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SYSTEMATIC AND APPLIED MICROBIOLOGY

Species Concepts in the Cylindrocladium floridanum and Cy. spathiphylli Complexes (Hypocreaceae) Based on Multi-allelic Sequence Data, Sexual Compatibility and Morphology

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Summary

Much attention has recently been devoted to the delimitation of species units in Cylindrocladium (Cy.). In this regard the present study focuses on the taxa within the unresolved Cy. floridanum and Cy. spathiphylli species complexes. Maximum parsimony analyses of DNA sequences of ITS, b-tubulin and histone regions of rRNA genes, and mating experiments revealed a geographically isolated species of Cylindrocladium in the Cy. spathiphylli (teleomorph: Calonectria spathiphylli) species complex. Cy. pseudospathiphylli sp. nov. (teleomorph: Ca. pseudospathiphylli sp. nov.) is described as a new phylogenetic, biological and morphological species. It is distinguished from Cy. spathiphylli by being homothallic, having smaller macroconidia, and distinct DNA sequences of b-tubulin and histone genes. Similarly, parsimony analysis of a combined data set also indicated several phylogenetic species to exist within Cy. floridanum (teleomorph: Ca. kyotensis). Based on differences in vesicle morphology and conidium dimensions, the Canadian population of Cy. floridanum, formerly known as Cy. floridanum Group 2, is described as Cy. canadense sp. nov., while a further collection from Hawaii is described as Cy. pacificum sp. nov.

Key words: Calonectria – ITS – b-tubulin – histone sequence analysis – mating studies – systematics

Introduction

The longstanding debate on species concepts for living organisms has attracted biologists from multiple disciplines (CLARIDGE et al., 1997). The recognition of species units in fungi has also recently received much attention (BRASIER, 1997), with significant progress being made in the field of DNA sequence-based phylogeny, and the delimitation of cryptic species (TAYLOR et al., 1999; O'DONNELL et al., 2000). The latter approach has also been taken in the hyphomycete genus Cylindrocladium Morgan (Cy.), where the comparison of molecular characters has successfully shown several morphological species to also contain genetically isolated cryptic species (CROUS et al., 1999; SCHOCH et al., 1999).

Cylindrocladium spathiphylli Schoult., El-Gholl & Alfieri was initially proposed as a species separate from Cy. floridanum Sobers & C.P. Seym. based on its globose vesicles and larger conidia (SCHOULTIES et al., 1982). UCHIDA and ARAGAKI (1992) later reported that isolates could have 1–3-septate conidia, with sphaeropedunculate, ellipsoid or spathulate vesicles. The teleomorph was described as Ca. spathiphylli El-Gholl, J.Y. Uchida, Alfenas, T.S. Schub. & Chase (EL-GHOLL et al., 1992), a species shown to have a biallelic, heterothallic mating system (EL-GHOLL et al., 1992; CROUS and PEERALLY, 1996). Recently, several isolates morphologically similar to Cy. spathiphylli were baited from soil samples (CROUS et al., 1997) collected in Ecuador. However, these isolates, as well as single-ascospore progeny, proved to be homothallic.

Cylindrocladium floridanum is characterised by having sphaeropedunculate vesicles (SOBERS and SEYMOUR, 1967), and lateral stipe extensions originating from sec-

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ondary and tertiary conidiophore branches (MORRISON and FRENCH, 1969). Calonectria kyotensis Terashita was described as its teleomorph, and has two known synonyms, namely Ca. floridana Sobers and Ca. uniseptata Gerlach (SOBERS, 1972). This synonymy was also supported when type cultures of the latter three species were compared using RAPD analysis and A+T-rich DNA polymorphisms (VICTOR et al., 1997). In a comparison of Cy. scoparium Morgan and Cy. floridanum, JENG et al. (1997) employed DNA fingerprinting with human minisatellite DNA as probe to show that although these two species were distinct, two groups existed within Cy. floridanum. The genetic variation within this species was further addressed by VICTOR et al. (1997), who found that the ex-type cultures representing Ca. kyotensis and its synonyms (ATCC 18882, ATCC 18834, CBS 413.67) formed a clade more closely related to Cy. scoparium than Canadian isolates of Cy. floridanum (Group 2), that appeared to be a separate, undescribed species. Furthermore, unlike Ca. kyotensis which is a well-known homothallic species, a few successful crosses with Canadian isolates suggested that the latter population could be heterothallic (A.C. Alfenas, pers. comm.), while terminal vesicles of the Cylindrocladium state also appeared to be intermediate between those of Cy. floridanum and Cy. scoparium.

Cylindrocladium floridanum and Cy. spathiphylli are both well-established species, known to be homothallic (Cy. floridanum), or to have a biallelic heterothallic mating system (Cy. spathiphylli) (EL-GHOLL et al., 1992; CROUS and PEERALLY, 1996). The present study reports on isolates that resemble the latter two species in general morphology. However, the populations reported on here appear to have been geographically isolated, and to have different mating systems. The Ecuadorean population of Cy. spathiphylli-like isolates are homothallic, while the Canadian population of Cy. floridanum-like isolates appear to be heterothallic, and the mating strategy of the Hawaiian Cy. floridanum-like isolates remain unresolved. The aim of the present study was thus to address the species concepts in these taxa by phylogenetic analysis of multi-loci DNA sequence data of b-tubulin, histone and ITS regions of rRNA genes. These data would help to determine if the taxa within the Cy. spathiphylli and Cy. floridanum complexes, which are distinguishable based on the morphological and biological species concepts, could also be separated based on a phylogenetic species concept.

Materials and Methods

Isolates

Cylindrocladium isolates were either obtained from symptomatic material, or baited from soil samples. Soil samples were collected and treated as explained in CROUS et al. (1997). Type specimens are lodged in the National Collection of Fungi in Pretoria (PREM), and ex-type cultures maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa (STE-U) and the Centraalbureau voor Schimmelcultures in the Netherlands (CBS).

Table 1. Isolates of *Cylindrocladium* species studied.

Species	Accession no.	Host	Location	Collector	Date	Mating strategy	GenBank (ITS, β-tub., H3)
Cy. canadense[a]	UFV 76	*Pinus* sp.	Canada	A.C. Alfenas	1990	Undetermined	AF348256, 348224, 348240
	STE-U 499[b]	*Picea* sp.	Canada	S. Greifenhagen	1992	Undetermined	AF348244, 348212, 348228
Cy. floridanum	ATCC 18834	*Robinia pseudoacacia*	Japan	Terashita	1963	Homothallic	AF348247, 348215, 348231
	ATCC 18882[b]	*Prunus persica*	Florida, U.S.A.	–	1965	Homothallic	AF348250, 348218, 348234
	CBS 413.67	*Paphiopedilum callosum*	Germany	W. Gerlach	1967	Homothallic	AF348251, 348219, 348235
	STE-U 500	*Picea* sp.	Canada	S. Greifenhagen	1992	Homothallic	AF348243, 348211, 348227
Cy. pacificum[a]	STE-U 682	Soil	Thailand	M. J. Wingfield	1993	Homothallic	AF348252, 348220, 348236
	A1568[b] = STE-U 2534	*Araucaria heterophylla*	Hawaii	M. Aragaki	1987	Undetermined	AF348254, 348222, 348238
	A1569 = STE-U 2535	*Araucaria heterophylla*	Hawaii	M. Aragaki	1987	Undetermined	AF348255, 348223, 348239
Cy. pseudo-spathiphylli[a]	STE-U 1623	Soil	Ecuador	M. J. Wingfield	1997	Homothallic	AF348257, 348225, 348241
	STE-U 1624	Soil	Ecuador	M. J. Wingfield	1997	Homothallic	AF348245, 348213, 348229
	STE-U 1641[b]	Soil	Ecuador	M. J. Wingfield	1997	Homothallic	AF348249, 348217, 348233
Cy. spathiphylli	ATCC 44730[b]	*Spathiphyllum* sp.	Florida, U.S.A.	C. L. Schoulties	1982	Heterothallic	AF348246, 348214, 348230
	P86-0210	*Heliconia* sp.	Florida, U.S.A.	N. E. El-Gholl	1986	Heterothallic	AF348248, 348216, 348232
	P87-0167	*Heliconia* sp.	Florida, U.S.A.	N. E. El-Gholl	1987	Heterothallic	AF348258, 348226, 348242
	STE-U 2188	*Spathiphyllum* sp.	Mpumalanga, R.S.A.	A. Thompson	1998	Heterothallic	AF348253, 348221, 348237

[a] Newly described species. [b] Ex-type cultures.

Gene amplification and sequencing

A total of 16 isolates were selected for sequencing (Table 1). Three genomic areas including the ITS regions adjacent to the 5.8S rRNA gene, and portions of the b-tubulin and histone genes were sequenced. Genomic DNA was isolated from fungal mycelium collected from the plates using the isolation protocol of LEE and TAYLOR (1990). Template DNA (20 ng) was amplified in a 25 µl PCR reaction mixture consisting of 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 6 mM $MgSO_4$, 500 µM each of dATP, dCTP, dGTP, and dTTP, with 60 pmols ITS1 and ITS4 primers (WHITE et al., 1990) or T1 (O'DONNELL and CIGELNIK, 1997) and bt2b (GLASS and DONALDSON, 1995) or H3-1a and H3-1b (GLASS and DONALDSON, 1995) primers, and 1.5 units Biotaq (Bioline, London, U.K.) DNA polymerase. The reaction was set up as follows: initial denaturation at 96 °C for 2 min, followed by 30 cycles of denaturation at 96 °C for 15 s, annealing at 55 °C for 30 s, extension at 75 °C for 35 s, and final extension at 75 °C for 2 min in a Rapidcycler (Idaho Technology Idaho, U.S.A.). A negative control using water instead of template DNA was set up for each experiment. PCR products were analysed by electrophoresis at 75 V for 2 h in a 0.8% (w/v) agarose gel in 0.5 æTAE buffer (0.4 M Tris, 0.05 M NaAc, 0.01 M EDTA, pH 7.85) and visualised in the gel documentation system (Gene Genius, Syngene, Synoptics Ltd.) following ethidium bromide staining. The PCR products were purified using Wizard PCR Preps (Promega Corporation, Madison, Wisconsin). Both strands of the PCR product were sequenced using the ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut). The cycle sequencing reaction with 20 to 40 ng of DNA template and 3.2 pmol primer in a total volume of 10 µl was carried out with a Dye Terminator Cycle Sequencing Ready Reaction Kit containing AmpliTaq DNA Polymerase (Perkin-Elmer). The reaction was set up as follows: initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 96 °C for 10 s, annealing at 55 °C for 10 s, extension at 60 °C for 4 min in a GeneAmp PCR System 2400 (Perkin-Elmer). The resulting fragments were finally purified using Centri-Sep Spin columns (Princeton Separations, Adelphia, New Jersey, U.S.A.) and loaded onto the sequencing gel.

Phylogenetic analysis

Nucleotide sequences of ITS rRNA, b-tubulin and histone genes of the isolates studied (Table 1), and GenBank retrievals of Fusarium nygamai L.W. Burgess & Trimboli (accession no.: X94174, U34426 and AF150855) as outgroups, were assembled using the Tex-Edit Plus programme (tombb@aol.com). Sequences were primarily aligned with Clustal W (THOMPSON et al., 1994) and optimised manually. Alignment gaps were coded as a fifth state in the analysis. Phylogenetic analysis of aligned DNA sequences was performed using PAUP* Version 4.0b2a (SWOFFORD, 1999). The most parsimonious trees were determined from the individual and combined data sets using the branch and bound and the heuristic search options with 1000 random sequence input orders with MULPARS on and TBR branch swapping for the exact solution. The unconstrained topologies of the equally parsimonious trees were compared using the Kishino-Hasegawa test. The best topology was selected as the most parsimonious tree topology. The tree stability was evaluated by 1000 parsimony bootstrap replicates accommodated in PAUP*. 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, retention, rescaled consistency and homoplasy indexes (TL, CI, RI, RC and HI) were also calculated. The best fit maximum likelihood tree was also calculated with 10 random sequence input orders and global rearrangement to test the parsimonious tree topology. A partition homogeneity test in PAUP* was conducted for the ITS, b-tubulin and histone sequences to examine the possibility of a joint analysis of the three data sets.

Sexual compatibility

Cylindrocladium spathiphylli-like single conidial isolates, and Cy. floridanum-like isolates were respectively mated in all possible combinations. This was achieved by removing 3 mm diam. agar plugs from the periphery of actively growing cultures and placing them on carnation leaf agar (CLA) plates as described by CROUS et al. (1993). The mating protocol used, and conditions followed are those explained in SCHOCH et al. (1999). Successful matings were regarded as those isolate combinations that produced perithecia with fertile, exuding ascospores.

Morphological and cultural comparisons

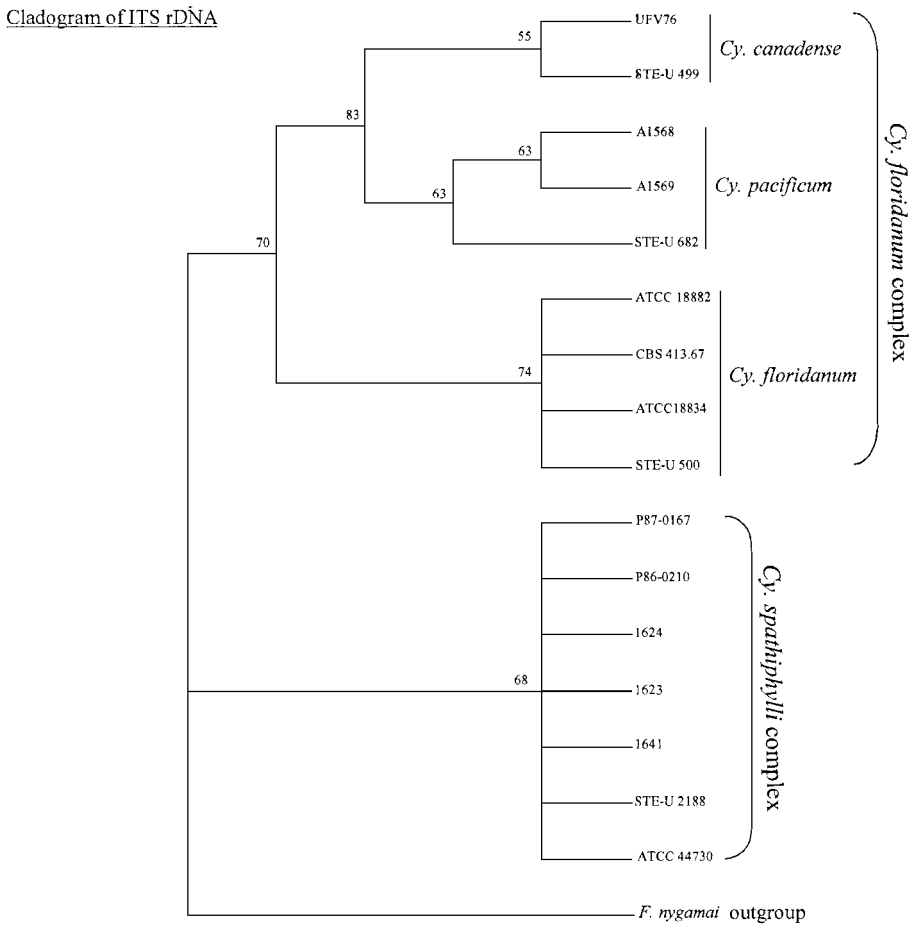
Morphological comparisons were made on divided Petri dishes containing 2% malt extract agar (MEA; Biolab, Midrand, Johannesburg, S.A.) on one side, and carnation-leaf agar (CLA; FISHER et al., 1982; CROUS et al., 1992) on the other. Plates were incubated at 25 °C under near-ultraviolet light, and examined after 7 d. Only cultures sporulating on carnation leaves were examined. Mounts were prepared in clear lactophenol, and measurements made at æ1000. Thirty observations were made of conidia and ascospores, the 95% confidence limits determined, and extremes given in parentheses. Cardinal temperature for growth (5–35 °C at 5° intervals) and cultural characteristics were determined after 6 d in the dark on MEA, using procedures described by CROUS and WINGFIELD (1994). Colony colours were coded according to RAYNER (1970).

Results

Phylogenetic analysis

The optimised nucleotide sequence alignments of ITS, b-tubulin and histone span 583, 619 and 477 sites, and contain 27, 190 and 138 parsimony-informative characters respectively. The result of the partition homogeneity test using heuristic search and 1000 replicates ($P = 0.113$, where $P \geq 0.05$ was significantly incongruent) indicated the compatibility of ITS, b-tubulin and histone data sets in evolutionary phylogeny. The joint alignment has been lodged in Treebase (http://www.herbaria.harvard.edu/treebase/index.html) with accession numbers: S571 & M864.

Maximum parsimony analysis of the ITS data set using the heuristic search option in PAUP* with 1000 randomisations of sequence input orders generated 2 equally most parsimonious trees (MPTs). The best tree topology of the 2 MPTs was selected as the final tree (Fig. 1) through the Kishino-Hasegawa likelihood test (data not shown) and evaluated with 1000 bootstrap replications in a heuristic search for clade stability. The ITS gene tree (Fig. 1) generated two major clades representing the Cy. floridanum and Cy. spathiphylli species complexes, with three subclades within the Cy. floridanum complex. The relatively low bootstrap values (55–83%) in the tree and indistinguishable relationship between the heterothallic and homothallic isolates in the Cy. spathiphylli complex resulted from the limited number of parsimony-informative characters in the ITS data set.



Figs. 1–4. Phylogeny of species in the Cy. floridanum and Cy. spathiphylli complexes.

Fig. 1. One of two most-parsimonious trees inferred from sequences of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions using maximum parsimony analysis with heuristic search option and 1000 random sequence input orders. TL = 401 steps, CI = 0.935, RI = 0.933, RC = 0.919, HI = 0.015.

A single MPT was obtained from the b-tubulin data set using the branch and bound search of maximum parsimony in PAUP*. The clade stability of the MPT was assessed by 1000 bootstrap replicates in a heuristic search. The b-tubulin gene tree (Fig. 2) segregated the same two major clades for the Cy. floridanum and Cy. spathiphylli species complexes, with high bootstrap support (87% and 100%), similar as to that observed in the ITS gene tree. The Cy. floridanum complex formed three clades representing three monophyletic lineages: the homothallic isolates (ATCC 18882, ATCC18834, CBS 413.67, STE-U 500), the heterothallic Canadian isolates (STE-U 499, UFV76), and the homothallic Thailand isolate (STE-U 682) and infertile Hawaiian isolates (STE-U 2534, 2535). Each clade received strong bootstrap support (100%). Furthermore, the Cy. spathiphylli complex was divided into two clusters containing the heterothallic (ATCC 44730, P86-0210, P87-0167, STE-U 2188), and homothallic (STE-U 1623, 1624, 1641) isolates. Both clusters had relatively high bootstrap support (77% and 82%), indicating two phylogenetically divergent lineages.

The branch and bound search of maximum parsimony for the histone data set also produced one single MPT. The histone gene tree (Fig. 3) evaluated by 1000 bootstrap replications in a heuristic search defined the same clades as those in the b-tubulin gene tree (Fig. 2). The heterothallic Cy. spathiphylli group received strong bootstrap support (100%). The major Cy. floridanum clade, however, collapsed. This resulted in three independent clades with strong bootstrap support (100%).

In accordance with the result of the partition homogeneity test, the joint alignment of ITS, b-tubulin and histone data sets were subjected to maximum parsimony analysis using the heuristic search with a 1000 random sequence input orders and the branch and bound search for an exact solution. Both approaches generated 2 MPTs. The resulting 2 MPTs from the heuristic search matched respectively with those from the branch and bound search. One of the MPTs was selected as the phylogenetic tree (Fig. 4) through the Kishino-Hasegawa likelihood test (data not shown) and evaluated with 1000 bootstrap replications in the branch and bound search for clade stability. The robustness of the branches of the tree were further tested by decay indices calculated using AutoDecay (Eriksson, 1998). Neighbor-joining and maximum-likelihood analyses were also performed in PAUP* for the joint alignment of ITS, b-tubulin and histone data sets, which produced an identical tree topology as in the phylogenetic tree (Fig. 4). In the phylogenetic tree, three clades were recognised in the Cy. floridanum complex representing: (1) the homothallic Cy. floridanum sensu stricto (ATCC 18882, ATCC18834, CBS 413.67, STE-U

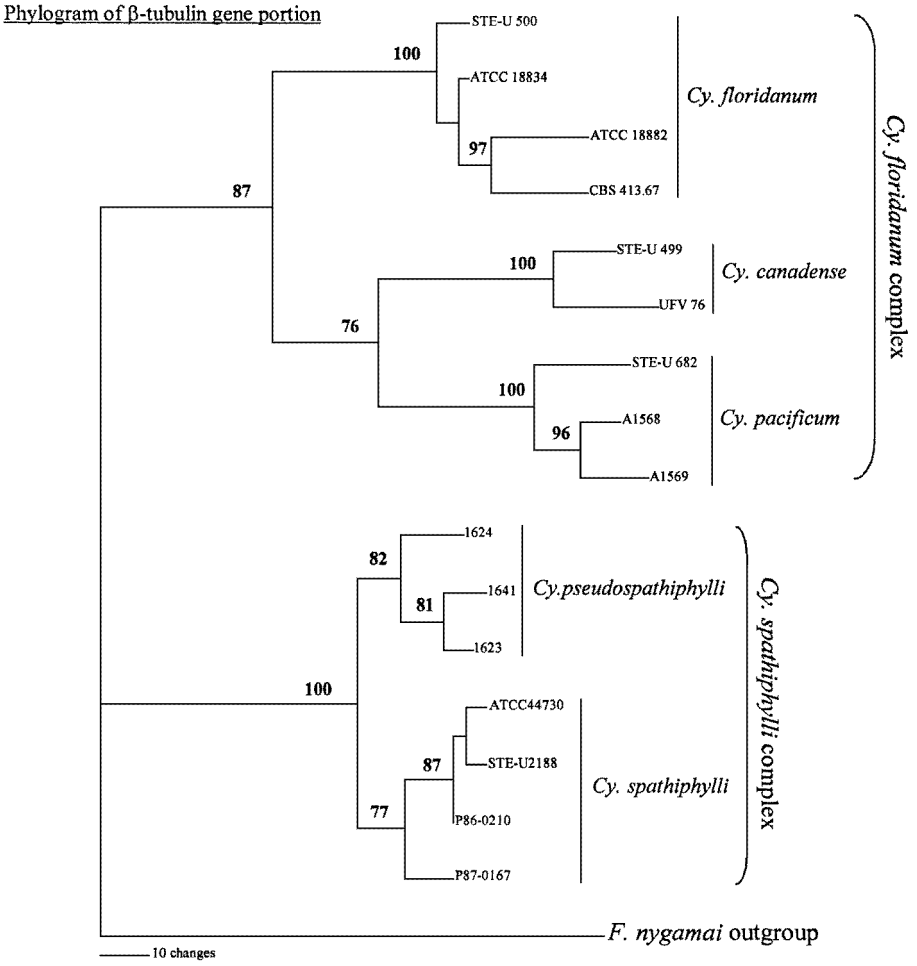


Fig. 2. The single most-parsimonious tree derived from the b-tubulin gene data set using the branch and bound search of maximum parsimony. TL = 555 steps, CI = 0.744, RI = 0.812, RC = 0.604, HI = 0.256. The values of 1000 bootstrap replicates are indicated.

500), (2) the heterothallic Canadian isolates (STE-U 499, UFV 76), and (3) the homothallic Thailand isolate (STE-U 682) and infertile Hawaiian isolates (STE-U 2534, 2535). All clades received strong bootstrap and decay indices support (100%/35, 100%/51 and 100%/33), respectively. The two intermediate nodes clumping the clades procured only low bootstrap and decay indices (62%/4 and 65%/4).

The major Cy. spathiphylli clade split into two clades representing the homothallic and heterothallic groups which respectively received high bootstrap support (93% and 97%) but relatively low decay indices (5 and 9). The intermediate node linking the two clades obtained strong bootstrap and decay indices support (100%/47).

Sexual compatibility

All isolates of Cy. floridanum (Group 1) proved to be homothallic. No isolates of the Canadian population (Cy. floridanum Group 2) could, however, be induced to form fertile progeny in culture. Although the homothallic Thailand isolate (STE-U 682) clustered with the Hawaiian isolates (STE-U 2534, 2535), the latter proved to be infertile. Isolates of Cy. spathiphylli proved to be heterothallic as reported earlier (EL-GHOLL et al., 1992; CROUS and PEERALLY, 1996). Isolates of the Cy. spathiphylli-like population from Ecuador, as well as their single ascospore progeny proved to be homothallic.

Morphological and cultural comparisons

Based on these results, three species are recognised in the Cy. floridanum and two species in the Cy. spathiphylli complexes, respectively. The new taxa described below are recognised as separate biological, morphological and phylogenetic species.

Cylindrocladium canadense J.C. Kang, Crous et Schoch, sp. nov. (Figs. 5, 13)

; Cylindrocladium floridanum Group 2 sensu Victor et al., Syst. Appl. Microbiol. 20: 268–285 (1997).

• Macroconidiophora: Stipes septatus, hyalinus, in vesiculam sphaeropedunculatam ad pyriforme 6–10 µm diam. terminans. Conidiophororum rami primarii non septati, vel raro 1-septati, 20–30 æ4–6 µm; rami secundarii non septati, 10–20 æ3–5 µm; rami tertiarii non septati, 10–15 æ3–5 µm. Phialides doliiformes ad reniformes, hyalinae, non septatae, 10–15 æ3–5 µm. Conidia cylindrica, hyalina, recta vel curvata, utrinque obtusa, 1-septata, (38–)48–55(–65) æ4(–5) µm.

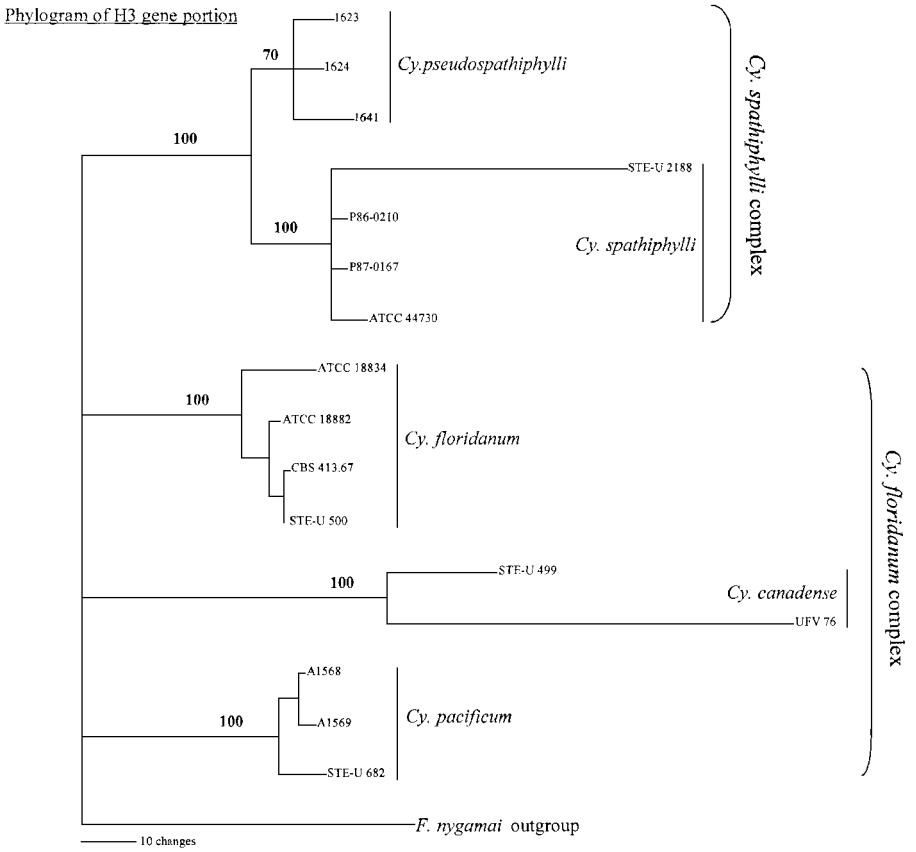


Fig. 3. The single MPT derived from the histone gene data set using the branch and bound search of maximum parsimony. TL = 485 steps, CI = 0.779, RI = 0.751, RC = 0.585, HI = 0.221.

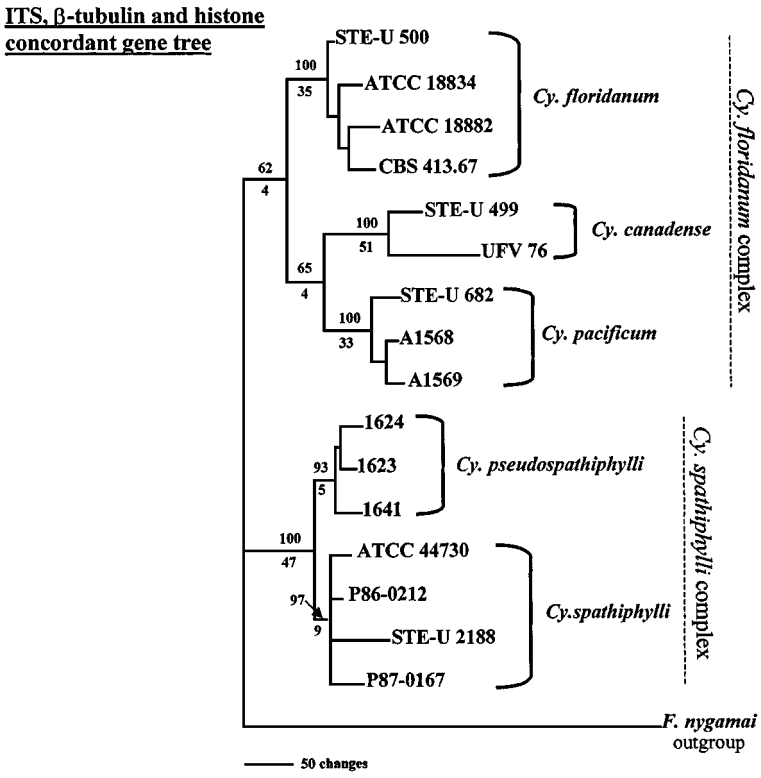
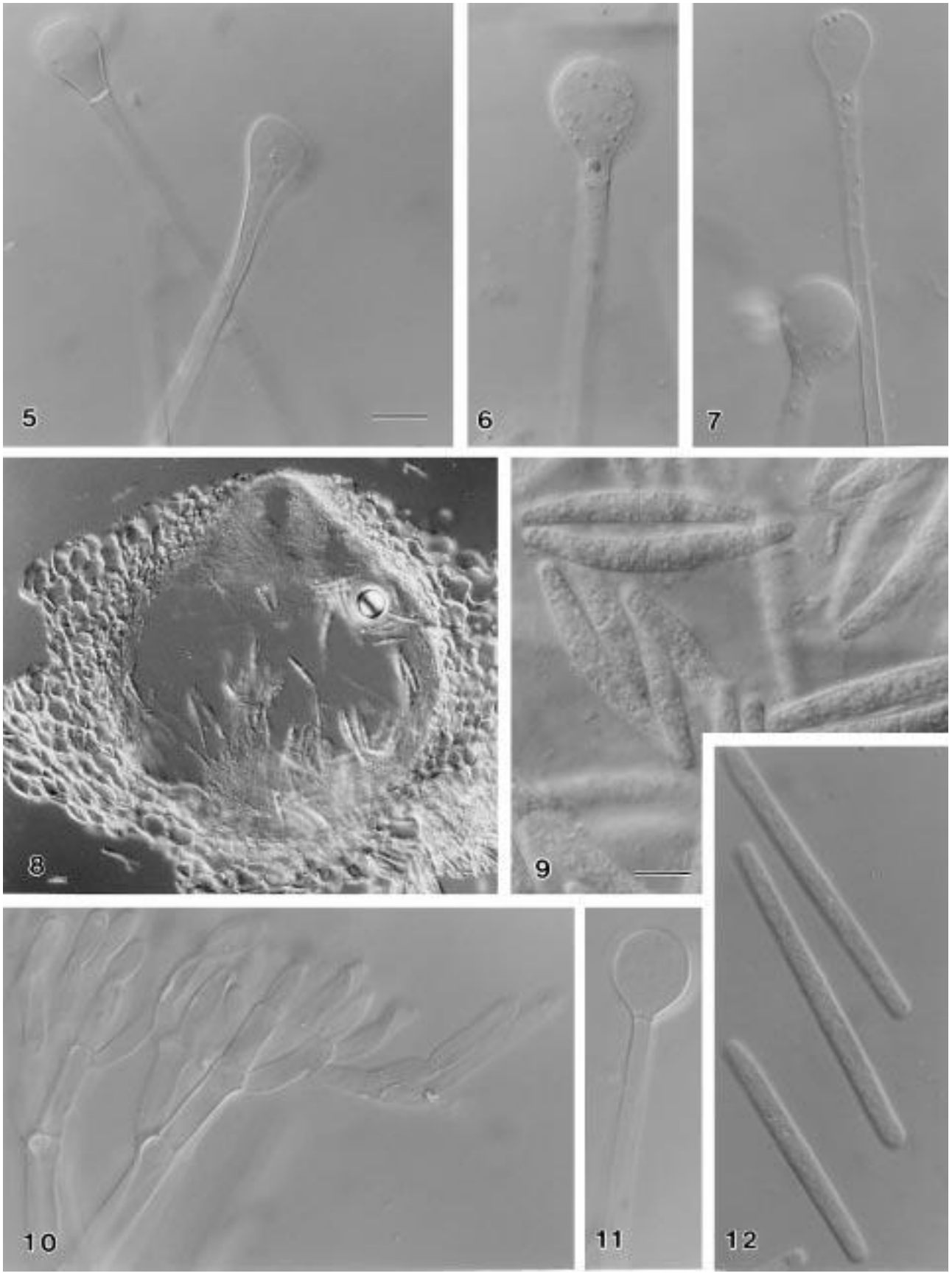


Fig. 4. One of two MPTs obtained from the joint alignment of ITS, b-tubulin and histone data sets using the heuristic search with a 1000 random sequence input orders and the branch and bound search of maximum parsimony. TL = 1863 steps, CI = 0.783, RI = 0.759, RC = 0.594, HI = 0.217. Bootstrap values obtained from 1000 replicates, and decay indices, are shown above and below the tree branches, respectively.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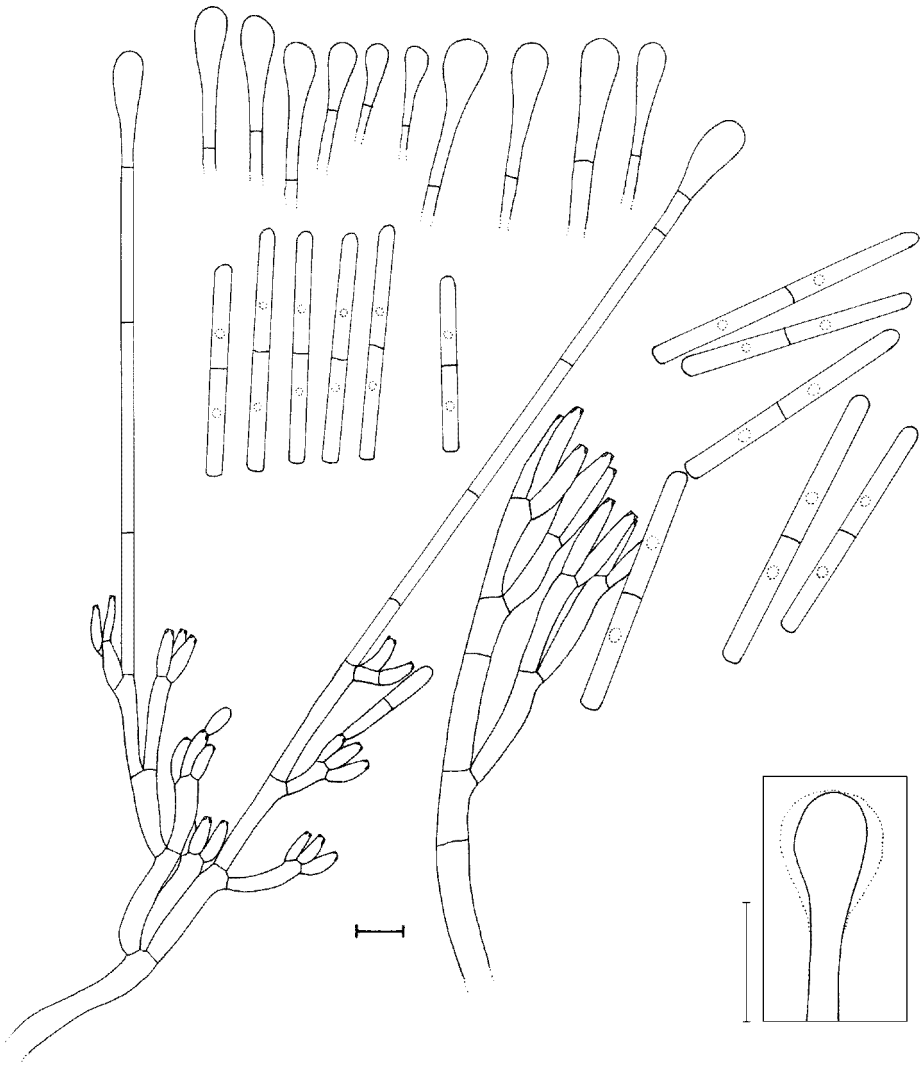

Fig. 13. Cylindrocladium canadense, conidiophores, conidia and vesicles. Range of variation in vesicle morphology indicated in box. Scale bars = 10 µm.

• Etymology: Refers to its geographic distribution, which is chiefly in Canada, north of the Great Lakes of North America.

• HOLOTYPE: Canada, Picea sp., 1992, S. Greifenhagen, PREM 57195 (holotype), STE-U 499 (culture ex-type).

• Macroconidiophores comprised of a stipe, a penicillate arrangement of fertile branches, a stipe extension, and a terminal vesicle. Stipe septate, hyaline, smooth, 50–80 æ 4–6 µm. Primary branches of conidiogenous apparatus aseptate or 1-septate, 20–30 æ4–6 µm; secondary branches aseptate, 10–20 æ 3–5 µm; tertiary branches aseptate, 10–15 æ 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 10–15 æ3–5 µm, apex with minute periclinal thickening and inconspicuous collarette; stipe extensions septate, straight to flexuous, 100–180 µm long, 3–4 µm wide at the apical septum, terminating in a pyriform to sphaeropedunculate vesicle, 6–10 µm diam. Conidia cylindrical, rounded at both ends, straight, (38–)48–55(–65) æ4(–5) µm, (mean = 50 æ 4 µm), 1-septate, lacking a visible abscission scar, held in cylindrical clusters by colorless slime. Megaconidiophores, microconidiophores and teleomorph unknown.

• Cultures: Colonies on MEA sienna, 13i (reverse); chlamydospores extensive, throughout medium, arranged in radiating chains; aerial mycelium extensive, dense, off-white; sporulation distributed throughout colony, but more concentrated in center.

• Cardinal temperatures for growth: Minimum above 10 °C, optimum 30 °C, maximum above 35 °C. This is a high temperature species, with medium sporulation on aerial mycelium.

Figs. 5–12. Species of Cylindrocladium and Calonectria.

Fig. 5. Vesicles of Cy. canadense.

Figs. 6, 7. Vesicles of Cy. pacificum.

Figs. 8–12. Calonectria pseudospathiphylli and its anamorph, Cy. pseudospathiphylli.

Fig. 8. Vertical section through a perithecium.

Fig. 9. Ascospores.

Fig. 10. Conidiogenous apparatus.

Fig. 11. Vesicle.

Fig. 12. Conidia. [Bars = 10 µm for Figs. 5–7, 8, 9–12].

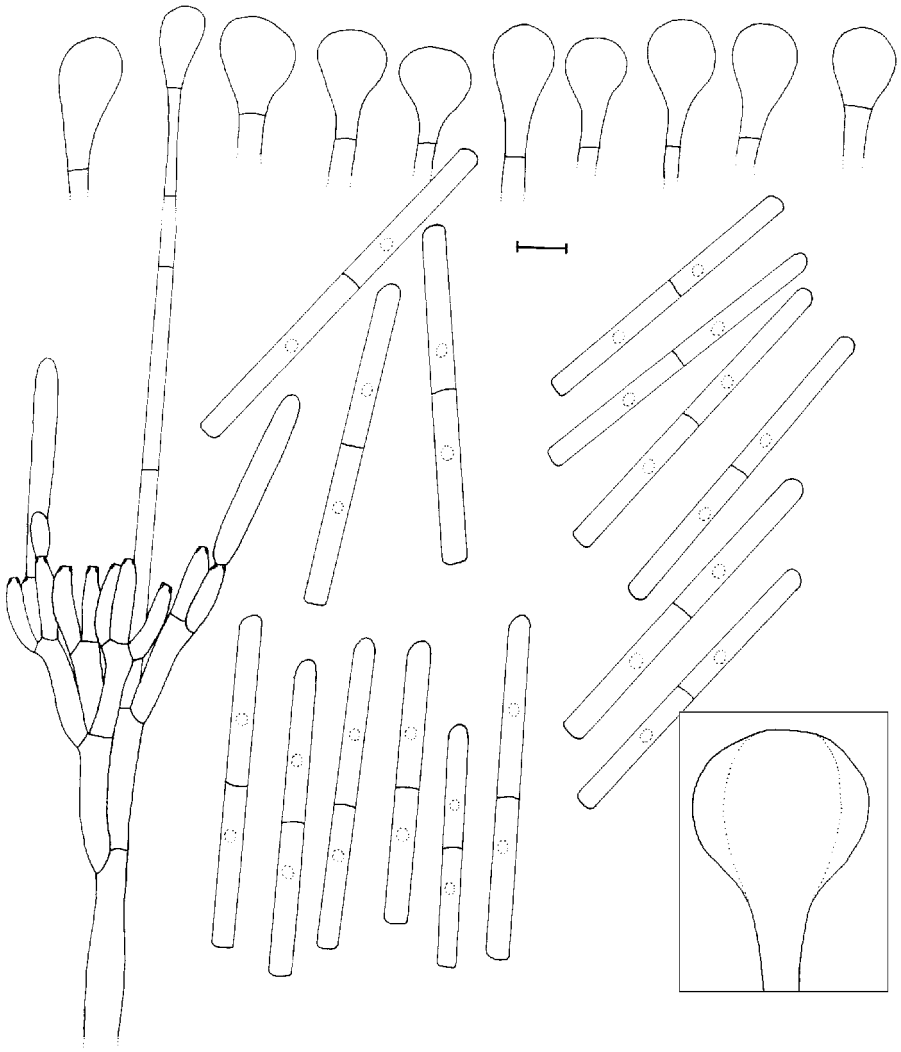

Fig. 14. Cylindrocladium pacificum, conidiophore, conidia and vesicles. Range of variation in vesicle morphology indicated in box. Scale bars = 10 µm.

• Substrate: Mimosa meansii, Picea spp., Pinus spp., Prunus sp., soil (VICTOR et al., 1997).
• Distribution: North America (Canada, but also U.S.A., predominantly above the great lakes), Brazil.

Cylindrocladium pacificum J.C. Kang, Crous et Schoch, sp. nov. (Figs. 6, 7, 14)
• Macroconidiophora: Stipes septatus, hyalinus, in vesiculam sphaeropedunculatam 7–15 µm diam. terminans. Conidiophororum rami primarii non septati, vel raro 1-septati, 20–30 æ 5–6 µm; rami secundarii non septati, 10–30 æ5–6 µm; rami tertiarii non septati, 12–20 æ3–4 µm. Phialides elongatae, doliiformes ad reniformes, hyalinae, non septatae, 12–20 æ3–4 µm. Conidia cylindrica, hyalina, recta vel curvata, utrinque obtusa, 1-septata, (40–)55–68(–75) æ4–5 µm.

Macroconidiophores comprised of a stipe, a penicillate arrangement of fertile branches, a stipe extension, and a terminal vesicle. Stipe septate, hyaline, smooth, 30–70 æ 6–7 µm. Conidiogenous apparatus 30–80 µm long, 20–60 µm wide; primary branches aseptate or 1-septate, 20–30 æ5–6 µm; secondary branches aseptate, 10–30 æ 5–6 µm; tertiary branches aseptate, 10–20 æ 5–6 µm, each terminal branch producing 2–4 phialides; phialides elongate doliiform to reniform, hyaline, aseptate, 12–20 æ3–4 µm, apex with minute periclinal thickening and inconspicuous collarette; stipe extensions septate, straight to flexuous, 150–250 µm long, 3–4 µm wide at apical septum, terminating in a sphaeropedunculate vesicle, 7–15 µm diam.; lateral stipe extensions also arising from terminal branches and phialides. Conidia cylindrical, rounded at both ends, straight, (40–)55–68(–75) æ 4–5 µm, (mean = 58 æ 4.5 µm), 1-septate, lacking a visible abscission scar, held in cylindrical clusters by colorless slime. Megaconidiophores, microconidiophores and teleomorph unknown.
• Etymology: Refers to its geographic distribution.
• HOLOTYPE: U.S.A. Hawaii, Araucaria heterophylla, 1987, M. Aragaki, PREM 57209 (holotype), A1568 = IMI 354528 = STE-U 2534 (culture ex-type).
• Cultures: Colonies luteous to sienna, 17b–15i (reverse); chlamydospores extensive, throughout medium, very dense, forming microsclerotia; aerial mycelium extensive, dense.

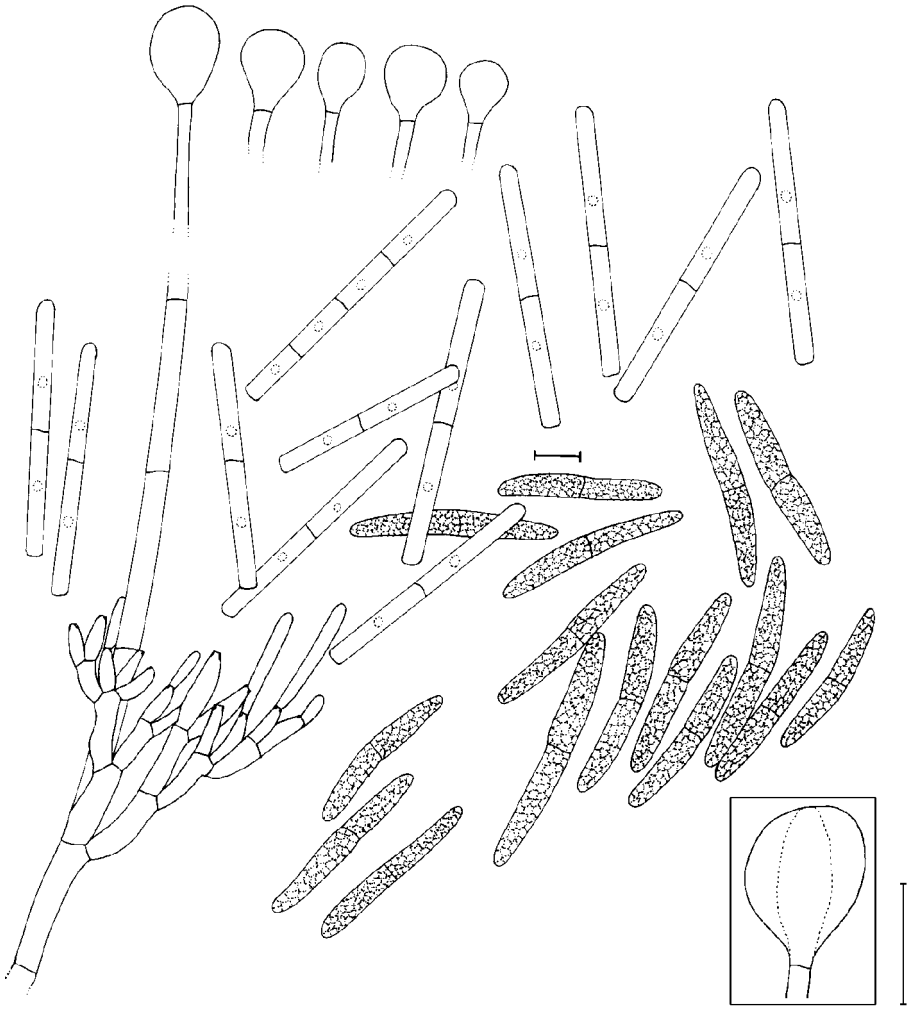

Fig. 15. Calonectria pseudospathiphylli and its anamorph, Cy. pseudospathiphylli. Conidiophore, conidia, vesicles and ascospores. Range of variation in vesicle morphology indicated in box. Scale bars = 10 µm.

• Cardinal temperatures for growth: Minimum above 10 °C, optimum 25–30 °C, maximum below 35 °C. This is a moderate temperature species, with medium sporulation on aerial mycelium throughout the colony.
• Substrate: Araucaria heterophylla, soil.
• Distribution: USA (Hawaii), Thailand.

Calonectria pseudospathiphylli J.C. Kang, Crous et Schoch, sp. nov. (Figs. 8–13, 15)

Anamorph. Cylindrocladium pseudospathiphylli J.C. Kang, Crous et Schoch, sp. nov.

Perithecia globosa ad subglobosa, 350–550 µm alta, 300–500 µm diam., crocea ad rubra, pariete exteriore verrucosa, ostiolo papillato. Asci clavati, in stipitem longum tenuem gradatim angustatae, 90–150 æ7–15 µm, 8-spori. Ascosporae hyalinae, fusoideae, 1(–3)-septatae, leviter ad septum constrictae, (30–)38–45(–55) æ5–6 µm. Conidiophororum filum septatum, hyalinum, 100–250 æ2.5–3.5 µm, in vesiculam sphaeropedunculatam ad ellipsoideam, 8–12 µm diam. terminans. Conidia cylindrica, hyalina, 1(–3)-septata, utrinque obtusa, (40–)47–55(–60) æ4–5 µm.
• Etymology: Refers to the morphological similarity with Cy. spathiphylli.
• HOLOTYPES: Ecuador, soil, Jun. 1997, M.J. Wingfield, PREM 57196 (type of Ca. pseudospathiphylli), PREM 57197 (type of Cy. pseudospathiphylli), STE-U 1641 (ex-type culture).

Perithecia solitary or in groups, orange to red under the dissection microscope, globose to subglobose, 350–550 µm high, 300–500 µm diam., turning red (KOH+); perithecial apex consisting of flattened, thick-walled hyphal elements with rounded tips forming a palisade, discontinuous with warty outer layer, gradually becoming thinner towards the ostiolar canal, and merging with the outer periphyses. Perithecia rough-walled, wall consisting of two thick-walled layers: outside layer of textura globulosa, 20–65 µm thick, becoming more compressed towards the inner layer of textura angularis, 10–15 µm thick, becoming thin-walled and hyaline towards center, outer cells in section 15–30 æ15–35 µm, inner cells 8–18 æ3–6 µm; perithecial base up to 200 µm wide, consisting of dark red, angular cells, merging with an erumpent stroma, cells of the outer wall layer continue into the pseudoparenchymatous cells of the erumpent stroma. Asci 8-spored when immature, becoming

4-spored when mature, clavate, 90–150 æ 7–15 µm, tapering into a long thin stalk. Ascospores aggregated in the upper third of the ascus when mounted in lactophenol, hyaline, guttulate, fusoid with rounded ends, straight to slightly curved, 1(–3)-septate, slightly constricted at the septa, (30–)38–45(–55) æ 5–6 µm (mean = 42 æ 5.5 µm). Macroconidiophores comprised of a stipe, a penicillate arrangement of fertile branches, a stipe extension, and a terminal vesicle. Stipe septate, hyaline, smooth, 100–350 æ 5–6 µm. Conidiogenous apparatus with primary branches that are aseptate or 1-septate, 20–35 æ 5–6 µm; secondary branches aseptate, 15–25 æ 5–7 µm; tertiary branches aseptate, 10–15 æ 4–5 µm; quaternary branches aseptate, 10–15 æ 3.5–4.5 µm, each terminal branch producing 2–4 phialides; phialides elongate doliiform to reniform, hyaline, aseptate, 10–18 æ 3–4.5 µm, apex with minute periclinal thickening and inconspicuous collarette; stipe extensions septate, straight to flexuous, 100–250 µm long, 2.5–3.5 µm wide at the apical septum, terminating in a sphaeropedunculate to ellipsoidal vesicle, 8–12 µm diam. Conidia cylindrical, rounded at both ends, straight, (40–)47–55(–60) æ 4–5 µm, (mean = 52 æ 4 µm), 1(–3)-septate, lacking a visible abscission scar, held in cylindrical clusters by colorless slime. Megaconidiophores and microconidiophores unknown.

• Cultures: Colonies umber in the center, 15m, sienna in the middle, 13i, and luteous in the outer circle, 17b (reverse); chlamydospores extensive, throughout medium, forming microsclerotia.

• Cardinal temperatures for growth: Minimum above 10 °C, optimum 25 °C, maximum below 35 °C. This is a moderate temperature species, with medium sporulation on aerial mycelium throughout the colony.

• Substrate: Soil.

• Distribution: Ecuador.

Discussion

Until recently the distinction of taxa in Cylindrocladium was chiefly based on morphological features. However, CROUS et al. (1993) integrated the biological species concept with that of the morphological concept in Cylindrocladium, and in so doing attempted to resolve the identity of reproductively isolated populations within accepted morphological species. The final integration of DNA phylogenetic data into this framework helped to further elucidate cryptic species that would otherwise not have been recognised (CROUS et al., 1999; SCHOCH et al., 1999).

Although earlier studies have also alluded to the variation occurring in Cy. floridanum (JENG et al., 1997; VICTOR et al., 1997), the present study is the first to address the delimitation of species entities in the Cy. floridanum and Cy. spathiphylli complexes by integrating the morphological, biological and phylogenetic species concepts.

The genealogy revealed by the molecular phylogeny of ITS, b-tubulin and histone genes, sexual compatibility and morphology indicates that three sibling species exist in the Cy. floridanum complex, and two in the Cy. spathiphylli complex. The three individual trees (Figs. 1–3) and the combined analysis based of the ITS, b-tubulin and histone gene data sets (Fig. 4) substantiate unambiguously the phylogeny of these taxa. Furthermore, the strong bootstrap and decay indices derived for the respective clades indicate the stable evolutionary status of these species.

The phylogeny of the two species in the Cy. spathiphylli complex is supported by the b-tubulin and histone gene data sets. The relatively lower bootstrap and decay index support (Figs. 2–4) potentially reflect a more recent divergence in the evolution of these lineages. Because of the unequal numbers of parsimony-informative characters, the values of ITS, b-tubulin and histone genes as phylogenetic tools to discover genealogy of microbial diversity vary. A previous phylogenetic study on species of Cylindrocladium (SCHOCH et al., 2000) reported the lower resolution power of ITS rDNA sequence due to the limited phylogenetically informative characters, which has once again been observed in this study. In spite of a potential alternative gene pool, the b-tubulin gene data, on the other hand, contained a much higher number of phylogenetically informative characters, enabling strong resolution at lower taxonomic levels. The histone gene was newly applied for the phylogeny of Cylindrocladium in the present study. In comparison with the other data sets, histone DNA sequences possessed a dominant number of phylogenetically informative characters, providing the highest phylogenetic resolution. This gene clearly separated the taxa in the Cy. floridanum, as well as Cy. spathiphylli complexes.

The phylogenetic species presently acknowledged in this complex can also be distinguished based on morphology and mating strategy. Perithecia of Ca. pseudospathiphylli are more globose in shape than the subglobose perithecia of Ca. spathiphylli. Furthermore, the former species and its progeny were shown to be homothallic, whereas Ca. spathiphylli is heterothallic. Ascospores of Ca. pseudospathiphylli are (30–)38–45(–55) æ 5–6 µm, and conidia (40–)47–55(–60) æ 4–5 µm, thus being shorter than those of Ca. spathiphylli [ascospores (22–)40–52 (–65) æ (3–)4.5–5.5(–7) µm, conidia (45–)65–80(–120) æ (5–)6(–7) µm]. These two taxa are therefore not only phlogenetically distinct, but also represent distinct morphological and biological species.

The three species presently known to occur in the Cy. floridanum complex all share the same vesicle morphology, and have relatively small, 1-septate conidia. Conidiophores of Cy. canadense tend to be less branched, and conidia slightly longer (38–)48–55(–65) æ 4(–5) µm, than those of Cy. floridanum (35–)45–50(–55) æ 3–4(–5) µm. Vesicles of Cy. canadense also tend to be more pyriform than those of Cy. floridanum, which are more sphaeropedunculate. Cy. pacificum also has sphaeropedunculate vesicles, thus more closely resembling Cy. floridanum. Its conidia, however, are the largest of the three, (40–)55–68(–75) æ 4–5 µm. The Hawaiian ex-type strain of Cy. pacificum was never observed to form a teleomorph in culture. It was surprising, therefore, when they clustered with an isolate from Thailand (STE-U 682), which is homothallic, and readily produces a teleomorph in culture morphologically similar to that of Ca. kyotensis. Morphologically the latter isolate also has shorter conidia (38–)45–55(–66) æ 4–5 µm than

other strains of Cy. pacificum. When its mating strategy and morphology is taken into account, the presence of this isolate in the Cy. pacificum clade is unexpected. One possibility is that this isolate resembles an outlier of the population, and that Cy. pacificum is morphologically more variable than accepted here, and that the ex-type strains have simply lost their ability to be sexual, which is not altogether unusual in Cylindrocladium. An alternative hypothesis, however, is that the Thailand isolate represents yet another sibling species closely related to Cy. pacificum, and that more isolates and other genes will have to be investigated to suitably resolve this issue.

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