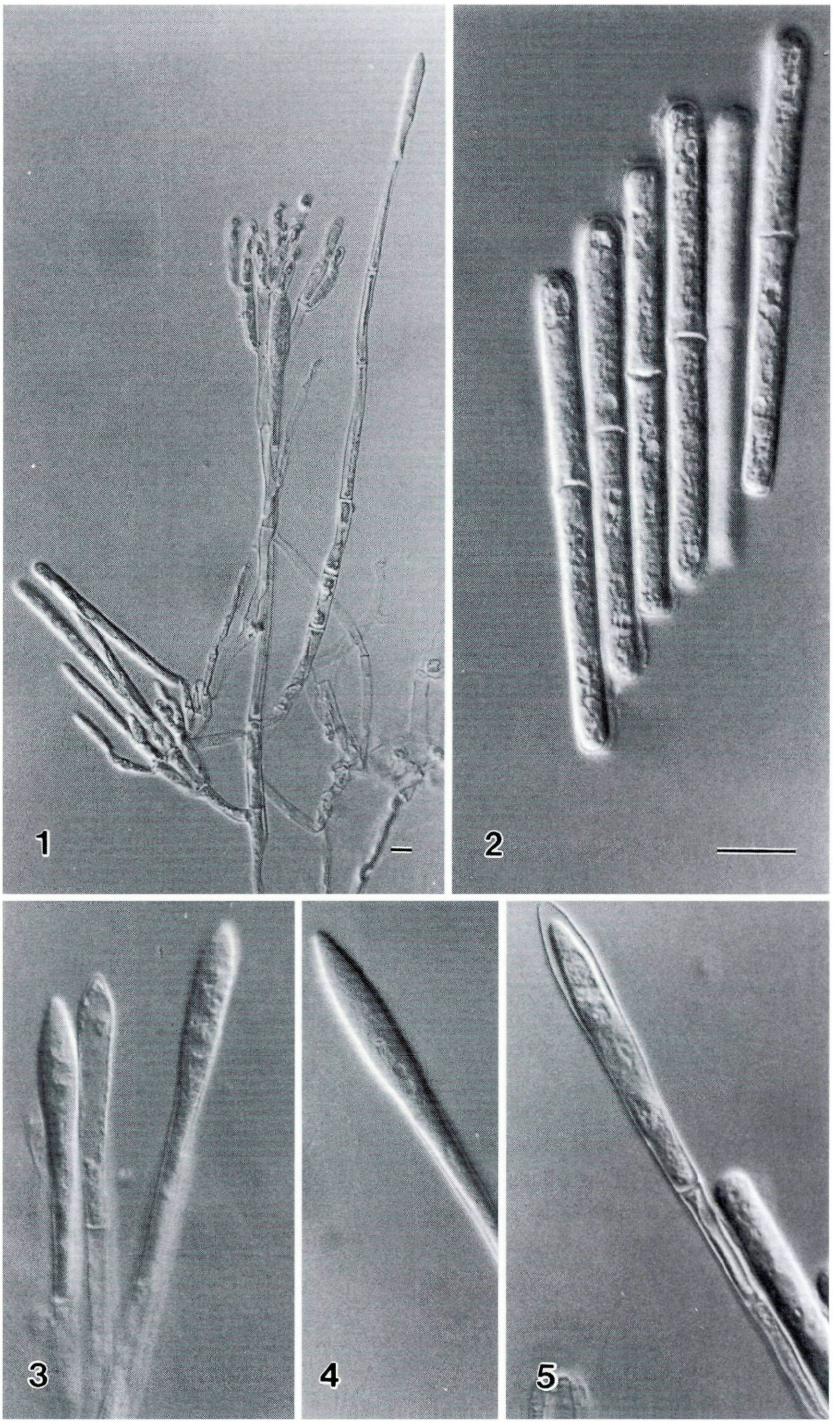
Anamorph	Teleomorph	Accession no.[a]	Collector	Substrate	Origin	GenBank no.
Cy. candelabrum	*Ca. scoparia*	STE-U 1674[b]	A.C. Alfenas	*Eucalyptus* sp.	Bahia, Brazil	AF210857
Cy. flexuosum	*Ca. clavata*	STE-U 2536[b]	N.E. El-Gholl	*Callistemon viminalis*	Florida, U.S.A.	AF333396
Cy. floridanum	*Ca. kyotensis*	ATCC 18882[b]	R.H. Morrison	Peach roots	Florida, U.S.A.	AF320193
Cy. gordoniae	Unknown	STE-U 3136[b]	D. Chiappini	*Gordonia lasianthus*	Florida, U.S.A.	AF449449
Cy. gracile	Unknown	PC 551197[b]	Bugnicourt	*Argyreia splendens*	Vietnam	AF232855
		STE-U 623	M.J. Wingfield	Soil	Amazonas, Brazil	AF333405
		STE-U 1586	P.W. Crous	Soil	Amazonas, Brazil	AF232863
Cy. graciloideum	*Ca. gracilipes*	STE-U 1153[b]	M.J. Wingfield	Soil	Colombia	AF333406
Cy. hawksworthii	Unknown	MUCL 30866[b]	A. Peerally	*Nelumbo necifera*	Mauritius	AF333407
Cy. insulare	*Ca. insularis*	STE-U 616	M.J. Wingfield	Soil	Amazonas, Brazil	AF210860
		STE-U 768[c]	P.W. Crous	Soil	Madagascar	AF210861
		STE-U 954	M.J. Wingfield	Soil	Veracruz, Mexico	AF210862
		STE-U 3211	M.J. Dudzinski & P.Q. Thu	*Eucalyptus camaldulensis*	Dong Nai, Vietnam	AF449451
		STE-U 3199	M.J. Dudzinski & P.Q. Thu	*Eucalyptus camaldulensis*	Binh Phuoc, Vietnam	AF449452
		STE-U 3219	M.J. Dudzinski & P.Q. Thu	*Eucalyptus camaldulensis*	Binh Phuoc, Vietnam	AF449450
Cy. mexicanum	*Ca. mexicana*	STE-U 927[b]	M.J. Wingfield	Soil	Yucatan, Mexico	AF210863
Cy. naviculatum	*Ca. naviculata*	STE-U 627[b]	M.J. Wingfield	Soil	Amazonas, Brazil	AF333409
		STE-U 628	M.J. Wingfield	Soil	Amazonas, Brazil	AF333410
Cy. parasiticum	*Ca. ilicicola*	CBS 190.50[b]	K.B. Boedijn & J. Reitsma	*Solanum tuberosum*	Java, Indonesia	AF333412
Cy. pauciramosum	*Ca. pauciramosa*	STE-U 416	S. de Buisson	*Eucalyptus grandis*	N. Province, R.S.A.	AF210869
		STE-U 972[b]	P.W. Crous	Soil	W. Cape, R.S.A.	AF210871
		STE-U 3207	M.J. Dudzinski & P.Q. Thu	*Eucalyptus saligna*	Lam Dong, Vietnam	AF449448
Cy. pseudonaviculatum	Unknown	STE-U 3570	Unknown	*Buxus sempervirens*	Waikato, New Zealand	AF449453
		STE-U 3613	R. Coutts	*Buxus sempervirens*	Auckland, New Zealand	AF449454

Tab. 1 (cont.). – Isolates of *Cylindrocladium* spp. studied.

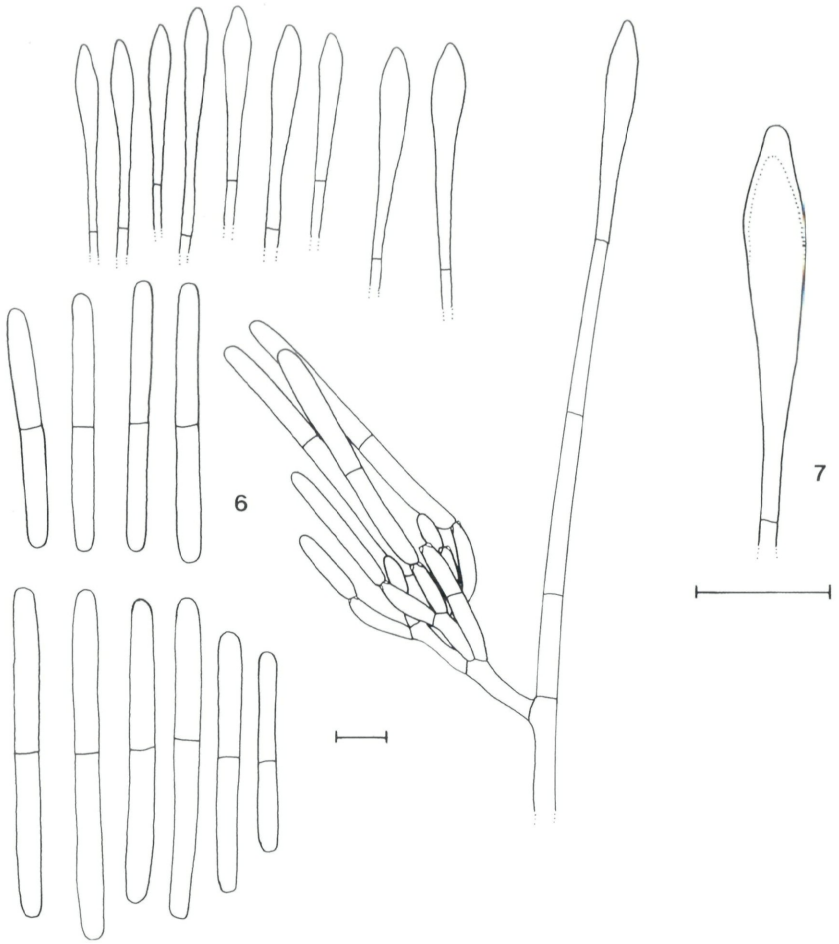
Anamorph	Teleomorph	Accession no.[a]	Collector	Substrate	Origin	GenBank no.
		STE-U 3399[b]	R. McDiarmid	*Buxus sempervirens*	West Auckland, New Zealand	AF449455
Cy. pseudogracile	*Ca. gracilis*	AR 2677[b]	A.Y. Rossman	*Manilkara* sp.	Amazonas, Brazil	AF232858
Cy. quinqueseptatum	*Ca. quinqueseptata*	STE-U 516	M.J. Wingfield	*Eucalyptus* sp.	Thailand	AF232870
Cy. scoparium	*Ca. morganii*	ATCC 38227	S.A. Alfieri	*Mahonia bealei*	Florida, U.S.A.	AF210872
		ATCC 46300[b]	D.M. Benson	*Leucothoe catesbaei*	North Carolina, U.S.A.	AF210873
		STE-U 1720	N.E. El-Gholl	*Rosa* sp.	Florida, U.S.A.	AF210874
		STE-U 1722	N.E. El-Gholl	*Dodonea viscosa*	Florida, U.S.A	AF210875
Cy. spathulatum	*Ca. spathulata*	ATCC 62616[b]	N.E. El-Gholl	*Eucalyptus viminalis*	Brazil	AF308463

[a] ATCC – American Type Culture Collection, Virginia, U.S.A.; AR – A. Y. Rossman, United States Department of Agriculture, A.R.S., Beltsville, Maryland, U.S.A.; MUCL – Mycothèque, Laboratoire de Mycologie Systématique et Appliqée, Université Louvain-la-Neuve, Belgium; PC – (P) Laboratoire de Cryptogamie, Paris, France; STE-U – Department of Plant Pathology, Univ. of Stellenbosch, Stellenbosch, South Africa.

[b] Ex-type cultures.



Figs. 1–5. – *Cylindrocladium pseudonaviculatum* (holotype). – 1. Conidiophore with conidia and stipe extension. – 2. Conidia. – 3–5. Variation in vesicle morphology. – Bars = 10 µm.



Figs. 6, 7. – *Cylindrocladium pseudonaviculatum* (holotype). – 6. Conidiophore, vesicles and conidia. – 7. Variation in vesicle morphology. – Bars = 10 µm.

mary branches aseptate or 1-septate, 20–30 × 4–5 µm; secondary branches aseptate, 15–20 × 3–4 µm; tertiary and additional branches –4, aseptate, 10–15 × 4 µm, each terminal branch producing 2–4 phialides; phialides doliiform to reniform, hyaline, aseptate, 12–20 × 3–4 µm, apex with minute periclinal thickening and inconspicuous collarette. – Conidia cylindrical, rounded at both ends, straight, (50–)55–65(–80) × 4–5(–6) µm, 1(–3)-septate, lacking a visible abscission scar, held in cylindrical clusters by colourless slime. – Megaconidiophores and microconidiophores unknown.

Cultural characteristics. – Colonies amber brown to Saccardo's umber, 13k – 17"k (reverse), with a pale luteous outer margin

(19d) (Rayner, 1970); chlamydospores extensive, dense, throughout medium, forming microsclerotia.

Cardinal temperatures for growth. – Min. above 5 °C, max. below 35 °C, opt. 25 °C, reaching 52–60 mm diam. after 6 d on MEA in the dark at 25 °C. This is a low- and high-temperature species, with medium sporulation on the aerial mycelium.

Symptoms. – Leaf spots.

Substrate and distribution. – *Buxus sempervirens* L., New Zealand.

Specimens examined. – NEW ZEALAND. West Auckland, Kumeu, *B. sempervirens*, 1998, R. MacDiarmid, PREM 57313 (holotype), culture ex-type (Lynfield 824 = STE-U 3399); Auckland, Orakei, *B. sempervirens*, 1998, R. Coutts, Lynfield 823 = STE-U 3613; Waikato, Cambridge, *B. sempervirens*, 1999, unknown collector, Lynfield 908 = STE-U 3570.

Results obtained in the present study support those of Crous (2002) and Schoch & al. (2001b), namely that minute differences in vesicle and conidium morphology are frequently indicative of different morphological and phylogenetic species in *Cylindrocladium*. *Cylindrocladium pseudonaviculatum* can easily be distinguished morphologically from all other species of *Cylindrocladium* except *C. naviculatum* (teleomorph: *Calonectria naviculata* Crous & M. J. Wingfield), which also has naviculate vesicles. Conidia of *Cy. pseudonaviculatum*, which measure (50–)55–65(–80) × 4–5(–6) µm, are significantly longer and wider than those of *Cy. naviculatum*, which are (40–)45–50 × 3(–4) µm (Crous & al., 1997). Apart from being morphologically and phylogenetically distinct (Fig. 8), crosses with tester mating strains of *Cy. naviculatum* (Crous & al., 1997; Crous, 2002) also proved negative, as did crosses between isolates of *Cy. pseudonaviculatum*.

Several isolates with 1-septate conidia and obpyriform to ellipsoidal to clavate vesicles were also obtained from leaf spots of *Eucalyptus* spp. collected in Vietnam. Based on morphology, they could be accommodated in the species described by Schoch & al. (1999) to represent the *Cy. candelabrum* Viégas species complex, namely *Cy. insulare* C. L. Schoch & Crous, *Cy. pauciramosum* C. L. Schoch & Crous and *Cy. mexicanum* C. L. Schoch & Crous. All four taxa have relatively small 1-septate conidia, and are primarily distinguished by their vesicle morphology, and compatibility with various mating type strains. Unfortunately, none of the isolates from Vietnam produced fertile progeny with any of the tester strains, and hence we had to resort to molecular techniques to support our morphological identifications. Isolate STE-U 3207 clustered with reference strains of *Cy. pauciramosum* with high bootstrap support.

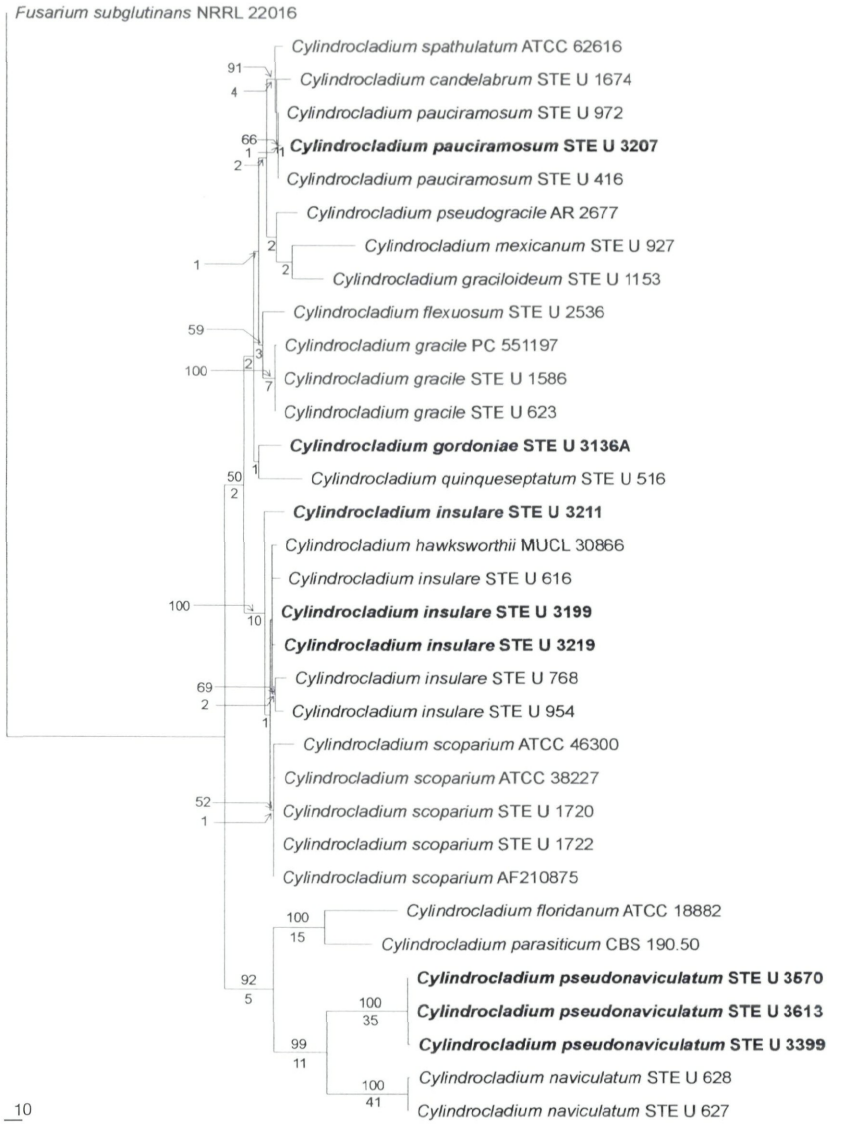


Fig. 8. – One of 5 most parsimonious trees obtained from a heuristic analysis of the complete data set (679 steps, CI = 0.700, RI = 0.790, RC = 0.553). The bar indicates 10 steps. The numbers above the branches represent bootstrap values based on 1000 resamplings, and the numbers below indicate the decay indices. The sequence of *Fusarium subglutinans* (Genbank: U34417) was included as outgroup.

Cylindrocladium pauciramosum is common in South Africa and Australia, and has recently also been introduced into the USA and Europe (Schoch & al., 2001a). This is the first report, however, of this species occurring in Vietnam.

Isolates STE-U 3211, 3199 and 3219 had a similar vesicle morphology to that of *Cy. pauciramosum* (STE-U 3207), but could be distinguished due to the presence of numerous conidiophore branches. Based on the phylogenetic analysis derived in this study, they clustered with isolates of *Cy. insulare* (Fig. 8). The latter species is known from Asian countries such as Indonesia and Malaysia (Crous, 2002), and its occurrence in Vietnam is, therefore, not totally unexpected.

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