Two new aquatic hyphomycetes

Two new *Tricladium* species from streams in Alaska

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**Abstract:** Two new species of aquatic hyphomycetes in the genus *Tricladium* are described from streams in Alaska, USA. Both species were isolated from submerged decaying sedges. *Tricladium kelleri* has blackish colonies and typical tricladioid conidia formed on sympodial conidiogenous cells. *Tricladium alaskense* has conidia with fine elements and 0–4 lateral branches; conidia are formed on sympodial conidiogenous cells. The two species are compared to other species in the genus and related genera using morphological characters and/or rDNA sequencing data (ITS and 28S). Molecular phylogenetic analysis placed both species in the Helotiales.

**Key words:** aquatic hyphomycetes, molecular systematics, morphology

**INTRODUCTION**

During a trip to Alaska in Jul 2004, Dr Keller Suberkropp collected material including samples of submerged plant litter from streams around Toolik Lake Field Station on the North Slope. Several cultures were isolated by the first author from that material that appeared to belong to an as yet undescribed species of aquatic hyphomycetes. Here we describe two new species that
belong to the largest genus of aquatic hyphomycetes, Tricladium, which forms several clades in the Helotiales, Leotiomycetes (Campbell et al. 2009). To our knowledge this is the first report and description of new aquatic hyphomycetes from tundra streams above the Arctic Circle.

**MATERIALS AND METHODS**

Plant material was collected from two streams near Toolik Lake Field Station, 68°37'39"N, 149°35'51"W. Both tundra streams are oligotrophic, circumneutral and bordered mainly by sedges and grasses with some dwarfed riparian vegetation (mostly Salix sp.). A detailed description of one of the streams (Hershey Creek) is given by Benstead et al. (2005). Single-spore isolates were obtained in the laboratory as described by Descals (1997). Plant material was incubated in sterile distilled water in Petri dishes at 15 C to induce sporulation. Further processing (isolation, cultivation, submergence of pure cultures) were performed at 15 C. Individual conidia were transferred onto 0.1% malt extract agar (MA, Difco) with 200 mg/L streptomycin and 126 mg/L penicillin G (potassium salt). After 24 h, germinating conidia were transferred to 0.1% MA plates supplemented with the same antibiotics and incubated for several days. Colonies were subcultured on 2% MA, and sporulation was induced by submerging colony slivers in standing sterile distilled water in Petri dishes.

Fungal morphology was examined with an Olympus BX51 compound microscope equipped with Nomarski optics. Microphotographs of conidia and conidiogenous structures mounted in water (fresh material) were taken with a digital camera (Q-Color3, Olympus). Permanent slides were prepared in lactic acid with 0.05% trypan blue or lactofuchsin (lactic acid with 0.1% acid fuchsin).

For molecular analyses, mycelia were harvested directly from MA plates. DNA was extracted with UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, California) according to manufacturer's instructions. The ITS region of fungal rDNA was amplified by PCR with primers SR6R (5' AAGWAAAAGTCGTAACAAGG 3') and LR1 (5' GGTGGTTTCTTTTCTCTCTCT 3') (Vilgalys and Hester 1990) that resulted in a fragment of approximately 600 bp. PCR was performed with PCR Master Mix (Promega, Madison, Wisconsin), 0.5 µM of each primer and 1 µL DNA extract in MJ mini thermal-cycler (Bio-Rad, Hercules, California). Initial denaturation began at 94 C for 2 min, followed by 30 cycles of denaturation for 30 s at 94 C, primer annealing at 50 C for 40 s and extension at 72 C for 1 min. Final extension occurred at 72 C for 5 min. Size and quality of PCR products were checked by electrophoresis on 1% agarose gels with a 100 bp ladder (Promega) as
a reference. The PCR products were cleaned up with QIAquick PCR Purification Kit (QIAGEN, Valencia, California) according to manufacturer's instructions. Bidirectional sequencing was done with SR6R and LR1 primers on ABI 3730 DNA analyzer (Applied Biosystems, Carlsbad, California) at the University of South Carolina EnGenCore facility. Sequences were assembled with ChromasPro (Technelysium, Queensland, Australia). In addition to isolates of two new Tricladium species from Alaska, we also sequenced ITS rDNA from Tricladium minutum (S.H. Iqbal) Marvanová & Descals, isolate CCM F-10203 and Varicosporium delicatum S.H. Iqbal, isolate CCM F-19494. These two strains are morphologically somewhat similar to our new Tricladium isolates. All sequences were used as queries in the GenBank sequence similarity query tool BLAST (http://blast.ncbi.nlm.nih.gov) with normal stringency. The top-scoring sequences from the BLAST queries were included in phylogenetic analyses (TABLE I).

Phylogenetic relationships were analyzed with the ARB software package (Ludwig et al. 2004). Alignments were performed with the ARB 1.03 Fast Aligner, examined and manually optimized according to primary and secondary structure information. Ambiguously aligned nucleotide characters were excluded before phylogenetic analyses. The final dataset was 461 nucleotides long. Neighbor joining, parsimony and maximum likelihood analyses were performed with ARB. Heuristic searches for most parsimonious trees (Swoford et al. 1996) were performed with parsimony informative characters in which gaps were treated as missing data. Most parsimonious tree topologies were optimized by nearest neighbor interchange and global Kernighan-Lin (KL) tree modifications (Kernighan and Lin 1970). Maximum likelihood analyses were performed applying the GTR+I+G model of sequence evolution. Searches were performed with random sequence addition and 100 replicates. Branch support was assessed with 1000 bootstrap replications of the dataset.

RESULTS

The alignments and the final tree resulted from the molecular phylogenetic analyses were deposited to TreeBASE (http://www.treebase.org) under study accession number S12536. The final alignment included 23 taxa and was 461 base pairs long. The dataset comprised 249 constant characters, 79 parsimony uninformative and 133 parsimony informative characters. Neighbor joining, parsimony and maximum likelihood (Fig. 1) analyses yielded identical tree topologies. Leotia lubrica was chosen as an outgroup. The sequences of T. kelleri and T.
*alaskense* were placed within the Helotiales and appeared as sister clades. The sequence similarity between the two new *Tricladium* species was 96%. Much lower was the sequence similarity of the new *Tricladium* species with morphologically similar *T. angulatum* and *T. minutum* (84 and 75–77% respectively). Closest BLAST hits were sequences of *Cadophora* and *Oculimacula*, which showed 90–94% sequence similarity to *T. kelleri* and *T. alaskense*.

Furthermore, the new *Tricladium* species show close relationships with *Leptodontidium* sp. (endophyte), *Mycochaetophora* sp. (plant pathogen) and sequences of uncultured endophytic and mycorrhizal fungi.

**TAXONOMY**

**Tricladium kelleri** Gulis & Marvanová., sp. nov.  
*Figs. 2–3*

MycoBank: MB564246

Colonies (MA, 2%) dark gray to black, moderately fast growing, aerial mycelium dark gray, low, funiculose in the center. Sporulation underwater (15 C). Hyphae branched, hyaline to brown, 1–5 µm wide, with slightly thickened walls, septate. Mycelial funicles up to 35 µm wide.

Conidiophores semimacronematous, single or in groups, terminal, septate, hyaline, simple or sparsely branched. Conidiogenous cells terminal, integrated, cylindrical to subclavate, 7–20 × 2.5–3.5 µm, monoblastic to polyblastic sympodial, proliferations few. Conidia acropleurogenous, mostly solitary or in pairs, hyaline. Main axis long-fusoid, sigmoid or arcuate, 77–140 × 3–4(–5) µm, septate, base truncate or with a short, typically eccentric or percurrent extension developing after secession, distal part subulate. Branches (0–1)2, 14–26 µm apart, straight or slightly curved, mostly perpendicular to the main axis or divergent, 33–55 × 2.5–3 µm, at the base, subulate, insertion unconstricted or only slightly constricted. Conidial secession schizolytic. Teleomorph unknown.
Specimens examined: USA. ALASKA: Hershey Creek near Toolik Lake Field Station, 68°37'26"N, 149°27'41"W, ca. 870 m. On submerged decaying leaves of sedges (Carex sp.), 3 Jul 2004, collected by K. Suberkropp, VG-68-2 (HOLOTYPE, K(M) 172049 (microscopic preparation derived from isolate VG 68-2), ex-type culture CBS 130985); same locality, substrate, date and collector, VG 68-1.

Etymology: Derived from the name of Prof Keller Suberkropp, a lifelong student of aquatic fungi.

Tricladium alaskense Gulis & Marvanová, sp. nov. Figs. 4–5

MycoBank: MB564247

Colonies (MA, 2%) dark gray to black, moderately fast growing, aerial mycelium low, dark gray. Sporulation underwater. Hyphae hyaline, branched, septate, some fuscous to dark brown with slightly thickened walls; hyphal coils are common in culture. Conidiophores semimacronematous, single or in groups, terminal, septate, hyaline, simple or rarely sparsely branched. Conidiogenous cells terminal, integrated, cylindrical to subclavate, 12–20 × 2–3 µm, monoblastic or polyblastic, proliferations sympodial. Conidia acropleurogenous, solitary or in groups of up to three, hyaline. Main axis sigmoid or arcuate, (52–)80–115(–178) × 2–3 µm, septate, tapering distally, base truncate, slightly thickened, sometimes with a short, percurrent or eccentric extension. Primary lateral branches 0–4, 18–32 µm apart, straight or slightly curved, arranged in different planes, mostly perpendicular to main axis, (16–)30–62(–98) × 1.5–2 µm, subulate, insertion often on a little shoulder, slightly constricted. One or two secondary branches sometimes develop on the proximal primary branch. Conidial secession schizolytic. Teleomorph unknown.

Specimens examined: USA. ALASKA: Qannik Stream, 69°05'31"N, 148°50'07"W, ca. 320 m. On submerged decaying leaves of sedges (Carex sp.), 3 Jul 2004, collected by K. Suberkropp, VG 69-2 (HOLOTYPE, K(M) 172054 (microscopic preparation derived from isolate VG 69-2), ex-type culture CBS 130986); same locality, substrate, date and collector, VG 71-7.
**Etymology:** From the location of the type specimen.

**Discussion**

Conidia of *T. kelleri* in field samples (either captured from stream water from membrane filters or after inducing conidial production from plant litter) initially were identified as *T. minutum*, however careful examination of conidia and conidiogenesis in pure culture resulted in recognition of new species. Although *T. minutum* produces conidia of similar size, its colonies are not blackish, but pale brown to brown (Iqbal 1974, Marvanová and Descals 1996), the conidiogenous cells are percurrent, conidia have a narrower main axis and less strongly tapering branches and the distal part of the main axis. The two species clearly are separated phylogenetically based on ITS sequences (Fig. 1); they also were placed in two distinct *Tricladium* clades by Campbell et al. (2009) based on 28S rDNA (*T. kelleri* as *Tricladium* sp. 1). Conidia of *T. angulatum* Ingold, a common species in temperate streams (Gulis et al. 2005, Baschien et al. 2006), also may resemble those of *T. kelleri*; in addition it has polyblastic sympodial conidiogenesis. However, *T. angulatum* forms white to yellowish colonies in culture and, based on ITS (Fig. 1, Baschien et al. 2006) and 28S sequence data (Campbell et al. 2009), it belongs to a different clade within the Helotiales.

Conidia of *T. alaskense* in field samples can resemble parts of, or less complex conidia of *Varicosporium delicatum*, an aquatic hyphomycete described from submerged leaf litter (Iqbal 1971). However, conidia of the latter typically have more numerous secondary branches, tertiary branches also occur, conidial elements apparently do not taper toward apices, and constrictions at branch insertions are more distinct. A characteristic feature of *V. delicatum* conidia is that the elements consist of subfusoid segments. *V. delicatum* is distant from *T. alaskense* based on molecular data (Fig. 1). *T. angustum* K. Ando has smaller conidia that are somewhat similar to
those of *T. alaskense*. *T. angustum* was isolated from decaying tree leaves in a terrestrial habitat, from a locality at ca. 2000 m (Ando and Kawamoto 1985). However, the conidiophores in this species are mostly intercalary or only up to 7 μm long when lateral and the colonies are pale. Some conidia of *T. alaskense* may resemble those of *Tricladiella pluvialis* K. Ando & Tubaki, known from rainwater from trees (Ando and Tubaki 1984), although conidia of the latter arise on micronematous conidiophores, are considerably smaller and have cylindrical elements. *Lambdasporium lushanense* J.L. Chen, W.S. Lin & Tzean, which is morphologically very similar to *Tricladiella pluvialis*, has darker colonies and significantly larger conidia and may be more similar to *T. alaskense*, but the conidial elements of *L. lushanense* are cylindrical and conidiophores are micronematous (Chen et al. 2000). As far as we are aware, no phylogenetic studies of the three taxa mentioned above based on DNA sequences were published, hence their relationships to *T. alaskense* remain unknown. Another nonaquatic fungus with conidia somewhat similar to those of *T. alaskense* is *Ramulispora sorghicola* E. Harris, known from tropical localities as a causal agent of *Sorghum* leaf spot disease (Harris 1960). *Tricladium sorghicola* P.C. Gupta & S.K. Gandhi (as ‘sorghicolum’), described from India in terms not much different from the description of *R. sorghicola*, is probably conspecific (Gupta and Gandhi 1979). According to phylogenetic studies (Crous et al. 2009a, b), the type of *Ramulispora, R. sorghi* (Ellis & Everh.) L.S. Olive & Lefebvre, is placed within the Mycosphaerellaceae, Dothideomycetes, which is a strong argument for doubting the relationship of *T. alaskense* to *Ramulispora*.

Of interest, a *Tricladium* sp. was reported from the Alaskan Arctic by Kobayasi et al. (1969). According to the brief description (no illustration) it is impossible to identify it with *T. alaskense*, although the reported conidial dimensions may not exclude it.
Despite significant differences in conidial morphology, *T. kelleri* and *T. alaskense* are phylogenetically close (Fig. 1). We found 96% sequence similarity of ITS rDNA between the isolates of the two species. Both species also showed close relationships to *Oculimacula* and *Cadophora* (Helotiales, Dermateaceae) in our ITS analyses. The anamorph genus *Tricladium* is polyphyletic with up to six clades distributed among several families of the Helotiales, Leotiomyceses (Campbell et al. 2009). Based on 28S rDNA data, *T. kelleri* and *T. alaskense* also clustered together within the same major *Tricladium* clade (as *Tricladium* sp. 1 and sp. 2 respectively) (Campbell et al. 2009). Species of this clade (*T. kelleri*, *T. alaskense*, *T. chaetocladium* Ingold, *T. curvisporum* Descals, *Varicosporium delicatum*) are diverse with respect to their conidial morphologies, but all have a sigmoid main axis and mostly form dark gray to black colonies in culture (with the exception of *T. curvisporum*). Of interest, four of the five species above (except *T. chaetocladium*) have been recorded from the Alaskan streams sampled in this study.

We found that Alaskan tundra streams from which our two new *Tricladium* species are described have unique assemblages of aquatic hyphomycetes. To our knowledge, there is a single publication that dealt with aquatic hyphomycetes in the tundra streams. Engblom et al. (1986) reported 19 species from stream foam in Greenland, but only three species matched the taxa found in the Alaskan tundra: *Dendrospora polymorpha* A. Roldán & Descals (Fig. 2i, as unknown), *Tricladium curvisporum* and *Ypsilina graminea* (Ingold, P.J. McDougall & Dann) Descals, J. Webster & Marvanová (as *Volucrispora graminea*). In Alaskan streams, along with the two new *Tricladium* species, we found some rare or infrequently reported species, such as *Anguillospora* cf. *curvula* S.H. Iqbal, *D. polymorpha*, *Flagellospora fusarioides* S.H. Iqbal, *Fontanospora fusiramosa* Marvanová, P.J. Fisher & Descals, *T. curvisporum*, *Varicosporium*
delicatum, Variocladium giganteum (S.H. Iqbal) Descals & Marvanová, V. rangiferinum
(Descals) Descals & Marvanová and Y. graminea. D. polymorpha (as Dendrospora sp., Fig. 6B)
and Y. graminea (as V. graminea) also were recorded in a Swedish subarctic stream (Marvanová
and Müller-Haeckel 1980, 68°21′N). Six of the above mentioned rare species were described or
previously reported from submerged decaying rushes or grasses or found in water from moorland
streams in England that are largely devoid of trees in the riparian zone (e.g. Iqbal and Webster
1977). This suggests that aquatic fungal community may be strongly shaped by the character of
riparian vegetation and also lends support to the idea that some aquatic hyphomycetes may
exhibit substrate preferences (Gulis 2001).

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LITERATURE CITED


Japan 25:39–47.

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Benstead JP, Deegan LA, Peterson BJ, Huryn AD, Bowden WB, Suberkropp K, Buzby KM, Green AC, Vacca JA.


LEGENDS
FIG. 1. Phylogenetic tree showing relationships of two new *Tricladium* species based on ITS rDNA sequences using maximum likelihood (Ln likelihood = −3186.201197). Bootstrap support values ≥ 95% are at the corresponding nodes.


FOOTNOTE

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Tricladium alaskense VG 71-7 submerged Carex sp. JQ417291 ^a

^aSequences generated in this study.