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Freshwater ascomycetes: *Aquapoterium pinicola*, a new genus and species of Helotiales (Leotiomycetes) from Florida

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Abstract: As part of a survey of freshwater ascomycetes in Florida an unusual discomycete fungus belonging in the Helotiales was found on submerged *Pinus* needles. This fungus is described and illustrated as a new genus and species, *Aquapoterium pinicola*, based on morphological data. *Aquapoterium pinicola* is characterized by minute, hyaline apothecia with an excipulum one cell layer thick of parallel hyphae composed of chains of cells narrow at the basal end and enlarged at the apical end and aseptate ascospores that are surrounded by a gelatinous sheath. Analyses of the nuclear ribosomal large subunit DNA sequence data confirmed its placement within the Helotiales but failed to resolve its familial placement.

Key words: aquatic fungi, LSU, phylogenetics, systematics

INTRODUCTION

During a study of freshwater ascomycetes along the Florida peninsula, a small hyaline discomycetous fungus belonging to the Helotiales was found on submerged, decomposed *Pinus* L. needles. This species is characterized by white to cream colored, minute apothecia with a thin excipulum composed of a single layer of parallel, hyaline, septate hyphae composed of chains of cells narrow at the basal end and enlarged at the apical end; cylindric-clavate asci with a Melzer's negative (MEZ-) apical pore, and hyaline, smooth-walled, 1-celled, multiguttulate, short clavate-cylindric ascospores surrounded by a gelatinous sheath. The foregoing morphological features are characteristic of order Helotiales (Korf 1973, Kirk et al 2001).

Of the 538 freshwater ascomycetes currently reported from freshwater habitats, 112 are discomycetes, of which 101 belong to the Helotiales (Leotiomycetes) (http://www.life.uiuc.edu/plantbio/fungi/). Many of the discomycetes reported from freshwater also occur in terrestrial habitats, frequently on waterlogged wood (Dennis 1978, Pfister and Kimbrough 2001). However discomycete species that have been described originally from freshwater habitats thus far have not been reported from terrestrial habitats and might be aquatic species. The discomycete fungus collected from freshwater habitats in Florida is different from other genera in the Helotiales and therefore a new genus is established to accommodate this fungus.

The goals of this study were to fully characterize and describe the morphology of the new discomycete fungus and to use morphology and phylogenetic analysis of molecular data (large subunit rDNA, LSU) to determine its familial placement.

MATERIALS AND METHODS

Morphological study.—Collection, isolation and characterization. Methods for collection and characterization of the fungus are described in Fallah and Shearer (2001) and Raja and Shearer (2006). Water temperature, pH and latitude and longitude were recorded in the field. Morphological characterization of the fungus was done with apothecia found on *Pinus* needles collected from aquatic habitats in Florida and incubated in plastic boxes containing moist paper towels at ambient temperatures (ca. 24 C) under 12/12 h (light/dark) conditions. Single spore isolations were made according to the procedures of Shearer et al (2004). Sexual reproduction by axenic isolates was stimulated in water cultures following the methods outlined in Fallah et al (1998) and Fallah and Shearer (2001). The holotype specimen is deposited in the Herbarium of the University of Illinois at Urbana-Champaign (ILL). A holotype culture derived from the holotype specimen is deposited at the American Type Culture Collection (MYA 4213).

Molecular study.—DNA extraction, amplification, sequencing, and sequence alignment. Fungal isolates were grown on cornmeal agar (CMA, Difco) and peptone, yeast extract glucose agar (PYG; peptone 1.25 g, yeast extract 1.25 g, D-glucose 3.00 g; distilled water 1000 mL, agar 18 g). For extraction of genomic DNA, mycelium from axenic cultures was scraped from culture plates with a sterile scalpel and ground to a fine powder in liquid

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nitrogen with a mortar and pestle. About 400 μL of AP1 buffer from the DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California) was added to the mycelial powder and DNA was extracted following the manufacturer's instructions. Total genomic DNA was observed on a 1% TBE agarose gel stained with ethidium-bromide.

Partial large subunit (LSU) 28S nrDNA was amplified by the polymerase chain reaction (PCR) with puReTaq™ Ready-To-Go PCR beads (Amersham Biosciences Corp., Piscataway, New York) according to Huhndorf et al (2004). Primers LROR and LR6 (Vilgalys and Hester 1990) were used to amplify the LSU. PCR products were purified to remove excess primers, dNTP and nonspecific amplification products with the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, California). Purified PCR products were used in 11 μL sequencing reactions with BigDye® Terminators v3.1 (Applied Biosystems, Foster City, California) in combination with these LSU primers: LROR, LR3, LR3R, LR6 (Vilgalys and Hester 1990). Sequences were generated on an Applied Biosystems 3730XL high-throughput capillary sequencer, then assembled and aligned with Sequencher 4.7 (Gene Codes Corp., Ann Arbor Michigan), optimized by eye and manually corrected when necessary. The LSU nrDNA was chosen for this study because several sequences representing the order Helotiales are available in GenBank and it has been useful in determining phylogenetic placement above the species level among Helotialean fungi (Wang et al 2005, 2006a, b).

Taxon sampling.—A LSU sequence of the newly discovered fungus *Aquapoterium pinicola* was analyzed with 36 other LSU sequences obtained from GenBank representing 10 of the 13 families currently circumscribed within the Helotiales, Leotiomycetes (Eriksson 2005) based on studies by Wang et al (2006a, b). GenBank accession numbers follow taxon names on the tree. *Orbilia auricolor* and *Orbilia deliculata* were used as outgroup taxa.

Phylogenetic analyses.—The first 81 bp of the 5′ end were excluded from all analyses due to missing data in most taxa. Five ambiguously aligned regions were delimited, and characters in these regions were recoded and analyzed to recover their phylogenetic signal with the program INAASE (Lutzoni et al 2000). The remaining unambiguously aligned regions were subjected to a symmetric step-matrix generated with STMatrix ver. 2.2 (François Lutzoni and Stefan Zoller, Biology Department, Duke University, Durham, North Carolina). An unequally weighted maximum parsimony analysis was conducted with PAUP* 4.0b10 (Swofford 2002) as follows: constant characters were excluded, gaps were treated as missing, 1000 random-addition replicates were implemented with TBR branch-swapping, MULTREES option was in effect and zero-length branches were collapsed. Bootstrap support was estimated by performing 1000 bootstrap replicates (Felsenstein 1985) with the above settings.

Modeltest 3.06 (Posada and Crandall 1998) was used to determine the best-fit model of evolution for the dataset. Maximum likelihood analyses were performed with PAUP with 1000 stepwise random-addition replicates and TBR branch-swapping with a reconnection limit of 12 using the best-fit model, which was the TrN + I + G model with unequal base frequencies (freqA = 0.2567, freqC = 0.1928, freqG = 0.3009, freqT = 0.2496), a substitution rate matrix (A<–>C = 1.0000, A<–>G = 2.1297, A<–>T = 1.0000, C<–>G = 1.0000, C<–>T = 6.5517, G<–>T = 1.0000), a proportion of invariable sites = 0.5279 and a gamma distribution shape parameter = 0.7206.

Bayesian analyses employing Markov chain Monte Carlo (MCMC) was performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) as an additional means of assessing branch support. The TrN + I + G model was implemented and four chains were run for 10 000 000 generations with trees sampled every 100th generation resulting in 100 000 total trees. The first 10 000 trees, which extend beyond the burn-in phase in each analysis, were discarded and the remaining 90 000 trees were used to calculate posterior probabilities.

TAXONOMY

Aquapoterium Raja & Shearer, gen. nov.

Apothecium sessile vel stipitatum, album vel cremeum coloratum, cupulatum. Subhymenium et excipulum strato unicellulari, crasso, e hyphis parallelibus, constrictis ad septa compositum. Paraphyses hyalinae, filiformes, obtusae vel clavatae ad apices, septatae, glabro-tunicatae, simplices vel ramosae. Asci octospori, non jodi caerulescens, cylindrici-clavati, ad apicem rotundatum, inoperculati, apice incrassatulo. Ascosporae hyalinae, glabro-tunicatae in luce transmisso, unicellulares, multiguttulatae, breviclavati-cylindricae, cinctae vagina gelatinosa.

Apothecia sessile or stipitate, cupulate, white to cream colored. Subhymenium and excipulum one cell layer thick, composed of a single layer of parallel hyphae; cells of excipular hyphae composed of chains of cells narrow at the basal end and enlarged at the apical end. Paraphyses hyaline, filiform, obtuse to clavate at apex, septate, smooth-walled, simple or branched. Asci 8-spored, apex MEZ-, cylindric-clavate, rounded at apex, inoperculate, with an apical slit; apical wall slightly thickened. Ascospores hyaline, smooth-walled, 1-celled, short clavate-cylindric, surrounded by a gelatinous sheath.

Etymology. aqua = L. water, in reference to the habitat; ποτήρ [poter] Greek for cup (water cup).

Typus. Aquapoterium pinicola Raja & Shearer

Aquapoterium pinicola Raja & Shearer sp. nov. FIGS. 2–11

Fungus submersus, aquaticus apotheciis. Apothecia 95–200 × 125–240 μm, alba vel cremea colorata ubi vegeta, brunescens ubi exsiccata, convexa ad centra, cupulata, applanata versus ambitus, stipitata vel sessila. Stipes si praesens cylindricus, ca. 50–350 × 20–25 μm. Excipulum strato unicellulari, e hyphis parallelibus, constrictis ad septa compositum. Cellulae excipuli similis textura oblita, ca. 13–15 × 5–7 μm. Paraphyses 40–72 × 1–2 μm, hyalinae,

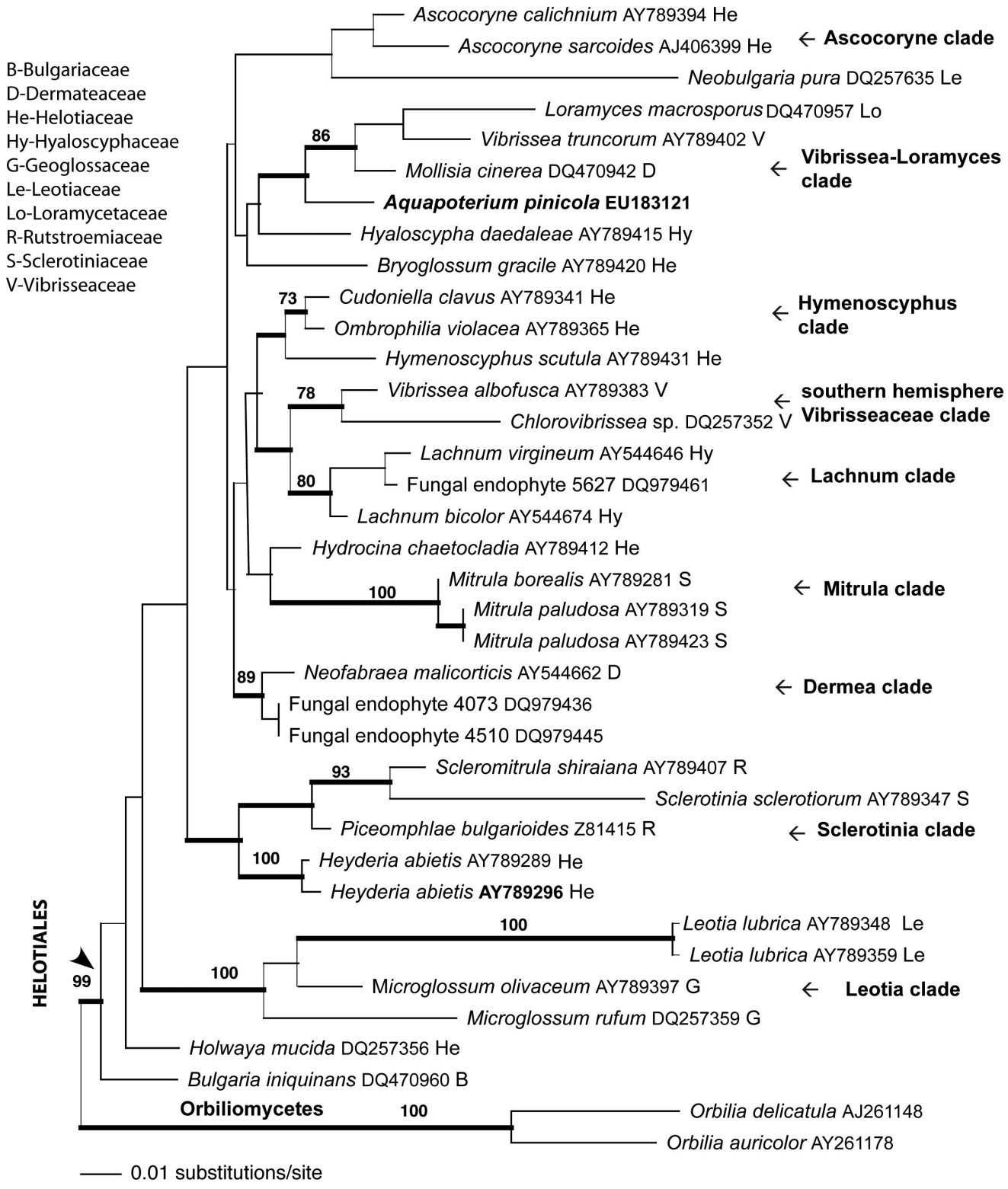
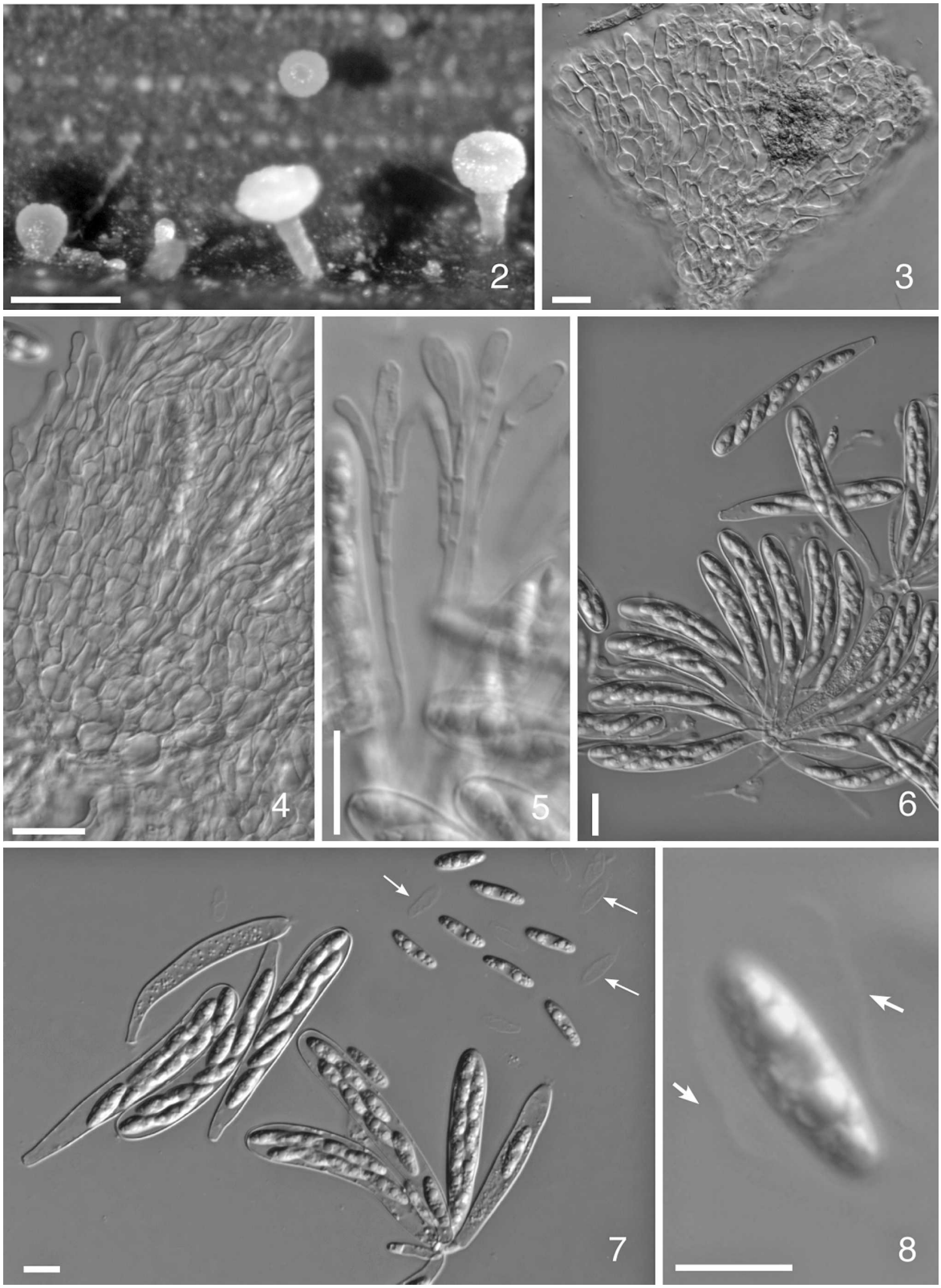
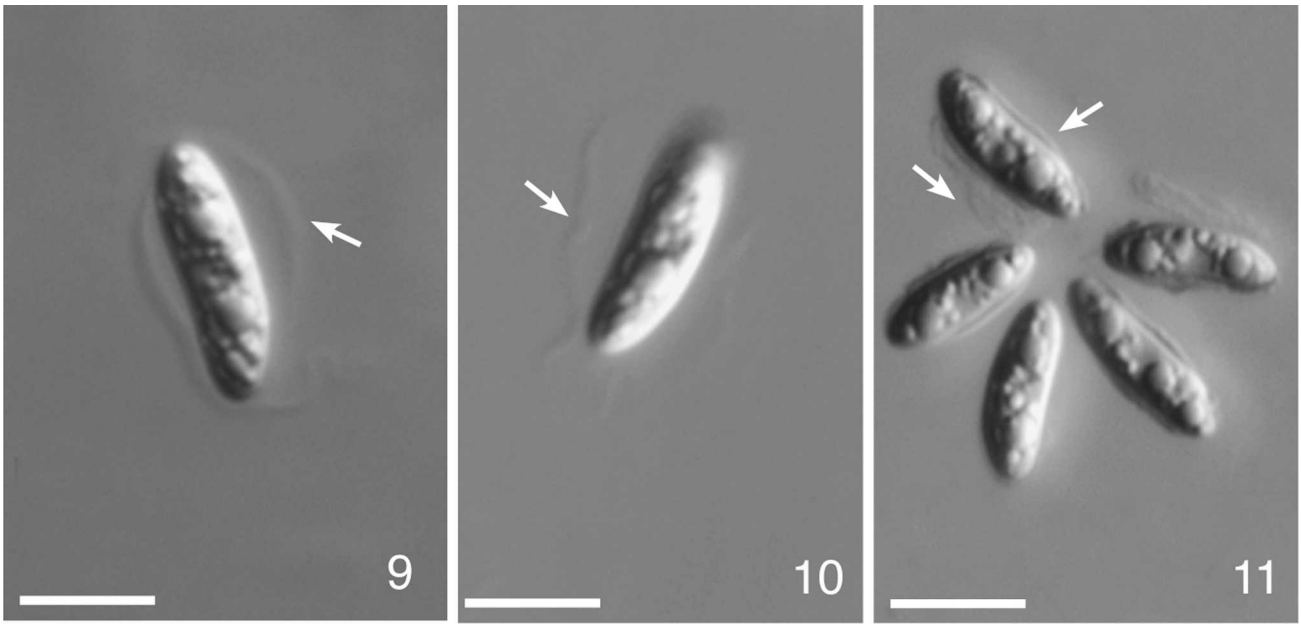


FIG. 1. Phylogram generated from a maximum likelihood analysis of 37 ascomycete large subunit (LSU) sequences. Thickened branches indicate Bayesian posterior probabilities ≥95%; numbers above branches refer to maximum parsimony bootstrap values ≥70%. *Orbilia delicatula* and *Orbilia auricolor* are used as outgroup taxa.



FIGS. 2–11. *Aquapoterium pinicola* from the holotype. 2. Various stages of apothecial development on *Pinus* needle. 3, 4. Squash mount of apothecia showing excipular cells one cell layer thick composed of chains of cells narrow at the basal end and enlarged at the apical end. 5. Branched paraphyses with enlarged apices. 6, 7. Asci, note empty ascus in 7 with ascospores released through a pore at the ascus apex; arrows indicate detached ascospore sheaths. 8–11. Ascospores with gelatinous sheath indicated by arrows. Bars: 2 = 500 μm, 3–11 = 20 μm.



FIGS. 2–11. Continued.

filiformes, obtusae vel subglobosae ad apices, ca. 2–5 µm latae, laeves, ramosae, aliquando matrice gelatinosa. Asci 50–80 × 7–10 µm (modus = 63 × 9 µm, n = 40), cylindrici-clavati, tenuitunicati, inoperculati, apice lato rotundato, non jodi caerulescens, ascosporis 8 uni- vel biseriatis. Ascosporae 12–18 × 4–5 µm (modus = 14 × 4 µm, n = 50), clavatae-cylindraceae, multiguttulatae, hyalinae, eseptata, vagina gelatinosa, ca. 1–3 µm lata.

Submerged aquatic discomycete. Apothecia on *Pinus* needles 95–200 × 125–240 µm, scattered, shiny white to cream colored when fresh (FIGS. 2–3), becoming brown on drying, superficial; cupulate, appearing convex in the center, becoming applanate toward the periphery, stipitate or sessile, if stipe present ca. 50–350 × 20–25 µm, cylindrical. Excipulum one cell layer thick, composed of a single layer of parallel hyphae composed of chains of cells narrow at basal end and enlarged at apical end, at the base they appear to be globose to isodiametric; cells ca. 13–15 × 5–7 µm, forming textura oblita (FIGS. 3–4). Paraphyses 40–72 × 2 µm, hyaline, filiform, obtuse to clavate at apex; apical region of paraphyses ca. 2–5 µm wide, smooth, branched (FIG. 5), occasionally in a gel matrix. Asci 50–80 × 7–10 µm (mean = 63 × 9 µm, n = 40), cylindric-clavate, thin-walled, inoperculate, with a broad, rounded apex; apical wall slightly thickened; apex MEZ-, with an apical slit, with 8 uniseriate or biseriate ascospores (FIGS. 6–7). Ascospores 12–18 × 4–5 µm (mean = 14 × 4 µm, n = 50), clavate-cylindric, multiguttulate, hyaline, aseptate, with a gelatinous sheath ca. 1–3 µm wide, which occasionally detaches in water (FIGS. 6–11), sheath staining blue with aqueous nigrosin; unstained sheath not seen in material preserved in glycerin or lactic acid. Colonies on CMA and PYG white to cream colored, growing slowly, reaching about 20 mm in 20 d. Mycelium composed of branched, septate, hyaline hyphae.

Etymology. From *Pinus*; substrate on which the fungus was found; cola L. = inhabiting. TYPE. UNITED STATES. FLORIDA: Liberty County, Apalachicola National Forest, unnamed swamp, 30°17′02″N, 84°50′25″W, water temperature 30 C, pH 6.4, on submerged *Pinus* needles, 12 Jul 2004, *Huzefa A. Raja* and *Christopher Brown, F47-1* (**HOLOTYPE designated here, ILL 40117**).

Additional specimens examined. UNITED STATES. FLORIDA: Ocala National Forest, unnamed swamp, 29°07′46″N, 81°37′34″W, water temperature 15 C, pH 5–5.5, on submerged *Pinus* needles, 20 Feb 2006, *Huzefa A. Raja* and *J.L. Crane, F47-2*; Chain O'Lakes, 29°07′57″N, 81°38′35″W, water temperature 17 C, pH 5–5.5, on submerged *Pinus* needles, 20 Feb 2006, *Huzefa A. Raja* and *J. L. Crane, F47-3*; Blackwater River State Forest, swampy area along Mason Branch Creek at Highway 191, 30°49′31″N, 86°54′53″W, water temperature 7 C, pH 5.5, on submerged *Pinus* needles, 12 Feb 2006, *Huzefa A. Raja* and *J.L. Crane, F47-4*; Callaway Swamp, 30°57′27″N, 86°59′48″W, water temperature 30 C, pH 5.5, on submerged *Pinus* needles, 7 Jul 2006, *Huzefa A. Raja* and *J.L. Crane, F47-5*; Big Coldwater Creek East Fork, 30°50′47″N, 86°59′02″W, water temperature 25 C, pH 5–5.5, on submerged *Pinus* needles, 7 Jul 2006, *Huzefa A. Raja* and *J.L. Crane, F47-6*; Archbold Biological Station on the Lake Wales Ridge, on submerged *Pinus* needles, 12 Jan 2000, *Carol A. Shearer, A424-1*.

Known distribution. USA (FL).

RESULTS

Maximum likelihood analyses of LSU sequences generated a single most likely tree (FIG. 1). This tree did not differ in topology from the single most parsimonious tree generated in unequally weighted maximum parsimony analyses (data not shown) except for the placement of *Bryoglossum gracile*. The placement of *A. pinicola* in the Helotiales was supported (FIG. 1) but its relationship to taxa in other genera and families in the Helotiales was not resolved.

DISCUSSION

Aquapoterium pinicola possesses several morphological characters that support its placement in order Helotiales based on molecular data. These characters include small, sessile to stipitate, white apothecia; interascal tissue of branched paraphyses with swollen tips; small, inoperculate, thin-walled asci with a MEZ-apical pore; and small, hyaline, smooth-walled ascospores (Kirk et al 2001). At present 13 families are recognized within the Helotiales (Eriksson 2005). Among these *A. pinicola* is most similar to taxa in the Helotiaceae in having small, glabrous, apothecia with an excipulum composed of parallel hyphae and ascospores that are hyaline and aseptate. *Aquapoterium pinicola* differs from the approximately 94 genera described in the family Helotiaceae (Eriksson 2005) in having small, white apothecia with a poorly developed subhymenium and an undifferentiated excipulum one cell layer thick and composed of parallel hyphae with cells narrow at the basal end and enlarged at the apical end (FIGS. 2–4). Although genera in the Helotiaceae have small apothecia and excipula composed of parallel hyphae, the ascomata frequently are brightly colored and the apothecial excipular tissue is made up of different texture types, characters not observed for *A. pinciola* (FIGS. 2–3).

Aquadiscula Shearer & J.L. Crane, *Crocicreas* Fr., *Cudoniella* Sacc., and *Hymenoscyphus* Gray are genera of Helotiaceae for which species have been reported previously from freshwater habitats (Tubaki 1966, Abdullah et al 1981, Fisher and Webster 1983, Descals et al 1984, Shearer and Crane 1985, Fisher and Spooner 1987, Sivichai et al 2003; see http://www.life.uiuc.edu/fungi/). Among these taxa *A. pinicola* is most similar to *Aquadiscula*, a freshwater discomycete genus reported from submerged leaves of *Acer rubrum* L. from a swamp in southern Illinois (Shearer and Crane 1985). *Aquadiscula appendiculata* Shearer & J.L. Crane has white to cream colored, stipitate apothecia composed of parallel septate hyphae with a poorly developed subhymenium, branched hyaline paraphyses and one-celled appendaged ascospores. *Aquapoterium pinicola* differs from *Aquadiscula appendiculata* in having a small apothecium (95–200 × 125–240 μm in *Aquapoterium pinicola* vs. 300–640 × 700–1300 μm in *Aquadiscula appendiculata*) with an excipulum that is composed of a single layer of parallel hyphae with cells narrow at the basal end and enlarged at the apical end. *Aquadiscula appendiculata* has an excipulum made up of textura porrecta and textura angularis tissues and cells of the excipulum are longer compared to *Aquapoterium pinicola*. Ascospores of *Aquapoterium pinicola* are surrounded by a gelatinous sheath, are single-celled, whereas in *Aquadiscula appendiculata* ascospores bear an apical gelatinous appendage and are 1-septate. In addition *Aquadiscula appendiculata* was collected from submerged leaves of an angiosperm, while *Aquapoterium pinicola* was found only on submerged *Pinus* needles. *Aquapoterium pinicola* is also similar to *Aquadiscula clavispora* Fallah & Shearer, a minute, hyaline freshwater discomycete reported from a Wisconsin lake (Fallah and Shearer 2001). However *Aquapoterium pinicola* differs from *Aquadiscula clavispora* in excipulum anatomy and ascospore morphology as well as the type of substrate on which it was found. *Aquadiscula clavispora* occurred on decaying stems of *Scripus atrovirens* Wild. whereas *Aquapoterium pinicola* was collected from submerged *Pinus* needles.

In our phylogenetic tree using LSU sequence data *Aquapoterium pinicola* groups with *Loramyces macrosporus*, *Vibrissea truncorum* and *Mollisia cinerea* (Vibrissea-Loramyces clade, sensu Wang et al 2006a) with a >95% Bayesian posterior probability but low bootstrap support. The Vibrissea-Loramyces clade consists of species belonging to three families, Loramycetaceae, Vibrisseaceae and Dermateaceae. *Aquapoterium pinicola* is morphologically dissimilar in all characters to species in these three families except Loramycetaceae, which includes species with ascospores that possess gelatinous sheaths and appendages. Although species in the Vibrissea-Loramyces clade are morphologically distinct from one another, species in these families are known from freshwater habitats (http://www.life.uiuc.edu/fungi/).

During our study we collected *Aquapoterium pinicola* seven times. It occurred in northern and central collection sites within the Florida peninsula and in one lotic and six lentic habitats at temperatures of 7–30 C and pH of 5–6.4. We did not observe an aquatic (Ingoldian), aeroaquatic or other anamorphic state for *A. pinicola*, unlike other taxa of discomycetes reported from freshwater (Webster 1992, Sivichai and Jones 2003) but the teleomorphic state might have adapted to the aquatic habitat by

being able to form apothecia in situ while submerged. Apothecia also developed on *Pinus* needles that were submerged in distilled water with an axenic culture of *A. pinicola* on CMA. Apothecia produced in submerged water culture were similar to those on natural substrates, except that stipes of the apotheica grew longer depending on the depth of distilled water used to submerge the *Pinus* needles in the deep glass Petri dishes (the deeper the water, the longer the stipes). We did not find *A. pinicola* on any substrate other than *Pinus* needles, suggesting that this fungus might be substrate specific. Because the *Pinus* needles on which the fungus was found were blackened, decomposed and detached into single needles, we were unable to determine the species identity of the substrate.

Aquapoterium pinicola was tested for the production of extracellular enzymes in vitro and was found positive for cellulase, endoglucanase, beta-glucosidase, xylanase, amylase, and polygalacturonase (Simonis, Raja and Shearer unpubl data), suggesting it might contribute to decay of *Pinus* needles in freshwater. Although positive for xylanase *A. pinicola* was negative for production of the lignin modifying enzymes, peroxidase and tyrosinase, and it did not cause soft-rot in balsawood.

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