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Evolutionary relationships between aquatic anamorphs and teleomorphs: *Tricladium* and *Varicosporium*

Jinx CAMPBELL[a,*], *Ludmila MARVANOVÁ*[b], *Vladislav GULIS*[c]

[a]*Department of Coastal Sciences, University of Southern Mississippi, Ocean Springs, MS 39564, USA*
[b]*Czech Collection of Microorganisms, Institute of Experimental Biology, Faculty of Science, Masaryk University, Tvrdého 14, 602 00 Brno, Czech Republic*
[c]*Department of Biology, Coastal Carolina University, Conway, SC 29528-6054, USA*

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ABSTRACT

Tricladium, with 21 accepted species, is the largest genus of aquatic hyphomycetes. It encompasses species with dematiaceous as well as mucedinaceous colonies. Conidiogenesis is thalloblastic; conidiogenous cells proliferate percurrently or sympodially. Conidia have typically two alternate primary lateral branches. *Fontanospora* and *Variocladium* are segregates of *Tricladium*, differing by conidial branching. *Varicosporium* comprises nine species, one not well known. Conidiogenesis is blastic or thalloblastic, conidiogenous cells proliferate sympodially or are determinate; conidia regularly produce primary and secondary branches and often fragment into part conidia. Molecular analyses on the 28S rDNA of 86 isolates, including 16 species of *Tricladium*, five species of *Varicosporium*, two species of *Fontanospora* and one species of *Variocladium*, place these hyphomycetes within *Helotiales*. *Tricladium* is polyphyletic and placed in six clades; *Varicosporium* is polyphyletic and placed in three clades; *Fontanospora* is polyphyletic within a single clade. *Variocladium* is placed with poor support as a sister taxon to *Varicosporium giganteum*, *Hymenoscyphus scutula* and *Torrendiella eucalypti*.

Introduction

Previously published results, based on teleomorph–anamorph relationships and on molecular analyses of various genes of rDNA, place the ascomycetous aquatic hyphomycetes in the subphylum *Pezizomycotina* in five classes, *viz Dothideomycetes*, *Leotiomycetes*, *Orbiliomycetes*, *Pezizomycetes* and *Sordariomycetes*, and the basidiomycetous aquatic hyphomycetes in two classes of *Basidiomycota*, *viz Urediniomycetes* and *Hymenomycetes* (Marvanová 2002, 2007). In the proposed new classification of fungi (Hibbett *et al.* 2007) they appear in the subkingdom *Dicarya*; the aquatic ascomycetous members (66 taxa) are distributed in the same classes as mentioned above. Most of them (42) show affinity to *Leotiomycetes*, eight to *Orbiliomycetes*, seven to *Sordariomycetes*, eight to *Dothideomycetes* and one to *Pezizomycetes*.

This contribution continues our study of phylogenetic relationships of aquatic hyphomycetes. Previously (Campbell *et al.* 2006), using molecular analyses of 28S rDNA, we have shown that species of *Lemonniera*, *Margaritispora* and *Goniopila* support the hypothesis of convergent evolution of the asexual propagules in aquatic hyphomycetes (Ingold 1975), very probably due to their specific adaptation to the life in a lotic water environment. Species of *Lemonniera*, a morphologically relatively homogeneous holoanamorphic taxon with phialidic conidiogenesis, appeared to belong to two quite distinct clades (*Leotiomycetes* and *Dothideomycetes*) of ascomycetes.

* Corresponding author. Tel.: +1 228 818 8878; fax: +1 228 872 4264.
E-mail address: jinx.campbell@usm.edu
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This time we have chosen two morphologically heterogeneous genera with thalloblastic (Hennebert & Sutton 1994) or blastic conidiogenesis: *Tricladium* (with some segregates) and *Varicosporium*. Conidia of both are ''open branching systems'' in the sense of Kendrick (2003), and can be understood as modified hyphae with the function of propagules (Descals 1985). The inter-generic differences are not quite clear; there are species whose classification in one or the other genera is debatable (e.g. *Tricladium indicum*, *Tricladium terrestre*, *Varicosporium tricladiiforme*, and the anamorph of *Hymenoscyphus varicosporoides*).

Tricladium, with 21 recognized species, is the largest anamorph genus among aquatic hyphomycetes. It is based on *Tricladium splendens* (Ingold 1942). The generic characters are branched conidia, typically consisting of an axis and two broadly divergent primary alternate branches, developing in acropetal succession. Conidiogenesis is thalloblastic, i.e. the conidial axis during its development is integrated with the conidiogenous cell. The species are delineated on: (1) the degree of conidiophore branching (from simple to profusely branched); (2) the conidiogenous cell proliferation (percurrent, sympodial or absent); (3) the shape of the conidial axis and branches (parallel-walled, tapering distally, nearly straight, curved or geniculate, apices rounded or acute, branch insertion constricted to varying extent or unconstricted); and (4) the presence of a parabasal axial appendage before conidial secession. Colonies may be mucedinaceous or dematiaceous. Some species of *Tricladium* produce occasional secondary branches in culture, but it was described as a specific character for *T. terrestre* as this character also occurs in specimens in nature (Park 1974).

Tricladium is morphologically heterogeneous. With an increasing number of species, some also with more than two primaries and with secondary branches, it became difficult to define clearly its scope and to delimit them against similar taxa. To reduce the morphological heterogeneity there were several attempts to segregate some species from *Tricladium* into new genera. Iqbal (1974) erected *Scorpiosporium*, based on *Scorpiosporium minutum*, where he also included *Tricladium angulatum*. The main distinguishing characters were the 'scorpioid' (geniculate) conidial axis and unconstricted branch insertion. However, these features are not always linked (cf. also Ando & Kawamoto 1985); there are species with geniculate axis and constricted branch insertion (e.g. *Tricladium patulum*) and, moreover, the proliferations of the conidiogenous cells are percurrent in *S. minutum*, but sympodial in *T. angulatum*. Therefore Marvanová & Descals (1996) recombined *S. minutum* in *Tricladium*.

Dyko (1978) segregated *Tricladium eccentricum* into a new genus, *Fontanospora*, together with a new species *Fontanospora alternibrachiata*. The main distinguishing features from *Tricladium* are the median constriction of the conidial axis and two branches, inserted closely to each other, one below and one above this constriction.

Variocladium was published by Descals *et al.* (1998) to accommodate two similar species, *Tricladium giganteum* and *Scorpiosporium rangiferinum*, with large conidia of variable branching pattern (alternate and opposite primaries, and also secondaries appearing *in situ*) and acute distal ends. The conidiogenous cells are percurrently proliferating in the former and determinate in the latter. A spermatial state resembling *Phialocephala dimorphospora* was reported in *Variocladium giganteum* by Willoughby & Minshall (1975) as well as in *Variocladium rangiferinum* (Descals & Webster 1982).

Three *Tricladium* species have known teleomorphs, all classified in *Helotiales*: *Hymenoscyphus splendens* (*Helotiaceae*) is the teleomorph of *T. splendens* (Abdullah *et al.* 1981), *Hydrocina chaetocladia* (*Hyaloscyphaceae*) is the teleomorph of *Tricladium chaetocladium* (Webster *et al.* 1991), and *Cudoniella indica* (*Helotiaceae*) was described as the teleomorph of *Tricladium indicum* isolate from South Africa (Webster *et al.* 1995), but the conspecificity of Webster's isolate with the type of this species described from Himalaya (India) was doubted by Sivichai *et al.* (2003, see below). Phialidic andromorphs have been described in *T. chaetocladium*, *Tricladium curvisporum*, *Tricladium minutum*, *Tricladium obesum*, *Tricladium robustum*, *T. splendens* and *T. terrestre*.

Varicosporium is based on *Varicosporium elodeae* (Kegel 1906). Nine species are accepted at present, one of them not well known and by some authors classified in *Tricladium* (Sivichai *et al.* 2003). *Varicosporium* is characterized by branched conidia with rarely nearly straight, but often variously curved conidial axis and by regularly formed, diverging primary and secondary (in some species also of higher order) conidial branches. Unlike in *Tricladium*, the conidia have a strong tendency to fragment into simpler part conidia, often similar to those of a *Tricladium*. Conidiogenesis in *V. elodeae* is blastic, whereas it is thalloblastic in the rest of *Varicosporium* species. There is no species of *Varicosporium* with percurrent proliferation of conidiogenous cells known at present. In *V. elodeae* the conidiogenous cells do not elongate after having produced the first conidium and the subsequent conidia arise retrogressively (or randomly) down the conidiophore. In the rest of the species there are sympodial elongations or no growth of the conidiogenous cells after release of the first conidium. The species are distinguished by the curvature of the conidial elements (nearly straight, arcuate, helical or geniculate) by the morphology of conidial elements (parallel-walled, subulate, slightly clavate), by the shapes of apices (rounded or acute) and by branch insertion (constricted or unconstricted).

The only published teleomorph in *Varicosporium* is *H. varicosporoides*, but the classification of the anamorph in *Varicosporium* was doubted by Sivichai *et al.* (2003) and Boonyuen *et al.* (2006). Marvanová (unpubl.) has obtained minute apothecia of a leotiomycetan discomycete in a mating experiment with two isolates of *V. giganteum* from Canada. A phialidic andromorph is known in this species (Crane 1968) and was discovered in *Varicosporium scoparium* (Marvanová unpubl.).

The aims of this study were: 1) to test the hypothesis that the morphologically defined taxa are supported by molecular analyses of LSU rDNA nucleotide sequences; 2) to establish phylogenetic relationships of the anamorphic species to ascomycete clades; 3) to establish the relationships of the pleomorphic aquatic taxa with their terrestrial relatives.

Materials and methods

Collection, isolation and characterisation

Collections were made from streams in Europe and North America. A loopful of fresh foam was streaked onto a microscope slide coated with a thin layer of malt extract agar

(0.1–2 % MEA plus 100 mg L^{-1} chloramphenicol or penicillin/streptomycin [Sigma, St. Louis, MO]) and kept at 10–15 °C. When isolated from submerged plant litter (USA isolates), material was first incubated in sterile distilled water in Petri dishes to induce sporulation and then suspended conidia were transferred onto 0.1 % MEA with 200 mg L^{-1} of streptomycin and 200 000 units/L of penicillin G. After 24–48 h germinating conidia were transferred to MEA plates supplemented with antibiotics and incubated at 10–18 °C (Marvanová & Bärlocher 1998; Marvanová & Gulis 2000). Cultures were checked for bacterial and fungal contaminants, subcultured to MEA plates and incubated at 15 °C. All isolates were monoconidial.

DNA extraction, sequencing and cladistic analysis

Mycelia were harvested directly from MEA plates, incubated with 200 units of lyticase at 30 °C for 4 h, and ground at hourly intervals during incubation using a micropestle. RNaseA (40 mg) was added and incubated for 20 min at 65 °C and genomic DNA extracted using Qiagen's DNeasy Plant Mini Kit (Qiagen. 2004) following the procedure by Raja *et al.* (2003). The 5′ end of the 28S ribosomal gene was amplified with Taq PCR Master Mix Kit (Qiagen. 2002a) using fungal primers LROR (Bunyard *et al.* 1994) and LR6 (Vilgalys & Hester 1990). The PCR products were cleaned up with Qiaquick PCR Purification Kit (Qiagen. 2002b) and sequenced directly. Sequences (Table 1) were aligned with published sequence data (Table 2) using Clustal X (Thompson *et al.* 1997), and then refined manually in Se-Al (Rambaut 1996).

The rationale for the taxon sampling followed Campbell *et al.* (2006). Additionally, the sequences derived in this study were entered into BLAST, NCBI (Altschul *et al.* 1997) to help identify sequence similarity to other taxa and to determine which orders and families should be included in the phylogenetic analyses.

Bayesian Metropolis coupled Markov chain Monte Carlo (B-MCMCMC) analyses were performed with the general time reversible model of substitution model (Rodriguez *et al.* 1990) with invariant sites and gamma distribution ($GTR + I + \Gamma$) using MrBayes 3.0 (Huelsenbeck & Ronquist 2001): searches were conducted for a total of 2 000 000 generations with phylogenetic trees sampled every 100 generations. Three independent B-MCMCMC analyses were conducted to verify likelihood convergence and burn-in parameter. Out of 20 001 resulting trees from each analysis, the initial 3111 trees were identified as burn-in prior to the convergence of likelihoods and were excluded from post-run analyses. The majority rule consensus tree from 16 890 trees was generated with average branch lengths (Fig 1).

Maximum parsimony (MP) analyses were performed using PAUP*4.0b 10 (Swofford 2002). A two-step search approach was employed in an attempt to avoid local optima (Olmstead *et al.* 1993). Step-one consisted of 100 heuristic replicates with random starting trees, random stepwise addition and tree-bisection–reconnection branch swapping with MulTrees off and a maximum of two trees saved per replicate. All of the shortest trees from these initial runs were saved and then used as starting trees for the second step, which consisted of searches with MulTrees on and the maximum number of trees set to 10 000. Gaps were treated as missing data.

Nodal support was assessed by non-parametric bootstrapping and Bayesian posterior probabilities. Bootstrap values (Felsenstein 1985) were calculated from 1000 replications using a heuristic search on 100 replicates with random starting trees, random stepwise addition and MulTrees off. Posterior probabilities were calculated from the majority rule consensus tree of the 20 000 trees from the Bayesian analyses, with the initial 3111 trees excluded as burn in prior to the convergence of likelihoods.

Results and discussion

Initial analyses were performed on ~250 species with representatives from 17 orders of ascomycetes, and with basidiomycetes as outgroup taxa (data not shown). All species of *Tricladium* (16), *Fontanospora* (2), *Variocladium* (1) and *Varicosporium* (5) treated in this study are placed among the *Leotiomycetes*. These placements were also supported in the BLAST searches. Further analyses were run with 86 taxa, including our 45 isolates; members of the *Pezizomycetes* were used as outgroup taxa.

The Bayesian tree (Fig 1) indicates that *Tricladium* is polyphyletic and placed in six clades; *Varicosporium* is polyphyletic and placed in three clades; *Fontanospora* is polyphyletic within a single clade; *Variocladium* is placed with poor support as a sister taxon to *Varicosporium giganteum*, *Hymenoscyphus scutula* and *Torrendiella eucalypti*.

The first group (Figs 1, 2) includes *Tricladium* species with dark colonies (*Tricladium* clade 1). These five species (*Tricladium castaneicola*, *Tricladium indicum*, *Tricladium obesum*, *Tricladium splendens* and *Tricladium terrestre*) form a relatively homogeneous group from the point of colony color and of conidial morphology. They all have dark colonies; conidiophores in culture are mostly lateral, relatively short, often unbranched (except in *T. splendens*, where they typically consist of a stipe with acrotonous branches); conidiogenous cell proliferation is percurrent (in *T. splendens* the type of the genus, and in *T. obesum*), sympodial (in *T. castaneicola* and *T. terrestre*) or they are determinate (in *T. indicum*); conidia are medium sized to large (span ca. 100–200 (400) μm, smaller in *T. obesum*), and the conidial elements are mostly 5–11 μm wide (except in *T. castaneicola*); secondary branches are occasional or regularly present (in *T. terrestre*), branch insertion is constricted to varying extent; phialidic andromorph is known in *T. obesum* (Marvanová 2004), *T. splendens* and *T. terrestre* (Descals & Webster 1982). However, four *Tricladium* species with dark colonies appear in at least three other clades (see below).

The two strains of *T. splendens* treated here were also sequenced by Baschien *et al.* (2006, Fig 3). Neighbor joining analyses on the 18S rDNA placed *T. splendens* in a well supported clade with the helotialean aquatic anamorphs *Anguillospora crassa*, *Anguillospora furtiva*, *Anguillospora fustiformis*, *Anguillospora mediocris* and *Geniculospora grandis*. The same grouping was found with another isolate of *T. splendens* by Belliveau & Bärlocher (2005, Fig 2) using maximum parsimony and weighted parsimony on the 18S rDNA. Baschien *et al.* (2006, Fig 15, ITS1–5.8S–ITS2 rDNA sequences, maximum likelihood) found a close relationship to *Zalerion varium* (which according to Bills *et al.* (1999) may be a member of *Helotiales*), and to

Table 1 – Fungal isolates used in this study.

Species	Culture no.[a]	Country of origin	GenBank accession no.
Fontanospora eccentrica	CCM F-11402	UK	GQ477304
Fontanospora eccentrica	CCM F-46394	Canada	GQ477305
Fontanospora fusiramosa	CCM F-03680b	Slovak Republic	GQ477306
Fontanospora fusiramosa	CCM F-12900	Czech Republic	GQ477307
Fontanospora fusiramosa	VG 66-6	USA	GQ477308
Hydrocina chaetocladia[b]	CCM F-10890	UK	GQ477309
Tricladium angulatum	CCM F-00282	Czech Republic	GQ477310
Tricladium angulatum	CCM F-14186	Czech Republic	GQ477311
Tricladium attenuatum	CCM F-06485	Switzerland	GQ477312
Tricladium attenuatum	CCM F-10103	Canada	GQ477313
Tricladium biappendiculatum	CCM F-13000	Czech Republic	GQ477314
Tricladium biappendiculatum	CCM F-19794	Canada	GQ477315
Tricladium castaneicola	CCM F-11296	Czech Republic	GQ477316
Tricladium castaneicola	CCM F-13005	Portugal	GQ477317
Tricladium caudatum	CCM F-13498	Czech Republic	GQ477318
Tricladium caudatum	CCM F-21299	Czech Republic	GQ477319
Tricladium chaetocladium	CCM F-03485	UK	GQ477320
Tricladium chaetocladium	VG 23-1	USA	GQ477321
Tricladium curvisporum	CCM F-23387	Canada	GQ477322
Tricladium curvisporum	VG 67-5w	USA	GQ477323
Tricladium indicum	VG 112-1	USA	GQ477324
Tricladium indicum	VG 113-4	USA	GQ477325
Tricladium minutum	CCM F-10203	UK	GQ477326
Tricladium obesum	CCM F-13798	Czech Republic	GQ477327
Tricladium obesum[b]	CCM F-14598	Czech Republic	GQ477328
Tricladium patulum	CCM F-15199	Czech Republic	GQ477329
Tricladium patulum	CCM F-17199	Czech Republic	GQ477330
Tricladium patulum	VG 8-1w	USA	GQ477331
Tricladium procerum[b]	CCM F-16786	Slovak Republic	GQ477332
Tricladium splendens	CCM F-16599	Czech Republic	GQ477333
Tricladium splendens	CCM F-19087	Canada	GQ477334
Tricladium terrestre	CCM F-10101	Portugal	GQ477335
Tricladium terrestre	CCM F-10201	Portugal	GQ477336
Tricladium sp.1	VG 68-1	USA	GQ477337
Tricladium sp.2	VG 69-2	USA	GQ477338
Varicosporium delicatum	CCM F-03977	Czech Republic	GQ477339
Varicosporium delicatum	CCM F-18499	Czech Republic	GQ477340
Varicosporium elodeae	CCM F-13589	UK	GQ477341
Varicosporium elodeae	CCM F-20087	Canada	GQ477342
Varicosporium giganteum	CCM F-10987	Canada	GQ477343
Varicosporium giganteum	CCM F-11287	Canada	GQ477344
Varicosporium scoparium[b]	CCM F-10303	Spain	GQ477345
Varicosporium trimosum	CCM F-14398	Czech Republic	GQ477346
Varicosporium trimosum	CCM F-32694	Canada	GQ477347
Variocladium giganteum	CCM F-16686	Slovak Republic	GQ477348

a CCM = Czech Collection of Microorganisms; VG = Culture Collection of Vladislav Gulis.
b Ex-isotype cultures.

A. furtiva, *A. crassa* and *Cudoniella* sp. AY89371, confirming partly the results of the SSU rDNA analyses (see above).

The teleomorphs *Hymenoscyphus splendens* and *Cudoniella indica* were respectively connected to *T. splendens* (Abdullah *et al.* 1981) and *T. indicum*, an isolate from S. Africa (Webster *et al.* 1995). We did not have sequences for *H. splendens* or *C. indica*, but we did have sequences for *H. scutula*, *Cudoniella clavus* and *Cudoniella* sp., however these were not placed in the clade with *T. splendens* and *T. indicum*. *H. scutula* was placed as sister taxon to *V. giganteum* in the second clade (see below), and *Cudoniella* sp. and *C. clavus* were placed in a clade with no anamorphic taxa (Fig 1). However, as pointed out in the Introduction, the anamorph–teleomorph connection between the type of *T. indicum* described from Himalaya by Sati & Tiwari (1992) and *C. indica* from South Africa (Webster *et al.* 1995) was questioned by Sivichai *et al.* (2003). They obtained an anamorph, similar to that isolated by Webster *et al.* (1995) from apothecia on wood baits in Thailand, which they identified as *Hymenoscyphus varicosporoides*. They suggested the *Varicosporium* anamorph of *H. varicosporoides* should be reclassified in *Tricladium*, but have not renamed it formally (cf. also Baschien *et al.* 2006). Later, Boonyuen *et al.* (2006) in their study based on analyses of the ITS rDNA of the ex-type cultures CBS 651.66 (*H. varicosporoides* from Japan derived from ascospores) and CBS 430.94 (*T. indicum* – here as *C. indica* – from Indian Himalaya derived from conidia), reported a high percentage

Table 2 – Sequences obtained from GenBank.

Species	GenBank accession no.
Baeomyces placophyllus	AF356658
Brasiliomyces trina	AB022350
Bulgaria inquinans	DQ470960
Chlorencoelia sp.	AY789351
Cudoniella clavus	DQ470944
Cudoniella sp.	AY789375
Cyathicula coronata	AF222491
Cystotheca wrightii	AB022355
Dermea acerina	DQ247801
Dibaeis baeomyces	AF279385
Erysiphe betae	AB079684
Fabrella tsugae	AF356694
Gyromitra esculenta	FJ176906
Helicodendron conglomeratum	AY856900
Helvella lacunosa	U42681
Heyderia abietis	AY789289
Hyaloscypha daedaleae	AY789415
Hydrocina chaetocladia	AY789412
Hymenoscyphus scutula	AY789431
Hymenoscyphus sp.	AF430278
Icmadophila ericetorum	DQ883694
Lachnum cf. *bicolor*	AY544674
Lachnum virgineum	AY544646
Lambertella tubulosa	AY616237
Leveillula taurica	AB022387
Microsphaera pulchra	AB022389
Morchella esculenta	AF279398
Neofabraea malicorticis	AY544662
Ombrophila violacea	AY789365
Ostropa barbara	AY584642
Pezicula carpinea	DQ470967
Phyllactinia moricola	AB022401
Podosphaera longiseta	AB022423
Potebniamyces pyri	DQ470949
Rhytisma acerinum	AF356696
Siphula ceratites	DQ986775
Spirosphaera floriformis	AY616238
Torrendiella eucalypti	DQ195800
Typhulochaeta japonica	AB022415
Uncinula septata	AB183532

(99.5 %) of bp similarity between these specimens (see their Table 2), and suggested this indicated that *H. varicosporoides* had a *Tricladium* anamorph.

Moreover, there is a third large-spored (holoanamorphic) species of *Tricladium*, *Tricladium marylandicum*, described from USA (Crane 1968) whose relationships with *T. indicum* and *H. varicosporoides* are also unresolved. All these three taxa are characterized by dark colonies and large conidia with 1–4 primary and (in culture) also secondary branches. Multi-gene molecular analyses employing all these species may help solve this problem.

This first group also contains *Lambertella tubulosa*, *Helicodendron conglomeratum* and *Spirosphaera floriformis*. They also have dark colonies, but with their helicoform conidia are morphologically and ecologically quite different. They are called aero-aquatic hyphomycetes and unlike aquatic hyphomycetes, they thrive in standing or slow-moving waters, with low dissolved oxygen, and their conidia have air-trapping shapes, resistant against submerging, and adapted to dispersal on the water surface. *Spirosphaera* appears to be polyphyletic however, as Voglmayr (2004, partial nuc 28S and ITS1–5.8S–ITS2 rDNA) and Baschien *et al.* (2006, the same gene regions) placed *Spirosphaera cupreorufescens* in a well supported clade with *Anguillospora longissima* in the *Dothideomycetes*.

The second group (Figs 1, 3, 4) contains *Tricladium p.p.-Varicosporium p.p.-Fontanospora*. It consists of two subclades:

The first subclade (Figs 1, 3) contains *Tricladium attenuatum*, *Tricladium biappendiculatum*, *Tricladium minutum*, *Tricladium patulum*, *Varicosporium elodeae*, *Varicosporium trimosum*, *Fontanospora eccentrica*, *Fontanospora fusiramosa*. If we exclude *T. minutum*, which is on a poorly supported branch within this subclade, then the unifying features here are pale colonies, conidial branch insertion constricted to various extent, relatively narrow (usually up to 4 μm wide) conidial elements, absence of spermatial synanamorph and unknown teleomorph. Except for *T. biappendiculatum*, the conidiophore usually consists of a stipe and a fertile head which, if fully developed, is a multibranched structure with numerous conidia in lateral as well as terminal position. However, the branching pattern of the conidia is different: in *Fontanospora* there are two nearly opposite laterals inserted near the middle of a bent axis; *V. elodeae* and *Varicosporium trimosum* have conidia with primary and secondary branches (in the latter the conidia are relatively simple, consisting of an axis bearing one primary and one secondary branch, both inserted at right angle to their parent structures); the two *Tricladium* species have typical conidia for this genus differing by geniculate axis and blunt conidial apices in *T. patulum* against nearly straight axis and acute conidial apices in *T. attenuatum*. *T. biappendiculatum* also has acute conidial ends, but its conidiophores are short, often simple, and conidiogenous cell proliferation is percurrent.

T. minutum is on a poorly supported branch within the above subclade. This may support its former segregation in *Scorpiosporium* by Iqbal (1974), but in morphology, there are no characters diagnostic enough on the generic level to justify its separation. *T. minutum* has brown colonies, percurrent conidiogenous cells, small to medium large conidia with geniculate axis, and typically two primary branches with unconstricted branch insertion. A phialidic andromorph was seen in one of the British isolates by E. Descals (Marvanová & Descals 1996).

V. elodeae was sequenced by Baschien *et al.* (2006, Fig 3, 18S rDNA, neighbor joining, maximum likelihood and parsimony analyses) and was placed in a poorly supported clade with *T. patulum*, which is congruent with our results. Both strains of *V. elodeae* sequenced by Baschien *et al.* (2006), as well as those sequenced in our study, produce extracellular green pigments in agar cultures, and the conidia have parallel-walled axis and branches, typical for isolates from water.

The second subclade differs from the species of the first subclade by having dark colonies. *V. giganteum* has large conidia (spanning over 200 μm), with geniculate axis produced on sympodial conidiogenous cells and primary and secondary branches with unconstricted insertion. *Variocladium giganteum* is placed with poor support as a sister taxon to the species within the second subclade. It is relatively isolated from other *Tricladium* species, indicating that its segregation might have been justified. It is unique in the large dimensions of conidia

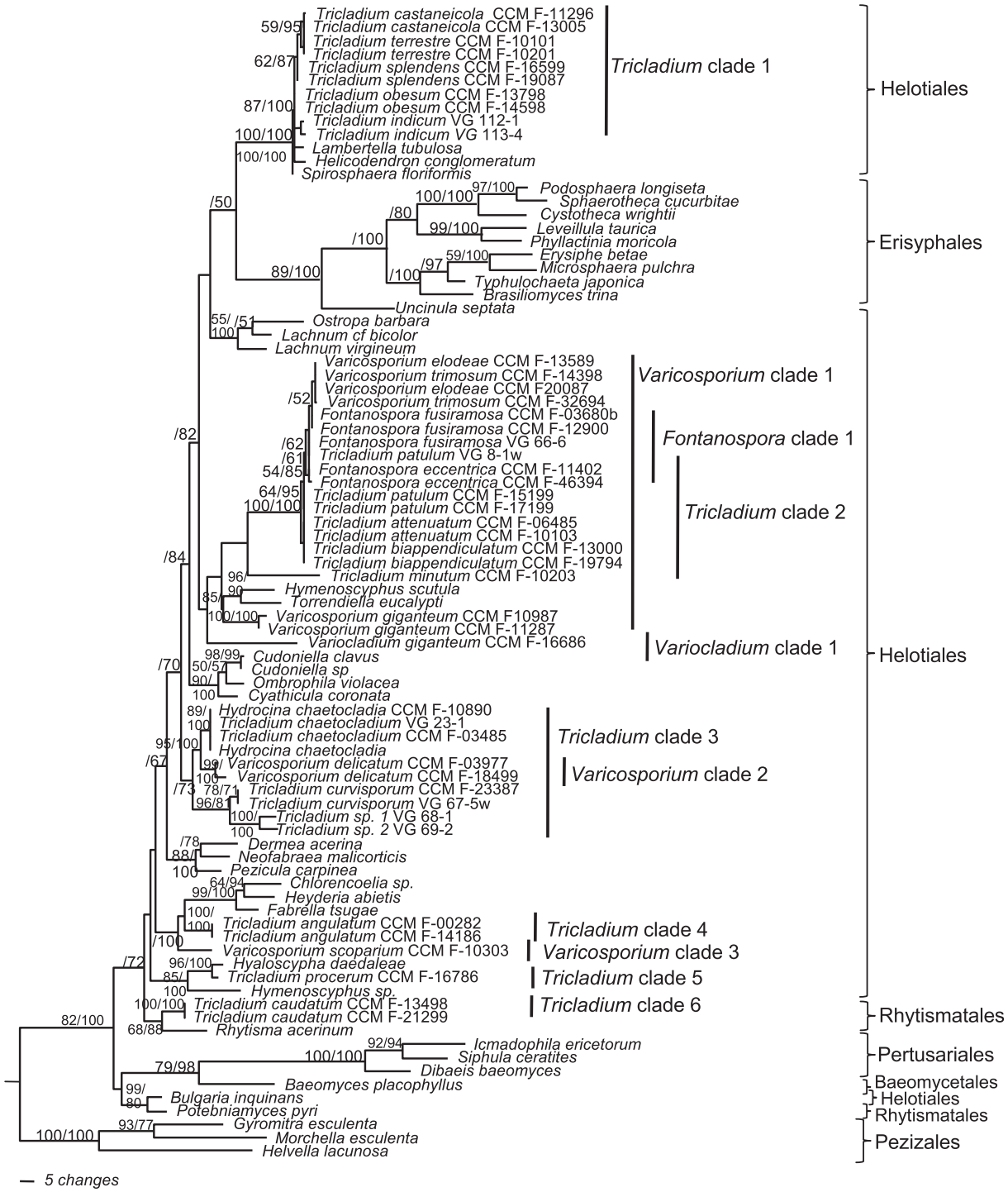

Fig 1 – B-MCMCMC majority rule consensus tree generated with average branch lengths from 16 890 trees inferred from Bayesian analyses on the 28S rDNA data. Maximum parsimony bootstrap supports and Bayesian posterior probability values greater than 50 % are given, respectively, at the corresponding nodes.

and variable branching pattern (alternate or opposite). Colonies are dark, conidiogenous cells percurrent and conidial branch insertion is unconstricted.

The third group (Figs 1, 5) contains *Tricladium chaetocladium* (including its teleomorph *Hydrocina chaetocladia*), *Tricladium curvisporum*, *Tricladium* sp.1 and sp.2 and *Varicosporium delicatum*. In our study, these species form a poorly supported clade within *Helotiales*. There are only two morphological characters which the four *Tricladium* species have in common: a sigmoid conidial axis and a phialidic andromorph in two of them. Otherwise they are culturally and morphologically quite different. *T. chaetocladium*, represented by two isolates from ascospores of the teleomorph and two from the anamorphic state, has *V. delicatum* as a sister taxon. However, their morphological

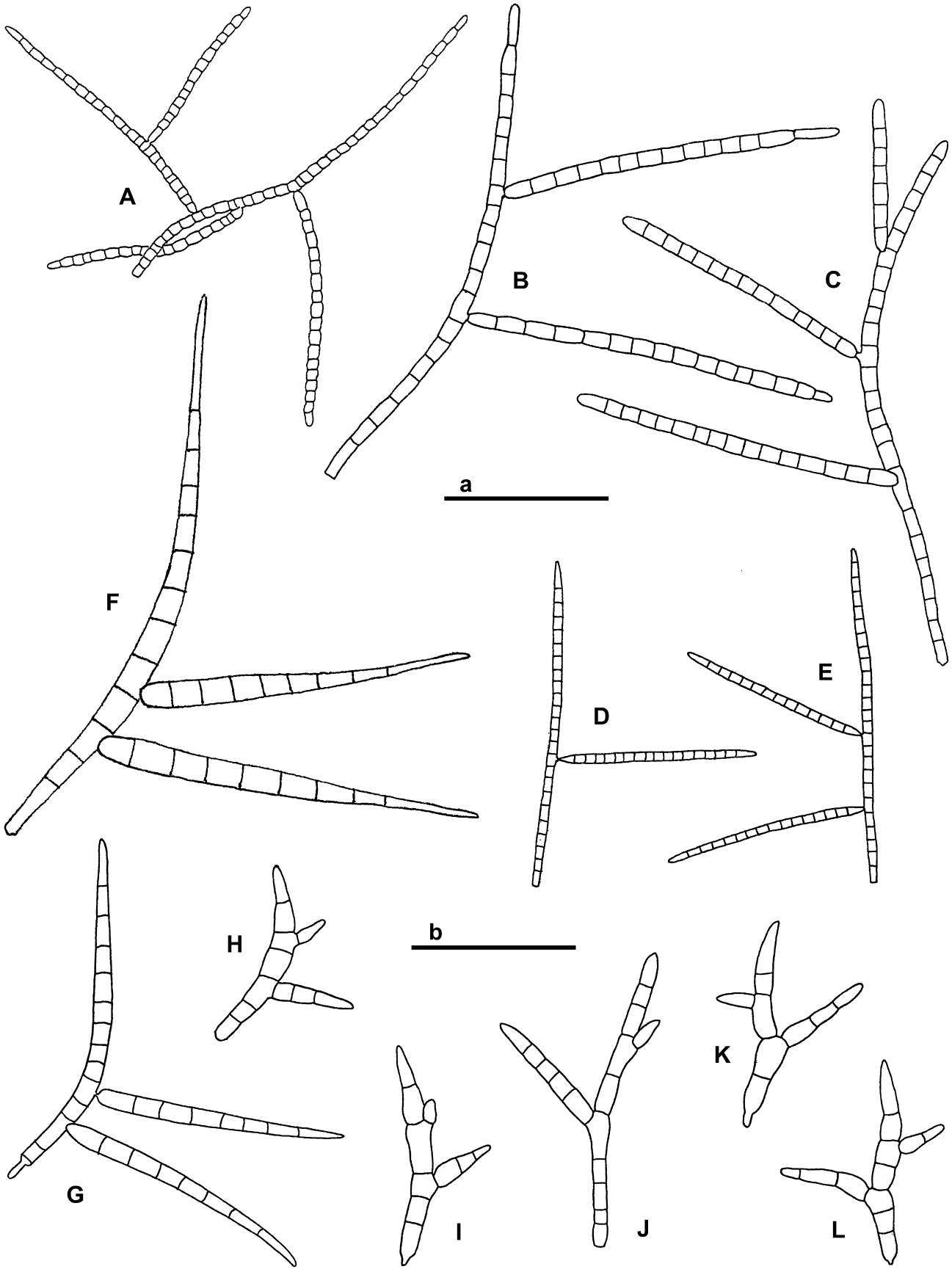

Fig 2 – Group 1. Conidia from *Tricladium* clade 1. (A) *Tricladium terrestre* CCM F-10101. (B–C) *Tricladium indicum* VG 112-1. (D) *Tricladium castaneicola* CCM F-11296. (E) *T. castaneicola* CCM F-10605. (F) *Tricladium splendens* CCM F-16599. (G) *T. splendens* CCM F-19087. (H–J) *Tricladium obesum* CCM F-14598. (K–L) *T. obesum* CCM F-13798. Scale bar a (A–C) = 100 μm, scale bar b (D–L) = 50 μm.

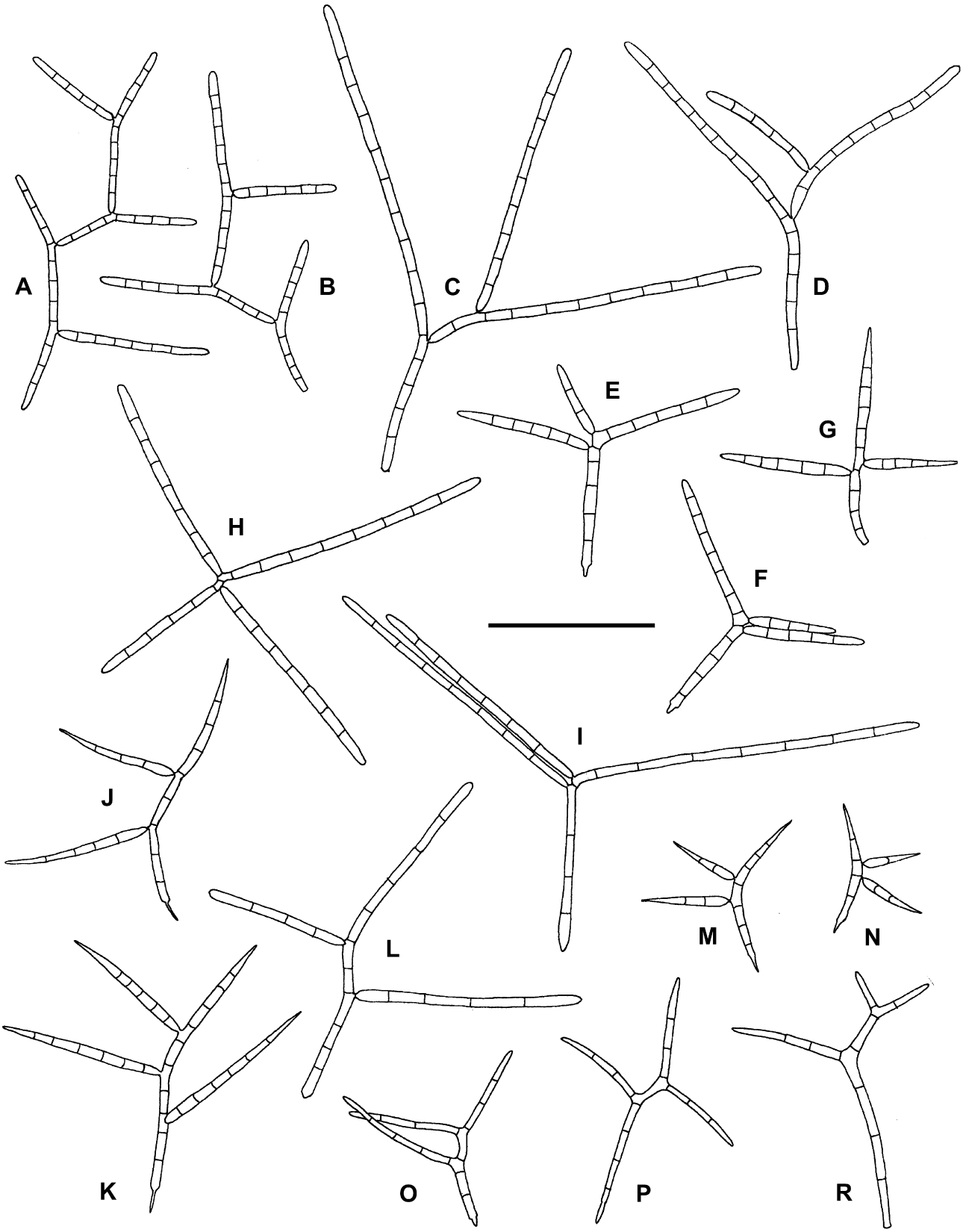

Fig 3 – Group 2, subclade 1. Conidia from *Varicosporium* clade 1. (A–B) *Varicosporium elodeae* CCM F-20087. (C) *Varicosporium trimosum* CCM F-14398. (D) *V. trimosum* CCM F-32694. Conidia from *Fontanospora* clade 1. (E–F) *Fontanospora fusiramosa* VG 66-6. (G) *F. fusiramosa* CCM F-03680b. (H) *Fontanospora eccentrica* CCM F-11402. (I) *F. eccentrica* CCM F-46394. Conidia from *Tricladium* clade 2. (J) *Tricladium attenuatum* CCM F-06485. (K) *T. attenuatum* CCM F-10103. (L) *Tricladium patulum* CCM F-17199. (M–N) *Tricladium biappendiculatum* CCM F-13000. (O–R) *Tricladium minutum* CCM F-10203. Scale bar = 50 μm.

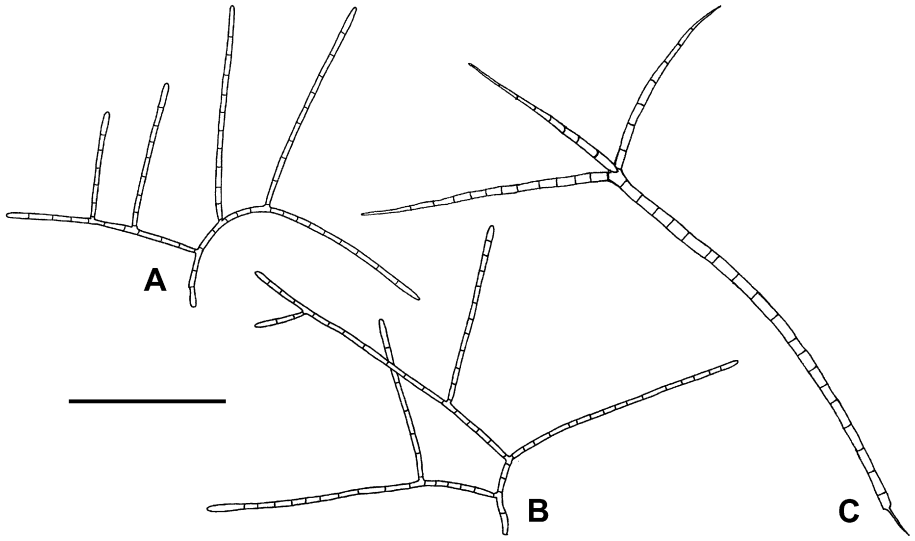

Fig 4 – Group 2, subclade 2. Conidia from *Varicosporium* clade 1. (A–B) *Varicosporium giganteum* CCM F-11287. Conidium from *Variocladium* clade 1. (C) *Variocladium giganteum* CCM F-16686. Scale bar = 100 μm.

similarity is low and can be seen only in the relatively large conidia with constricted or subconstricted branch insertion. Another small subclade linked to *T. chaetocladium*, consists of two isolates of *T. curvisporum* and two problematic taxa *Tricladium* sp.1 VG 68-1 and *Tricladium* sp.2 VG 69-2, both isolated from sedges submerged in fresh water and representing undescribed species. *Tricladium* sp.1 has conidia somewhat similar to those of *T. angulatum* or *T. minutum*, but it has black colonies, the conidial elements are wider, the conidial axis is sigmoid and axis and branches have conspicuously acute apices; elongation of conidiogenous cell is sympodial. *Tricladium* sp.2 is a similar isolate with dark colonies and sympodial elongation of conidiogenous cells, but conidia are much more delicate with 0–2(3) primary branches and an occasional secondary branch produced after release. Conidia may bear some resemblance to part conidia of *V. delicatum*.

H. chaetocladia (HME 4375, ex-isotype, teleomorph of *T. chaetocladium*) was found to be placed within a poorly supported, so called ''bsa'' clade (Wang *et al.* 2005, LSU, SSU, 5.8S rDNA) containing ecologically similar saprotrophic fungi, mostly with bright apothecia, preferring aquatic or at least humid habitats. In their parsimony analysis the closest neighbors are two species of *Mitrula* and two of *Vibrissea* (*Vibrisseaceae*), but in their maximum likelihood tree topology *Hydrocina* appears closest only to *Vibrissea* spp. (Wang *et al.* 2005). In a later study, however, (Wang *et al.* 2006a, parsimony analysis of LSU, SSU and 5.8S rDNA regions) they place this fungus into *Hyaloscyphaceae*, but with no bootstrap support. Finally, in an extensive study based on SSU, LSU and 5.8S rDNA sequences of 99 taxa (Wang *et al.* 2006b), it is placed in the so-called *Mitrula* clade among *Helotiaceae*, again without bootstrap support (cf. also Raja *et al.* 2008, LSU, maximum parsimony).

T. chaetocladium in the study of Belliveau & Bärlocher (2005, SSU rDNA) formed a poorly supported clade with *T. angulatum* and with *Monilinia fructicola* (*Sclerotiniaceae*) a fruit parasite on *Rosaceae*. The classification in *Hyaloscyphaceae*, suggested tentatively by Webster *et al.* (1991) remains questionable. The only *Hyaloscypha* hit in our blast search grouped with *Tricladium procerum*.

The remaining species treated in our study form rather small clades within *Helotiales*. The fourth group (Figs 1, 6) consists of *T. angulatum* and *Varicosporium scoparium*, both of which have pale colonies and sympodial conidiogenous cell proliferation, but otherwise there is very little morphological similarity between them. *V. scoparium* is a rare species, which differs from other *Varicosporium* species by ''scopiform'' (broom-like) conidia. This clade also contains the teleomorphs *Chlorencoelia* sp., *Heyderia abietis* and *Fabrella tsugae*, all placed in *Dermateaceae*, but this alignment is only poorly supported. The only other aquatic hyphomycetes having teleomorphs in that family are *Anguillospora crassa*, *Casaresia sphagnorum* and *Filosporella* sp. (Marvanová 2007); none of them are morphologically similar to *Tricladium* or *Varicosporium*.

Our results corroborate the distant placement of *T. angulatum* and *T. splendens*, the type of the genus, revealed by Belliveau & Bärlocher (2005) and by Baschien *et al.* (2006). Although in *T. splendens* the teleomorph was described in *Hymenoscyphus*, *Helotiaceae* (Abdullah *et al.* 1981), *T. angulatum* seems to have affinity to *Hyaloscyphaceae* (Belliveau & Bärlocher 2005, SSU rDNA; Baschien *et al.* 2006, SSU, ITS, rDNA). Both taxa differ considerably also in phenotypic characters: *T. splendens* has dark colonies, percurrent conidiogenous cells and constricted conidial branch insertion, whereas *T. angulatum* has pale colonies, polyblastic sympodial conidiogenesis and unconstricted conidial branch insertion.

The fifth group (Figs 1, 6) contains *T. procerum*, forming a small, but highly supported clade with members of two families of *Helotiales*: *Hyaloscypha daedaleae* and *Hymenoscyphus* sp. (*Hyaloscyphaceae* and *Helotiaceae*, respectively). It does not seem to be related to other species of *Tricladium*. *T. procerum* seems rare, known so far only from the type locality in the Slovak Republic and from a few streams in the Czech Republic, all

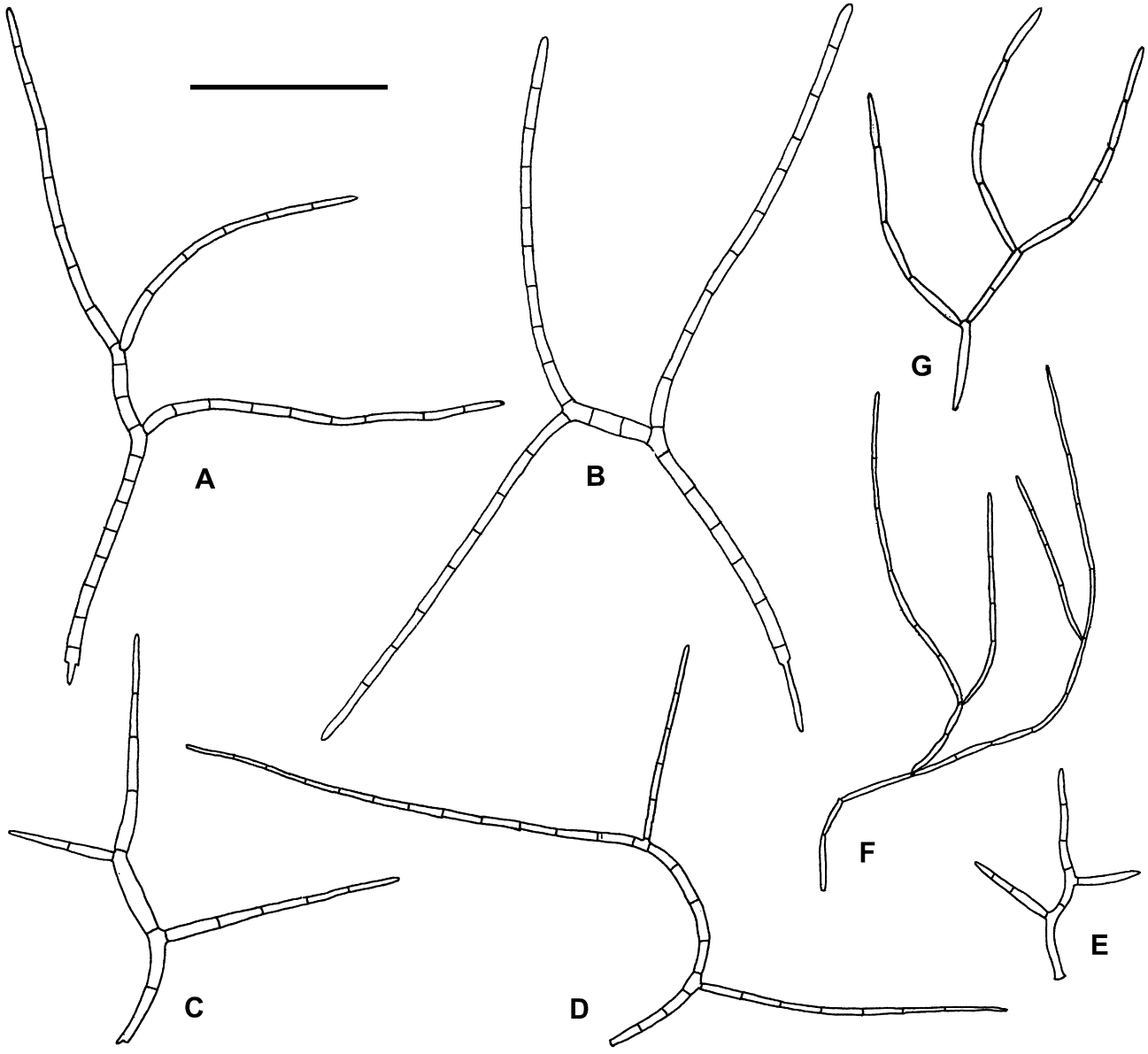

Fig 5 – Group 3. Conidia from *Tricladium* clade 3. (A) *Hydrocina chaetocladia* conidial state, CCM F-10890. (B) *Tricladium chaetocladium* CCM F-03485. (C) *Tricladium* sp. 1, VG 68-1. (D) *Tricladium* sp. 2, VG 69-2. (E) *Tricladium curvisporum* CCM F-23387. Conidia from *Varicosporium* clade 2. (F) *Varicosporium delicatum* CCM F-03977. (G) *V. delicatum* CCM F-18499. Scale bar = 50 μm.

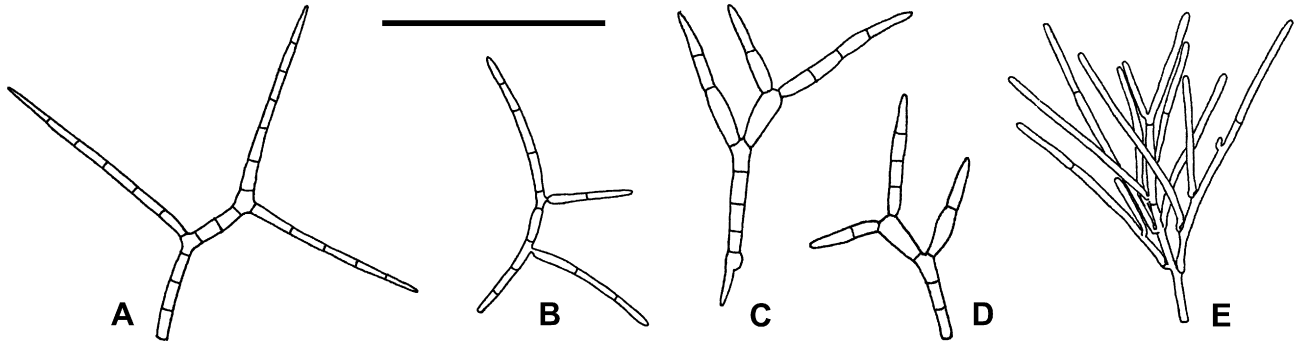

Fig 6 – Groups 4–6. Conidium from *Tricladium* clade 4. (A) *Tricladium angulatum* CCM F-00282. Conidium from *Tricladium* clade 5. (B) *Tricladium procerum* CCM F-16786. Conidia from *Tricladium* clade 6. (C–D) *Tricladium caudatum* CCM F-21299 (this culture was isolated from a conidium without parabasal extension, but it appears in some conidia). Conidium from *Varicosporium* clade 3. (E) *Varicosporium scoparium* CCM F-10303. Scale bar = 50 μm.

with soft water (Marvanová 1988). Affinity to *Hyaloscyphaceae* based on rDNA sequence data (Sokolski *et al.* 2006, ITS1–5.8S–1TS2) was found also in *Dwayaangam colodena*, an aquatic hyphomycete collected in foam on streams in the Canadian boreal forests and isolated from *Picea mariana* needles. Conidia of *D. colodena* have dichotomous and trichotomous branches on a stalk and are quite different from those of *T. procerum* or *T. angulatum*, mentioned above as possible hyaloscyphaceaean members.

The sixth group (Figs 1, 6) contains *Tricladium caudatum*, which appears with 88 % support in a small clade with *Rhytisma acerinum* (*Rhytismatales*). The only other aquatic hyphomycete with such affinity is *Tricladiopsis flagelliformis* which has quite different conidia. *Tricladium caudatum* is also the only species in our group of taxa with affinity outside *Helotiales*. It is unique within this genus by typical *in situ* production of an excentric caudal extension on conidia. CCM F-21299, isolated from a conidium lacking this extension, produces extensions only in a small percentage of conidia. The high similarity of nucleotide sequences with isolate CCM F-13498, collected in the same area and cultured from a conidium bearing this extension supports the conspecificity of both isolates.

Conclusions

Using molecular analyses of the partial 28S rDNA, we were not able to unequivocally support the classification of taxa based on conidiogenesis or on morphological similarity of conidia. The ambiguity in inter-generic classification of *Tricladium* and *Varicosporium* evident on the morphological level is not resolved on the basis of the LSU rDNA gene analysis. Neither the branching pattern nor the conidiogenesis seem to be diagnostic for generic separation.

Molecular phylogenetic studies in aquatic hyphomycetes have one limitation, which is not always fully recognized and in some individual cases may lead to misinterpretations: there are very few ex-type cultures (Marvanová 2007) and therefore we have to rely upon our ability to correctly identify specimens used for sequencing in accordance with the protologue. This may be sometimes difficult due to the phenotypic plasticity and genetic variability (Hebert *et al.* 2003), an insufficient protologue or a lack of experience in distinguishing morphological characters.

The molecular taxonomy is still in the stage of accumulating knowledge and search for suitable genes for sequencing. Presently, especially concerning the staurosporous and scolecosporous hyphomycetes, the amount of treated taxa and sequenced genes is small.

The inconsistencies between classifications based on morphology and phylogenetic analysis are especially striking in staurosporous anamorphs because our classification in this group is, besides conidiogenesis, predominantly based on conidial morphology. We face a disquieting problem: our visual approach to classification of objects forces us to group those with similar shapes together, but the phylogenetic analyses sometimes link taxa with markedly dissimilar conidia. Some recent results in other groups with conspicuous conidial configuration, e.g. *Spirosphaera cupreorufescens*, an aero-aquatic hyphomycete with complex conidia consisting of intertwined branched filament and closely related to scolecosporous *Anguillospora longissima* (Voglmayr 2004) or *Aquaphila albicans* with sickle-shaped conidia and close affinity to some helicosporous hyphomycetes (Tsui *et al.* 2007), raise a similar question.

Close phylogenetic relationships between aquatic hyphomycete taxa with branched and with unbranched conidia indicate that conidial branching might have arisen repeatedly and as the response to microhabitat conditions influencing distribution of species within a stream. A stream is a hydrologically complex habitat and offers various niches in its rapid and slow or deep and shallow parts. Branch number as well as their arrangement might represent advantages or disadvantages in leaf colonization within a particular microhabitat.

The majority of anamorphic aquatic hyphomycetes treated here show relationships to *Helotiales*, contributing so to the 23 species whose affinity to that order is already known (Marvanová unpubl.). Only one species is placed in *Rhytismatales*, an order housing among others the leaf parasite *Rhytisma acerinum*. However, *Helotiales* in the contemporary interpretation is considered paraphyletic (Hibbett *et al.* 2007) and not well resolved (Wang *et al.* 2006b). Its future refinement may link at least some aquatic hyphomycetes with possible smaller monophyletic lineages.

As in previous studies (Belliveau & Bärlocher 2005; Baschien *et al.* 2006), the teleomorphs described in some pleomorphic taxa on the basis of cultural studies, have not shown clear relationships to terrestrial representatives of the same genera. One of the reasons may be that not enough terrestrial teleomorphs were included in the study. The LSU gene region sequences are not frequently represented in GenBank.

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REFERENCES

Abdullah SK, Descals E, Webster J, 1981. Teleomorphs of three aquatic hyphomycetes. *Transactions of the British Mycological Society* **77**: 475–483.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

Ando K, Kawamoto I, 1985. New and interesting microfungi from fallen leaves in Japan: two *Tricladium* species. *Transactions of the Mycological Society of Japan* **26**: 39–47.

Baschien C, Marvanová L, Szewzyk U, 2006. Phylogeny of selected aquatic hyphomycetes based on morphological and molecular data. *Nova Hedwigia* **83**: 311–352.

Belliveau MJ-R, Bärlocher F, 2005. Molecular evidence confirms multiple origins of aquatic hyphomycetes. *Mycological Research* **109**: 1407–1412.

Bills GF, Platas G, Peláez F, Masurekar P, 1999. Reclassification of a pneumocandin-producing anamorph *Glarea lozoyensis* gen et

sp. nov., previously identified as *Zalerion arboricola*. *Mycological Research* **103**: 179–192.
Boonyuen N, Sivichai S, Worapong J, Hywel-Jones N, 2006. A molecular phylogenetic study of selected in goldian (sic) species. *BRT Research Reports* **2006**: 56–63.
Bunyard BA, Nicholson MS, Royse DJ, 1994. A systematic assessment of *Morchella* using RFLP analysis of the 28S ribosomal RNA gene. *Mycologia* **86**: 762–772.
Campbell J, Shearer C, Marvanová L, 2006. Evolutionary relationships among aquatic anamorphs and teleomorphs: *Lemonniera*, *Margaritispora*, and *Goniopila*. *Mycological Research* **110**: 1025–1033.
Crane JL, 1968. Freshwater hyphomycetes of the Northern Appalachian Highland including New England and three Coastal Plain states. *American Journal of Botany* **55**: 996–1002.
Descals E, 1985. Conidia as modified hyphae. *Proceedings of the Indian Academy of Sciences (Plant Sciences)* **94**: 209–227.
Descals E, Webster J, 1982. Taxonomic studies on aquatic hyphomycetes III. Some new species and a new combination. *Transactions of the British Mycological Society* **78**: 405–437.
Descals E, Marvanová L, Webster J, 1998. New taxa and combinations of aquatic hyphomycetes. *Canadian Journal of Botany* **76**: 1647–1659.
Dyko BJ, 1978. New aquatic and waterborne hyphomycetes from the Southern Appalachian Mountains in the United States. *Transactions of the British Mycological Society* **70**: 409–416.
Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.
Hebert PDN, Cywinska A, Ball SL, deWaard JR, 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London* **270**: 313–321.
Hennebert GL, Sutton BC, 1994. Unitary parameters in conidiogenesis. In: Hawksworth DL (ed), *Ascomycete Systematics. Problems and Perspectives in the Nineties*. Plenum Press, New York and London, pp. 65–76.
Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Thorsten Lumbsch H, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Gams W, Geiser DM, Griffith DM, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson K-H, Lichtwardt R, Longcore J, Miądlikowska J, Miller A, Moncalvo J-M, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüssler A, Sugyiama J, Thorn RG, Tibell L, Untereiner W, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y-J, Zhang N, 2007. A higher-level phylogenetic classification of the Fungi. *Mycological Research* **111**: 509–547.
Huelsenbeck JP, Ronquist F, 2001. MR BAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.
Ingold CT, 1942. Aquatic hyphomycetes of decaying alder leaves. *Transactions of the British Mycological Society* **25**: 339–417.
Ingold CT, 1975. Hooker Lecture 1974. Convergent evolution in aquatic fungi: the tetraradiate spore. *Biological Journal of the Linnean Society* **7**: 1–25.
Iqbal SH, 1974. *Scorpiosporium minutum* gen. nov., sp. nov., an aquatic hyphomycete. *Biologia (Lahore)* **20**: 17–21.
Kegel W, 1906. *Varicosporium Elodeae*, ein Wasserpilz mit auffallender Konidienbildung. *Berichte der Deutschen Botanischen Gesellschaft* **24**: 213–216.
Kendrick WB, 2003. Analysis of morphogenesis in hyphomycetes: new characters derived from considering some conidiophores and conidia as condensed hyphal systems. *Canadian Journal of Botany* **81**: 75–100.
Marvanová L, 1988. New hyphomycetes from aquatic environments in Czechoslovakia. *Transactions of the British Mycological Society* **90**: 607–617.
Marvanová L, 2002. Aquatic hyphomycetes – emerging outlines of their classification in the fungal system. In: Third International Meeting on ''Plant Litter Processing in Freshwater'', September 7–12, 2002, Szentendre, Hungary. (Abstract), p. 11.
Marvanová L, 2004. New hyphomycete species from streams in the Šumava National Park (Bohemian Forest, Czech Republic). *Czech Mycology* **56**: 193–202.
Marvanová L, 2007. Aquatic hyphomycetes and their meiosporic relatives: slow and laborious solving of a jig-saw puzzle. In: Ganguli BN, Deshmukh SK (eds), *Fungi Multifaceted Microbes*. Anamaya Publishers, New Delhi, pp. 128–152.
Marvanová L, Bärlocher F, 1998. Hyphomycetes from Canadian streams III. *Arcispora bisagittaria* anam. gen. et sp. nov. *Mycologia* **90**: 531–536.
Marvanová L, Descals E, 1996. Hyphomycetes from streams: new taxa and new combination. *Mycotaxon* **60**: 455–469.
Marvanová L, Gulis V, 2000. Notes on aquatic hyphomycetes and streamborne spora from Austria. *Österreichische Zeitschrift für Pilzkunde* **9**: 125–140.
Olmstead RG, Bremer B, Scott KM, Palmer JD, 1993. A molecular systematic analysis of the *Asteridae* sensu lato based on *rbc*L sequences. *Annals of the Missouri Botanical Garden* **80**: 700–722.
Park D, 1974. *Tricladium terrestre* sp. nov. *Transactions of the British Mycological Society* **63**: 179–183.
Qiagen, 2004. *DNeasyTM Plant Mini Handbook*. QIAGEN Inc., USA.
Qiagen, 2002a. *Taq PCR Handbook for Taq PCR Master Mix Kit*. QIAGEN Inc., USA.
Qiagen, 2002b. *QIAquick Spin Handbook for QIAquick PCR Purification Kit*. QIAGEN Inc., USA.
Raja H, Campbell J, Shearer CA, 2003. Freshwater ascomycetes: *Cyanoannulus petersenii* a new genus and species from submerged wood. *Mycotaxon* **88**: 1–17.
Raja HA, Miller AN, Shearer CA, 2008. Freshwater ascomycetes: *Aquapoterium pinicola*, a new genus and species of Helotiales (*Leotiomycetes*) from Florida. *Mycologia* **100**: 141–148.
Rambaut A, 1996. *Se-Al: sequence alignment editor. vers. 1.0 alpha 1.0*. University of Oxford, UK.
Rodriguez F, Oliver J, Marin A, Medina J, 1990. The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology* **142**: 485–502.
Sati SC, Tiwari N, 1992. A new species of *Tricladium* from Kumaun Himalaya, India. *Mycological Research* **96**: 229–232.
Sivichai S, Jones EBG, Hywel-Jones NL, 2003. Lignicolous freshwater Ascomycota from Thailand: *Hymenoscyphus varicosporoides* and its *Tricladium* anamorph. *Mycologia* **95**: 340–346.
Sokolski S, Piché Y, Laitung B, Bérubé JA, 2006. Streams in Quebec boreal and mixed-wood forests reveal a new aquatic hyphomycete species, *Dwayaangam colodena* sp. nov. *Mycologia* **98**: 628–636.
Swofford DL, 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). ver. 4*. Sinauer Associates, Sunderland, Massachusetts.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The Clustal-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.
Tsui KM, Sivichai S, Rossman AY, Berbee ML, 2007. *Tubeufia asiana*, the teleomorph of *Aquaphila albicans* in the Tubeufiaceae, Pleosporales, based on cultural and molecular data. *Mycologia* **99**: 884–894.
Vilgalys R, Hester M, 1990. Rapid genetic identification mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.
Voglmayr H, 2004. *Spirosphaera cupreorufescens* sp. nov., a rare aeroaquatic fungus. *Studies in Mycology* **50**: 221–228.
Wang Z, Binder M, Hibbett DS, 2005. Life history and systematics of the aquatic discomycete *Mitrula* (Helotiales, Ascomycota)

based on cultural, morphological, and molecular studies. *American Journal of Botany* **92**: 1565–1574.

Wang Z, Binder M, Schoch CL, Johnson PR, Spatafora JW, Hibbett DS, 2006a. Evolution of Helotialean fungi (*Leotiomycetes*, Pezizomycotina): a nuclear rDNA phylogeny. *Molecular Phylogenetics and Evolution* **41**: 295–313.

Wang Z, Johnson PR, Takamatsu S, Spatafora JW, Hibbett DS, 2006b. Toward a phylogenetic classification of the Leotiomycetes based on rDNA data. *Mycologia* **98**: 1065–1075.

Webster J, Eicker A, Spooner BM, 1995. *Cudoniella indica* sp. nov. (Ascomycetes, Leotiales), the teleomorph of *Tricladium indicum*, an aquatic fungus isolated from a South African river. *Nova Hedwigia* **60**: 493–498.

Webster J, Scheuer Ch, Om-Kalthoum Khattab S, 1991. *Hydrocina chaetocladia* gen. et sp. nov., the teleomorph of *Tricladium chaetocladium*. *Nova Hedwigia* **52**: 65–72.

Willoughby LG, Minshall GW, 1975. Further observations on *Tricladium giganteum* Iqbal. *Transactions of the British Mycological Society* **65**: 77–82.