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Cancellidium pinicola sp. nov. from *Pinus massoniana* and its phylogeny

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Abstract – *Cancellidium pinicola* sp. nov. is described with illustrations from leaf litter of *Pinus massoniana* collected in Hong Kong. The fungus sporulated on decaying pine needles incubating on the surface of agar plates, as black, shiny, fan-shaped, superficial multicellular conidia, comprising two wall layers. The morphology of the conidia are typical of *Cancellidium* and therefore a new species is established. Morphologically, the new species differs from *Cancellidium applanatum*, the only described species in this genus with respect to the conidial shape and color intensity. In order to investigate possible teleomorphic and phylogenetic relationships of this new taxon, two different regions of the ribosomal gene (18S rDNA and 28S rDNA) were sequenced and analyzed. Parsimony analyses indicate that the isolate may be closely related to other unitunicate ascomycetes especially *Podostroma*, *Hypocrea* and *Trichoderma* (order Hypocreales). Since the isolate was obtained from conidia transferred from the surface of baited plates however, it is equivocal whether this is a pure isolate of *C. pinicola*.

Anamorphic fungi / Hypocreales / phylogeny / pine needles / rDNA / systematics

INTRODUCTION

Cancellidium is a monotypic genus represented by *C. applanatum* and first described by Tubaki (1975) during a survey of aero-aquatic hyphomycetes in Japan. It was later found on submerged leaves of *Hevea* and *Dillenia suffruticosa* in Malaysia and on decaying leaves in Queensland (Webster and Davey, 1980; Shaw, 1994). It is common in freshwater (Goh, 1997; Fryar *et al*., 2004; Luo *et al*., 2004; Tsui and Hyde 2004). Conidia are dictyospores formed singly on conidiophores. They are strongly flattened, dark brown, shiny, obovate or obcordate (Tubaki, 1975) and internally contain chains of small cells (Shaw, 1994; Goh, 1997). To our knowledge, *Cancellidium applanatum* has not been recorded on pine needles (Farr *et al*., 2005). Fungal diversity studies in the South East Asia over the past decade have yielded numerous new hyphomycetous taxa (Zhao, et al. 2004; Somrithipol and Jones, 2005; Zhao and Zhang, 2005) Previous studies documenting fungal diversity on decaying pine needles have already shown that most fungi occurred on these substrates are anamorphic taxa (mostly hyphomycetes) (Tokumasu, 1996; Tokumasu *et al*., 1988, 1994, 1997; Tokumasu and Aoiki, 2002). The present study provides a morphological characterization of a new *Cancellidium* species. In addition, we also determine the phylogenetic relationships of our new taxon based on two sequence datasets (18S rDNA and 28S rDNA) to establish its affinities to other unitunicate ascomycetes.

MATERIALS AND METHODS

Morphological study

Fallen brown needles of *Pinus massoniana* were collected from the hillsides of Yung Shue O in Hong Kong. They were placed in Zip-lock bags and taken to the laboratory. The samples were treated using the washing method of Tokumasu (1994). They were first washed with 0.005% Aerozol OT solution (Di-iso-octyl sodium sulfosuccinate) using a vortical shaker at 300 oscillations per minute for one minute. The washing solution was then discarded and fresh solution added. This washing process was repeated five times, and then a further three times with sterilized water. Whole needles were then incubated individually on half-strength corn-meal agar plate (Tokumasu, 1994). Following incubation, isolates of *Cancellidium pinicola* were obtained by picking conidia from the surface of washed needles of *Pinus massoniana* and transferring them to fresh plates as outlined by Choi *et al.* (1999). Unfortunately, because of the large size of the conidia, it is uncertain whether the cultures grew from the *C. pinicola* conidia or contaminants stuck to the surface of the conidia. However we isolated mycelium growing out from the *Cancellidium* conidia several times and each time obtained the same type of colony. The morphology of specimens from the natural substrate was recorded. Conidia were transferred from the surface of baited plates onto three types of media (Malt Extract Agar, Water Agar and Potato Dextrose Agar). Colony description was recorded, however the cultures failed to produce any spores.

DNA extraction, amplification and sequencing

Cultures grown on Potato Dextrose Agar (PDA) medium was used for DNA extraction. Sources, designation and Genbank accession numbers used in this study are listed in Table 1. Total genomic DNA was extracted from mycelial cultures by using CTAB lysis buffer and phenol chloroform as outlined by Jeewon *et al.* (2003, 2004) and Cai *et al.* (2005). Partial sequences from two different regions of the rDNA molecule (characterised by different rates of evolution) were amplified. Primer pairs NS1 and NS4 as defined by White *et al.* (1990) was used to amplified part of the SSU of the rDNA (approximately 1200 nucleotides). Part of the LSU of the rDNA was amplified by PCR with primer pairs LROR and LRO5 as defined by Vilgalys and Hester (1990) (approximately 950 nucleotides). The amplification conditions were performed in a 50 µl reaction volume as follows: 10xPCR buffer, 0.2 µM dNTPs, 0.3 M of each primer, 1.5 mM $MgCl_2$, 1.5 units Taq polymerase, 0.1% Bovine Serum Albumin (BSA), 1% Polyvinylpyrrolidone (PVP), 10 ng DNA sample. PCR parameters for both regions was as follows: Initial denaturation 95ºC for 3 min, 29 cycles of 95ºC for 1 min, 52ºC for 50 sec, 72ºC for 1 min, final extension for 10 min. The PCR products were characterised via agarose gel electrophoresis on a TAE 1% agarose gel containing the staining agent ethidium bromide. Amplified products were purified using DNA purification kit (Amersham Biosciences GFX^{TM} PCR DNA and Gel Band Purification Kit) prior to automated sequencing (API Prism 3730, Applied Biosystems) at the Genome Research Centre (The University of Hong Kong).

Table 1. Fungi used in this study with their GenBank accession numbers

Taxa (18S, 28S)	*18S rDNA*	*28S rDNA*
Achaetomium strumarium		AY681170
Ambrosiella brunnea	AF348146	
Ambrosiella ips		AF282872
Ambrosiella macrospora		AF282873
Ambrosiella tingens		AF282871
Aniptodera chesapeakensis	U46870	
Aphysiostroma stercorarium	ASU32398	
Apiosporopsis carpinea	AF277110	
Ascovaginospora stellipala	U85087	
Bartalinia robillardoides		AF382366
Cancellidium pinicola (SSU)	DQ144049	
Cancellidium pinicola (LSU)		DQ144048
Catabotrys deciduum		AY346268
Chaetomium globosum	AY545725	AY545729
Cladobotryum obclavatum	AF049145	
Cladobotryum rubrobrunnescens		AF160228
Clohiesia corticola		AF132329
Coniochaeta ligniaria	AY198389	
Cryphonectria parasitica	AF277116	
Cryphonectria havanensis		AF408339
Diaporthe pustulata		AF408358
Didymostilbe echinofibrosa	AY489674	
Discula fraxinea	AF277138	
Discula quercina	AF277108	
Dryadomyces amasae	AY858660	
Haligena elaterophora		AY864846
Halosphaeria appendiculata		U46885
Halorosellinia oceanica	AY083803	
Hypocrea jecorina		AF510497
Hypocrea lutea	AB027338	
Hypocrea rufa		AY489726
Hypomyces chlorinigenus		AF213027
Lasiosphaeria ovina	AY083799	
Lepteutypa cupressi		AF382379
Leucostoma auerswaldii		AF408384
Lignincola laevis	LLU46873	U46890
Linocarpon pandanicola		AF452041
Linocarpon sp.		AF452042
Nais inornata	AF050482	
Nectria haematococca	AY489697	
Neurospora crassa		AF286411
Nimbospora effusa	U46877	
Nohea umiumi	U46878	U46893
Ophiodeira monosemeia		U46894
Ophiostoma africanum		AF221015
Ophiostoma piliferum	OPU20377	AF221625
Ophiostoma torulosum	AY497517	
Plagiostoma euphorbiae	AF277114	
Podostroma cordyceps	AY245667	
Rosellinia necatrix	AY083805	
Seimatosporium leptospermi		AF382373
Seynesia erumpens		AF279410
Sordaria fimicola	AY545724	AY545728
Sphaerostilbella aureonitens		AF160246
Thielavia cephalothecoides		AF286413
Trichoderma harzianum	AF548100	
Umbrinosphaeria caesariata		AF261069
Valsella salicis		AF408389
Xylaria acuta		AY544676
Xylaria hypoxylon	XHU20378	
Outgroups	*18S dataset*	*28S dataset*
Pleospora betae	U43466	
Pleospora herbarum		AF382386

Phylogenetic analysis

The sequences were assembled using Bioedit (Hall, 1999). BLAST search was performed for each consensus DNA sequences in GenBank. Fungal members from Halosphaeriales, Ophiostomatales, Sordariales and the Xylariales were included in the 18S and 28S datasets. *Pleospora betae* and *Pleospora herbarum* were used as the outgroup. Taxa used and their GenBank accession numbers are shown in Table 1. Multiple alignment was done in Bioedit (Hall, 1999) and Clustal X (Thompson *et al*., 1997). Phylogenetic analyses were conducted in *PAUP version 4.0b10 (Swofford, 2004). Sequences were edited manually prior to phylogenetic analysis. Ambiguous sequences at the start and the end were deleted and gaps adjusted so as to optimise alignment. Parsimony trees were obtained using heuristic searches. Single-position gaps were treated as missing data. Each homologous sequence position was treated as a discrete character with four possible unordered states (A, C, G or T) and unweighted parsimony (with a transition transversion ratio of 1: 1) were included in the parsimony analysis. Clade stability was assessed in a bootstrap analysis with 1000 replicates. Descriptive tree statistics (tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], homoplasy index [HI], and Log Likelihood [-ln L]) were calculated for all trees generated under different optimality criteria. Kishino-Hasegawa tests (Kishino and Hasegawa, 1989) and Templeton tests (Templeton, 1983), as implemented in PAUP*, were performed in order to determine whether trees were significantly different. Treeview (Page, 1996) was used to figure the trees.

TAXONOMY

Cancellidium pinicola S.Y. Yeung, R. Jeewon et K.D. Hyde, sp nov. (Figs 1a-h)

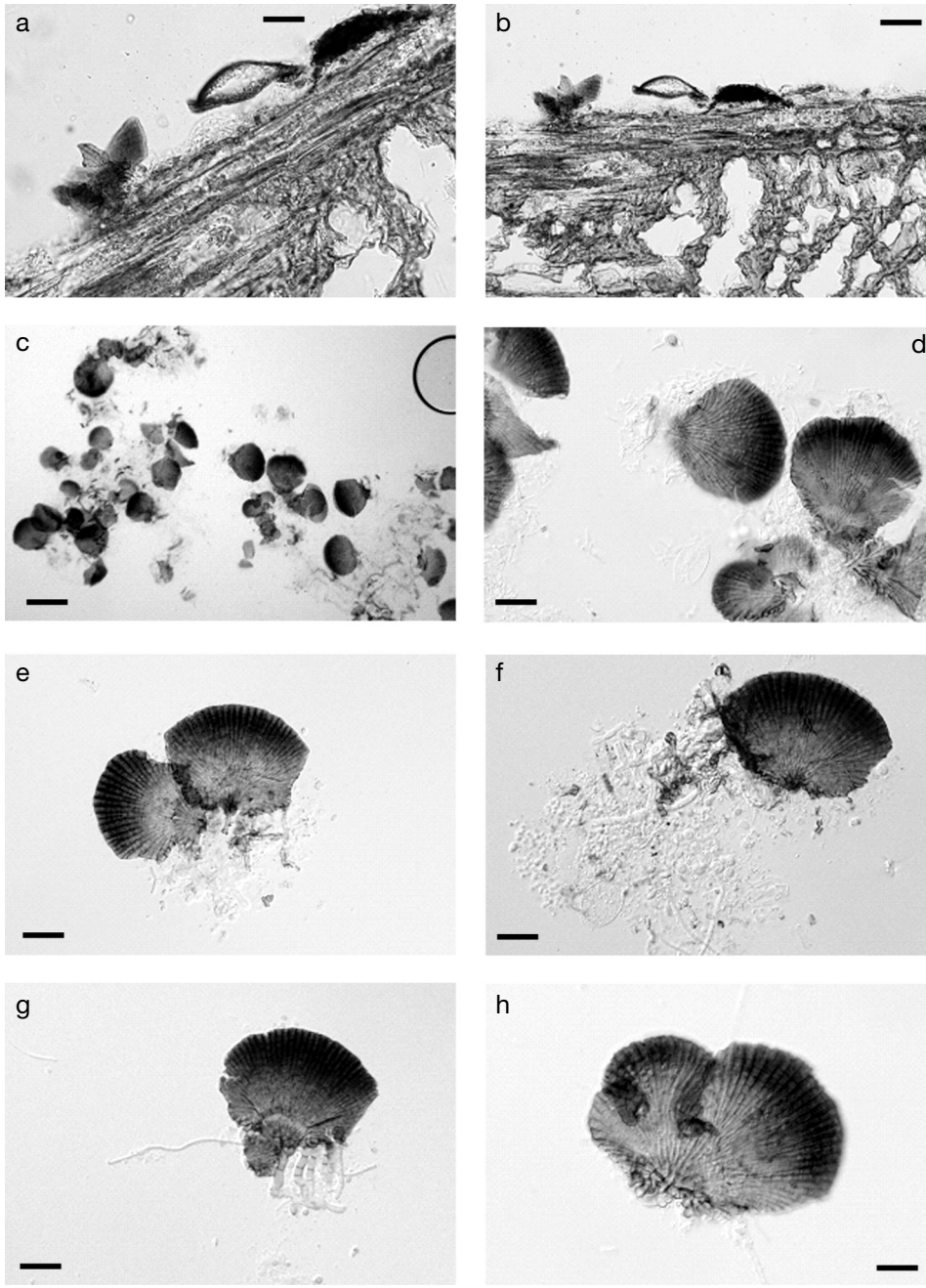
Coloniae substrati naturalis effusae, nigrae, nitentes. Mycelium substrati naturalis immersum vel superficiale; hyphae septatae, hyalinae vel subhyalinae, 2.5-3.75 µm crassum. Conidiophora micronematosa brevia vel absentia. Conidia dictyospora, composite ex apice hypharum, nitentia, valde applanata e lateribus, semicircularia ambitu; peroblata, et composite e seriebus ramorum septatorum ordinatorum lineis radiantibus e puncto affixo: 45-75 µm longa, 45-90 µm lata, 10-20 µm crassa.

Etymology: "*pinicola*" referring to the substrate *Pinus massoniana* where the fungus was found.

Holotype: China, Hong Kong SAR, New Territories, Yung Shue O, on partially decayed leaves of *Pinus massoniana*, 29 May 2003, S.Y. Yeung (HKU(M) 17167), extype living culture in HKUCC 10089.

Isotype: China, Hong Kong SAR, New Territories, Yung Shue O, on partially decayed leaves of *Pinus massoniana*, 29 May 2003, S.Y. Yeung HKU(M) 17168).

On natural substrate, colonies effuse, black and shiny. *Mycelium* immersed and superficial, composed of septate, subhyaline to hyaline, smooth-walled hyphae, 2.5-3.75 µm wide. *Conidiophores* micronematous, short (Figs 1f, g). *Conidia* dictyosporous, formed from the tips of multiple hyphal strands, strongly flattened, composed of many 20-30 parallel adherent rows of septate branches radiating from point of attachment, composed of two layers, 45-75 µm long, 45-90 µm wide and 10-20 µm thick (Figs 1a-h). Upper portion of the conidia dark;



Figs 1a-h. *Cancellidium pinicola* on *Pinus massoniana*. a-b. Section of conidia on host substrate. c-f. Conidia. g-h. Conidia with septate hyphae anchoring them to the substrate. Bars: a, b, d, e, f, g, h = 20 µm; c = 500 µm.

lower portion hyaline to subhyaline (Figs 1e, g, h). (Figs 1e, h), in cross-section peroblate (Figs 1a, b); mature conidia contain chains of small monilioid cells unbranched (Figs 1a, b, f).

Isolate from conidia on potato-dextrose agar (PDA) attained a diameter of 5.5 cm in 3 days at 23°C. Colonies appear as creamy white, velvety to cottony.Despite using different cultural media, one of the cultures produced any spores after 7 days.

DNA analysis

Small subunit (18S) dataset

This DNA matrix consists of 31 taxa with *Pleospora betae* as the outgroup. The dataset could be aligned confidently as there were no ambiguous regions. The final aligned dataset consisted of 976 characters, out of which 151 were parsimony informative (15%), 77 were parsimony uninformative and 748 were constant characters. Maximum Parsimony (MP) analysis treating gaps as missing state and equal weighting generated six trees which were topologically identical and not significantly different from each other. Tree length was 413 with a –lnL of 3884.550. A strict consensus tree was generated and showed in figure 2. It shows the relationships of *Cancellidium pinicola* with other Hypocreales members. Similar tree topologies were derived from the Neighbour Joining analyses (results not shown).

Large subunit (28S) dataset

This DNA matrix consists of 36 taxa with *Pleospora betae* as the outgroup. The final aligned dataset consisted of 837 characters, out of which 273 were parsimony informative which accounts for approximately 33%, 92 were parsimony uninformative and 472 were constant characters. Maximum Parsimony (MP) analysis treating gaps as missing state and equal weighting generated six trees which were topologically identical and not significantly different from each other. The new taxon was found to fit in the Hypocreales clade with 99% bootstrap confidence. Tree length was 1255 with a –lnL of 7013.403 A strict consensus tree was generated and showed in figure 3. It shows the relationships of our isolate of *Cancellidium pinicola* with other Hypocreales members. Similar results were obtained from Neighbour Joining analyses. Bootstrap values from 1000 replicates generated from Neighbour Joining analyses are shown below the nodes.

DISCUSSION

The new taxon possesses characters that fit within the generic concept of *Cancellidium* as proposed by Tubaki (1975). These include fan-shaped, black and shiny conidia which are multicellular i.e. dictyosporous with distinct rows of cells. Although the conidia share some characters with the genus *Mycoenterolobium* in having inconspicuous conidiophores (Goos, 1970), it is more similar to *Cancellidium* as its conidia have two wall layers with a different colour intensity between the upper and lower portion. Goh (1997) showed that the conidia of *C. applanatum* internally contain clusters of globose, monilioid cells which are formed in branched chains and enclosed by the double wall layers of the conidia. Similar cells were also present in *C. pinicola*. *Cancellidium pinicola* differs from

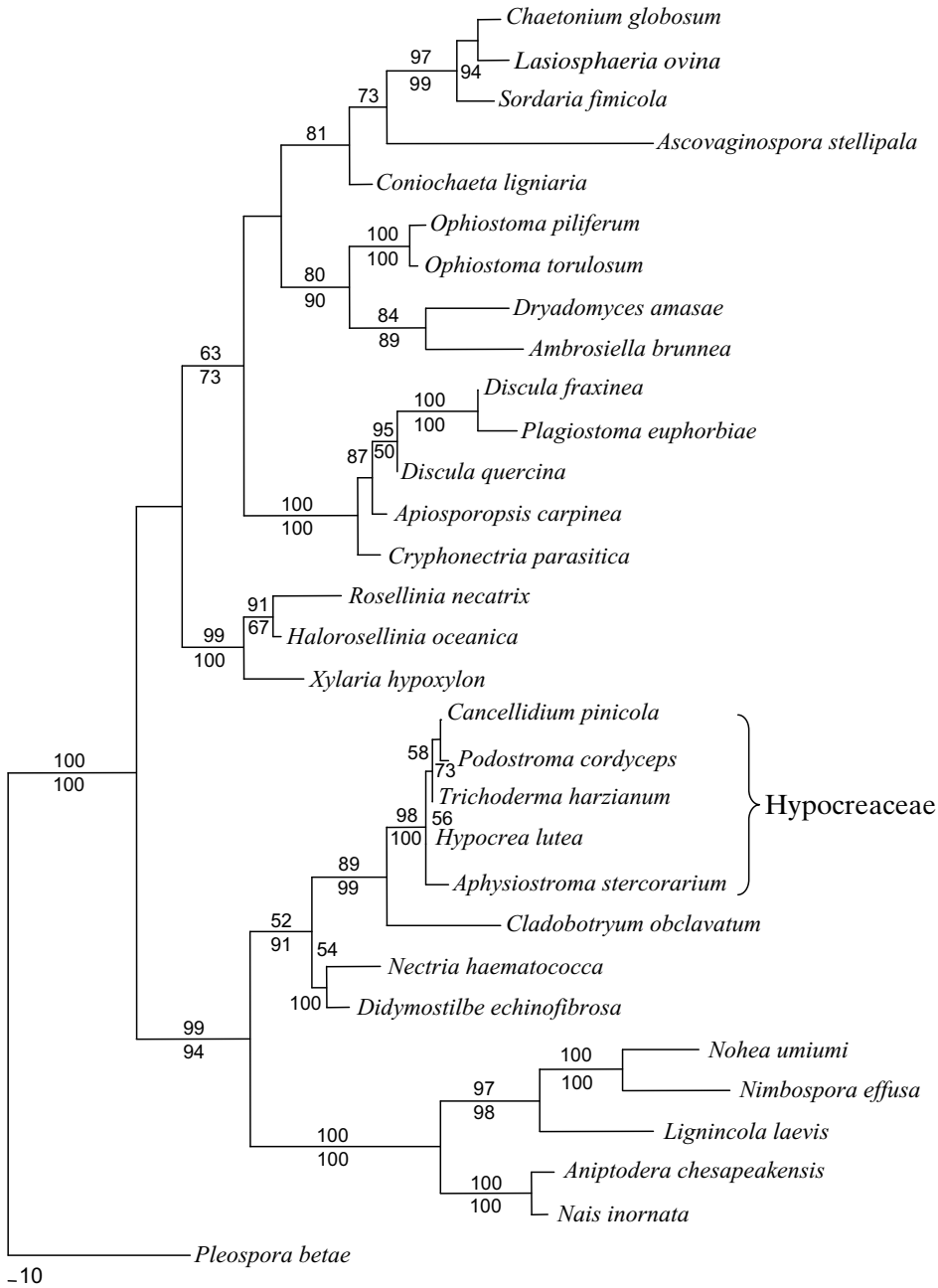


Fig. 2. Phylogenetic tree based on partial 18S DNA sequences. The tree was rooted with *Pleospora betae* and constructed under Maximum Parsimony criterion (unweighted parsimony). The number at each branch point represents percentage bootstrap support calculated from 1000 replicates. Bootstrap (BT) values of MP are shown above the branches while BT values of NJ are shown below the branches. Branch lengths are proportional to the number of nucleotide substitutions and are measured by scale bar.

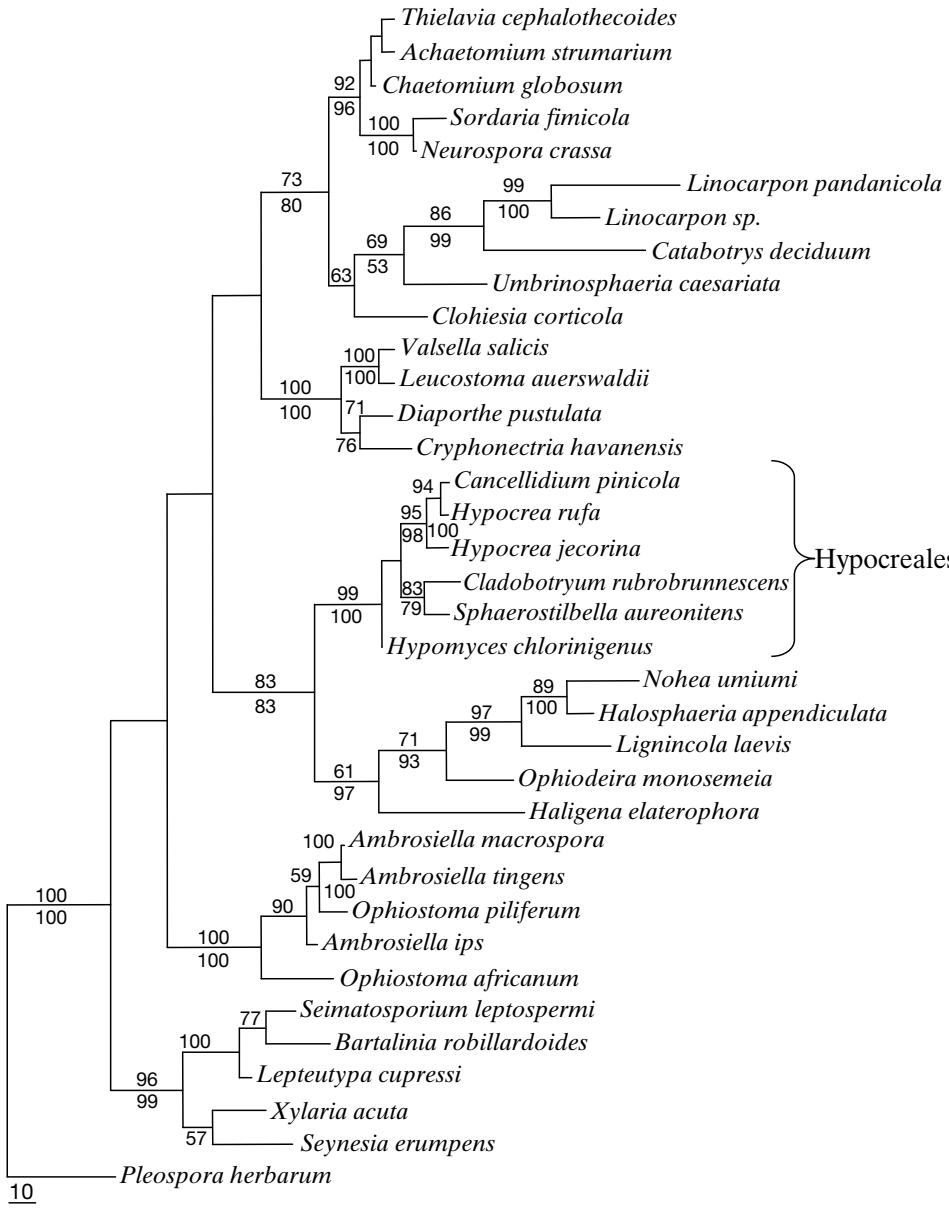


Fig. 3. Phylogenetic tree based on partial 28S DNA sequences. The tree was rooted with *Pleospora herbarum* and constructed under Maximum Parsimony criterion (unweighted parsimony). The number at each branch point represents percentage bootstrap support calculated from 1000 replicates. Bootstrap (BT) values of MP are shown above the branches while BT values of NJ are shown below the branches. Branch lengths are proportional to the number of nucleotide substitutions and are measured by scale bar.

C. applanatum (species examined: on submerged wood collected by C.K.M. Tsui on January 14, 1998 at Shing Mun Reservoir, New Territories, Hong Kong with HKU(M) numbers 8140 and 8159) in several aspects. The conidia of *C. pinicola* are, fan-shaped, wider at the top and narrower at the bottom, whereas. The upper portion is darker than the lower portion of the conidium while in *C. applanatum* the upper portion is lighter in colour. The conidia of *C. pinicola* are more transparent than those of *C. applanatum* as the small cells inside could be clearly seen under a light microscope. *Cancellidium applanatum* has a single distinct conidiophore whereas in *C. pinicola* the conidia arise from several strands of hyphae.

Since cultures of *C. pinicola* were obtained from conidia transferred from the surface of baited plates, where other fungi were present, it is equivocal whether this is a pure isolate of *C. pinicola*. Phylogenies generated using Maximum Parsimony and Maximum Likelihood from the two datasets (18S and 28S) gave similar results indicating that the isolate of *Cancellidium pinicola* we obtained is phylogenetically related to Hypocreales (Hypocreaceae members). The small subunit rDNA is a gene region which evolves relatively slowly (White *et al.*, 1990) and provided valuable systematic information about the familial placement of our isolate of *Cancellidium pinicola* at the ordinal and familial level. Based on blast search results, *Cancellidium pinicola* was highly similar to *Podostroma cordyceps* AY245667 and *Hypocrea rufa* AY489726 (99% sequence similarity). It nested in a clade with other hypocreaceous species (89% bootstrap confidence). The Hypocreaceae clade is connected to the clade with species from the families Nectriaceae and Bionectriaceae to form the Hypocreales clade (53% bootstrap support). The 28S rDNA sequence shared high similarity to *Hypocrea rufa* AY489726 (99% similarity) which form a clade with the Hypocreaceae species with 99% bootstrap support which further confirm the phylogenetic affinity of our isolate of *Cancellidium pinicola* to the Hypocreaceae. From the two datasets, the teleomorph of our isolate of *Cancellidium pinicola* might share close relationship with *Hypocrea* and *Podostroma*. It would be interesting to sequence *Cancellidium applanatum* to establish if it also belongs to the Hypocreaceae.

Hypocrealean fungi, as stated in Rehner and Samuels (1995) and Rossman (1996) are ubiquitous. From the morphological view, the number of anamorphs in the Hypocreales has increased considerably (Rossman, 2000). Anamorphs of hypocrealean fungi are usually morphologically distinctive (Rehner and Samuels, 1995) and it would not be surprising if *Cancellidium* was also an anamorph of the Hypoceales.

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REFERENCES

CAI L., JEEWON R. & HYDE K.D., 2005 — Phylogenetic evaluation and taxonomic revision of *Schizothecium* based on ribosomal DNA and protein coding genes. *Fungal Diversity* 19: 1-21.

CHOI Y.W., HYDE K.D. & HO W.W.H., 1999 — Single spore isolation of fungi. *Fungal Diversity* 3: 29-38.

GOOS R.D., 1970 — A new genus of the Hyphomycetes from Hawaii. *Mycologia* 63: 171-175.

FARR D.F., ROSSMAN A.Y., PALM M.E. & MCCRAY E.B., 2005 — *Fungal Databases*, Systematic Botany & Mycology Laboratory, ARS, USDA. Retrieved July 19, 2005, from http://nt.ars-grin.gov/fungaldatabases/.

FRYAR S.C., BOOTH W., DAVIES J., HODGKISS I.J. & HYDE K.D., 2004 — Distribution of fungi on wood in the Tutong River, Brunei. *Fungal Diversity* 17: 17-38.

GOH T.K., 1997 — *Tropical Freshwater Hyphomycetes. In* Biodiversity of tropical microfungi (ed. K. D. Hyde) Hong Kong University Press Hong Kong: 189-227.

HALL T.A., 1999 — Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.

JEEWON R., LIEW E.C.Y., SIMPSON J.A., HODGKISS I.J AND HYDE K.D., 2003 — Phylogenetic significance of morphological characters in the taxonomy of *Pestalotiopsis* species. *Molecular Phylogenetics and Evolution* 27: 372-383.

JEEWON R., LIEW E.C.Y. & HYDE K.D., 2004 — Phylogenetic evaluation of species nomenclature of *Pestalotiopsis* in relation to host association. *Fungal Diversity* 17: 39-55.

KISHINO H. & HASEGAWA M., 1989 — Evaluation of the maximum likelihood model estimates of the evolutionary tree topologies from sequence data and the branching order in *Homonoidea. Journal of Molecular Evolution* 29: 170-179.

LUO J., YIN J.F., CAI L., ZHANG K.Q. & HYDE K.D., 2004 — Freshwater fungi in Lake Dianchi a heavily polluted lake in Yunnan China. *Fungal Diversity* 16: 93-112.

PAGE R.D.M., 1996 — Treeview: An application to display phylogenetic trees on personal computers. *Computer Applications in Biosciences* 12: 357-358.

REHNER S.A. & SAMUELS G.J., 1995 — Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. *Canadian Journal of Botany* 73: S816-S823.

ROSSMAN A.Y., 1996 — Morphological and molecular perspectives on systematics of the Hypocreales. *Mycologia* 88: 1-19.

ROSSMAN A.Y., 2000 — Towards monophyletic genera in the holomorphic Hypocreales. *Studies in Mycology* 45: 27-34.

SHAW D.E., 1994 — The aero-aquatic fungus *Cancellidium applanatum* K. Tubaki in Queensland. *Mycologist* 8: 162-163.

SOMRITHIPOL S. & JONES E.B.G., 2005 — An addition to the hyphomycete genus *Melanographium* from Thailand. *Fungal Diversity* 19: 137-144.

SWOFFORD D.L., 2004 — *PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods)*. Version 4.0b10. Sinauer Associates Sunderland Massachusetts USA.

TEMPLETON A.R., 1983 — Phylogenetic inference from restriction endonuclease cleavage sites maps with particular reference to the evolution of humans and the apes. *Evolution* 37: 269-285.

THOMPSON J.D., GIBSON T.J., PLEWNIAK F., JEANMOUGIN F. & HIGGIN S.D.G., 1997 — The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876-4882.

TOKUMASU S., 1996 — Mycofloral succession on *Pinus densiflora* needles on a moder site. *Mycoscience* 37: 313-321.

ZHAO G.Z. & ZHANG T.Y., 2005 — Notes on dictyosporous hyphomycetes from China VIII. The genus *Nimbya. Fungal Diversity* 19: 201-215.

ZHAO R., YANG, Y. & ZHAO G. 2004 — *Pseudolachnella vermospora* sp. nov from *Yushania vigens* in China. *Fungal Diversity* 15: 255-260.