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Life history and systematics of the aquatic discomycete *Mitrula* (Helotiales, Ascomycota) based on cultural, morphological, and molecular studies[1]

Zheng Wang,[2] Manfred Binder, and David S. Hibbett

Department of Biology, Clark University, 950 Main Street, Worcester, Massachusetts 01610 USA

Mitrula species represent a group of aquatic discomycetes with uncertain position in the Helotiales and an unknown life history. *Mitrula* species were studied using a combination of cultural, morphological, and molecular techniques. Pure colonies were isolated from *Mitrula elegans*, and conidia were induced in vitro. Herbarium materials from Europe, Asia, and North America were studied. Sequences of rDNA, including partial small subunit rDNA, large subunit DNA and ITS, were used to infer phylogenetic relationships both within *Mitrula* and between *Mitrula* and other inoperculate discomycetes, with special attention to fungi that resemble *Mitrula* in morphology or ecology. Equally weighted parsimony analyses, likelihood analyses, constrained parsimony analyses, and Bayesian analyses were performed. Results suggest that (1) the anamorph of *M. elegans* produces brown bicellular conidia, (2) a new subalpine species *M. brevispora* is distinct, (3) more than six lineages and clades can be recognized in *Mitrula*, (4) the morphological species *M. elegans* is not monophyletic, (5) a close relationship between *Mitrula* and either Geoglossaceae or Sclerotiniaceae is not supported, (6) the Helotiaceae is paraphyletic, and (7) *Mitrula* belongs to a clade within the Helotiales that also includes other aero-aquatic genera, *Cudoniella*, *Hydrocina*, *Vibrissea*, *Ombrophila*, and *Hymenoscyphus*.

Key words: aquatic fungi; decomposition; ecology; mitosporic fungi; vernal pools.

Mitrula Fr. species, the so-called swamp beacons, represent a group of unique nonlichenized discomycetes in the Helotiales. Relatively common in northern temperate forests, they are distributed from Europe to Asia and North America (Redhead, 1977). *Mitrula* species produce yellow to pinkish-orange, club-shaped spore-producing bodies (apothecia), which mostly occur on submerged, decaying vegetation in standing or slowly moving, shallow water. The apothecia emerge above the water level and are termed ''aero-aquatic.'' In North America, *Mitrula* species have been collected from April to October (Seaver, 1951; Redhead, 1977).

Ascomycetes generally have a stable haploid anamorph stage, with the ability to produce asexual spores (conidia). The mycelium produced by a single conidium can recycle the anamorph, or it can fuse with another haploid individual to produce the dikaryotic teleomorph stage. A huge number of conidia can be produced in asexual life cycles, and thus anamorphs play an important role in the distribution, dikaryotization, and fertilization of ascomycetes. Anamorphs associated with aquatic members in the Helotiales have been treated as an ecological group, the aquatic hyphomycetes, which also are known as amphibious or Ingoldian fungi, because they were first described by Ingold (1942).

Fungi play a major role in decomposition of wood and leaf litter in forest ecosystems. Many wood- and litter-decaying fungi are members of the Basidiomycota (Alexopoulos et al., 1995). However, the extracellular wood-decaying enzymes produced by Basidiomycota require aerobic conditions, and substrates submerged under water do not favor the growth of these fungi (Kirk and Farrell, 1987; Pointing et al., 2004). Ingoldian fungi degrade large amounts of leaf litter and woody debris in woodland streams and rivers using a form of decomposition that has been classified as ''soft rot,'' and which has been reported in Ascomycota and anamorphic fungi (Blanchette, 1994). In one study, 75% of the fungal biomass on underwater substrates was made up of ascomycetes (Nikolcheva et al., 2003). Thus, *Mitrula* and other Ingoldian fungi play an important role in nutrient cycling in certain freshwater ecosystems. Because of their important ecological roles, Ingoldian fungi have been studied extensively, and more than 300 species have been described since 1942 (Harrington, 1997).

Despite the attention they have received, the teleomorphs of most aquatic anamorphs remain poorly known. In the families of the Helotiales, there is little correlation between the classifications of teleomorphs and anamorphs (http://www.fm5web.life.uiuc.edu:23523/mitosporic/; Sutton and Hennebert, 1994). Many anamorphic fungi have never been observed to have a sexual life cycle in the field, and it is possible that they may have completely lost the sexual state through evolution.

Molecular methods have been used to investigate the linkages between anamorphs and teleomorphs (Pfister, 1997; Goodwin, 2002; Harrington and McNew, 2003). In the Helotiales, aero-aquatic species of *Cudoniella*, *Hymenoscyphus*, *Hydrocina*, *Loramyces*, *Ombrophila*, and *Vibrissae* have been reported to have aquatic anamorphs in the form-genera *Anavirga*, *Anguillospora*, *Articulospora*, *Helicodendron*, and *Tricladium* (Abdullah et al., 1981; Fisher and Webster, 1983; Descals et al., 1984; Digby and Goos, 1987; Fisher and Spooner,

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[2] Author for correspondence (e-mail: zwang@clarku.edu) additional address: Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, 110016, China.

1987; Hamad and Webster, 1988; Webster et al., 1995; Marvanová and Descals, 1996). Thousands of ascocarps of *Mitrula* have been collected, but an anamorphic state has not been reported in the life history.

The systematic position of *Mitrula* in the Helotiales also is uncertain. Because of the club-shaped apothecia, *Mitrula* species have been accepted as members of the family Geoglossaceae, which contains fungi commonly referred to as ''earth tongues'' (e.g., *Geoglossum glutinosum* Pers.: Fr.) (Imai, 1941; Dennis, 1968; Korf, 1973; Benkert, 1983). The concept of the Geoglossaceae has been modified extensively by recent studies and classifications (Hawksworth et al., 1995; Kirk et al., 2001), and it seems likely that the family will be restricted and excluded from a monophyletic Helotiales in future classifications (Platt, 2000; Pfister and Kimbrough, 2001; Z. Wang et al., unpublished manuscript). Based on ultrastructure of the ascus (a saclike cell containing ascospores) from selected taxa in the Helotiales, Verkley (1994) concluded that *Mitrula paludosa* Fr. is closely related to members of the Sclerotiniaceae, and this placement was accepted by some recent classifications (Hawksworth et al., 1995; Eriksson et al., 2004). However, based on internal transcribed spacer (ITS) rDNA sequences, *Mitrula elegans* (Berk.) Fr. was placed as the sister group of *Chloroscypha* species (Helotiaceae) instead of having a close relationship with *Sclerotinia sclerotiorum* (Lib.) de Bary (Sclerotiniaceae) (Gernandt et al., 1997). The family Helotiaceae was resolved as paraphyletic in a study based on 18s rDNA phylogeny (Gernandt et al., 2001), and the position of *Mitrula* in the family was accepted with a question mark in *The Dictionary of the Fungi*, vol. 9 (Kirk et al., 2001).

Fifty-five species names have been recorded under the genus *Mitrula* (http://www.indexfungorum.org). Some species described in *Mitrula* have been transferred to other genera, such as *Bryoglossum*, *Scleromitrula* (= *Verpatinia*), and *Heyderia* (Imai, 1941; Maas Geesteranus, 1964; Dennis, 1968; Korf, 1973; Redhead, 1977; Schumacher and Holst-Jensen, 1997). Morphological characters used for defining *Mitrula* species are limited and include characters of asci, ascospores, and apothecia. Noting that the measurements of asci and spores by different authors were not consistent, Imai (1941), Maas Geesteranus (1964), and Korf (1973) suggested that *Mitrula* is a monotypic genus, with *M. paludosa* as the sole species.

The present concept of *Mitrula* (Redhead, 1977) includes four species that are united by possession of orange, clavate, fleshy apothecia, a white stipe, inflated stipe hyphae, hyaline ascospores, an amyloid apical ascal tip, and an aquatic habitat. In Redhead (1977), characters of spores and apothecia, and geographic distributions were used to distinguish species. Four species were recognized, *M. elegans*, *M. borealis* S.A. Redhead, *M. lunulatospora* S.A. Redhead, and one European species *M. paludosa*, which may be referred to Asian material as well (Redhead, 1977). *Mitrula borealis* has a boreal and subalpine distribution in North America and Europe, while the other three species are believed to be restricted to shallow water at a low altitude. In addition to *M. borealis* and *M. paludosa*, two *Mitrula* species with dark brown or pink hymenophores, *M. multiforme* (E. Henning) Massee and *M. omphalostoma* E.-H. Benedix, were accepted in Europe by Benkert (1983). The genus *Bryoglossum* was erected based on the bryophilous (moss-inhabiting) species *Mitrula gracilis* Karsten. *Bryoglossum gracilis* (Karsten) S.A. Redhead may be closely related to *Mitrula* species, but shows some significant differences from the four *Mitrula* species in producing minute cauloscales (scale-like stipe hairs), having a gelatinized free margin, a yellowish stipe with narrow stipe hyphae, and releasing a yellow to reddish brown pigment in 10% KOH solution (Kankainen, 1969; Redhead, 1977).

The goals of this study were to (1) isolate *Mitrula* strains from field collections and induce its anamorphic stage in vitro, (2) evaluate the number of species within *Mitrula*, and (3) position *Mitrula* in the Helotiales using data from three rDNA regions, small subunit rDNA (ssu-rDNA), large subunit rDNA (lsu-rDNA), and 5.8S rDNA, from diverse taxa representing the major groups and many aero-aquatic genera in the Helotiales.

MATERIALS AND METHODS

Morphological and cultural studies—Morphological descriptions are based on observations of fresh, dried, or rehydrated specimens. Microscopic studies use squashed tissues. Measurements were made in Melzer's reagent (Korf, 1973) using bright field microscopy (Olympus CH-2, Olympus, Tokyo, Japan). Methods for collecting, isolating, and culturing *Mitrula* species basically followed Goos et al. (1986). Ascospores were collected in petri dishes on 2% malt extract agar sealed with parafilm, and resulting colonies were kept at room temperature (16–20°C) under ambient light. ITS sequences of the colonies were checked to detect contamination. Colonies were transferred into flasks containing sterilized leaves of *Fagus americana* Sweet. submerged in water, and were incubated at room temperature to induce sporulation. Mycelia were removed from the flasks onto moist filter paper and incubated under ambient light at room temperature for 2 weeks.

The nuclear condition of the mycelia was examined using the fluorescent stain 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI, Sigma, St. Louis, Missouri, USA). Sterile cover slides were placed close to selected colonies for a week, and the fungal cells were allowed to grow onto the slides. Attached fungal cells were fixed with formaldehyde solution (10% formaldehyde in 0.1% Triton X-100) overnight and washed twice with phosphate-buffered saline (PBS; pH 7.4, 138 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The cells were then resuspended in DAPI solution (1 : 10 000 DAPI in PBS) for 10 min in the dark followed by two washes with PBS. Slides with attached fungal cells were mounted with 50% glycerol solution and sealed with nail polish. Images were captured on a Nikon E600 microscope (Nikon, Augusta, GA, USA) with bright field and epifluorescence optics and a SPOT RT Slider cooled colored digital (CCD) camera using SPOT software (Diagnostic Instruments, Sterling Heights, Michigan, USA).

Molecular techniques—DNA was isolated from dried fruiting bodies and cultures. Approximately 20–30 mg of fungal tissue (herbarium materials) or a small amount of mycelia from cultures was ground in liquid nitrogen and extracted in 600 μL of extraction buffer (1% SDS, 0.15 M NaCl, 50 mM EDTA) at 75°C for 1 h, purified with phenol-chloroform-isoamyl alcohol (25 : 24 : 1), and precipitated with 95% ethanol and 3 M NaCl overnight. Crude DNA extracts were purified with GeneClean (Bio 101, La Jolla, California, USA). Cleaned DNA samples were diluted with distilled water up to 500 fold for use as PCR templates.

Sequence data were generated from three regions: (1) partial nuclear small subunit (nuc-ssu) rDNA bounded by primers PNS1 and NS41 (White et al., 1990; Hibbett, 1996), from 36 isolates of 31 genera; (2) partial nuclear large subunit (nuc-lsu) rDNA bounded by primers JS-1 and LR5 (Vilgalys and Hester, 1990; Landvik, 1996), from 47 isolates of 31 genera; (3) complete internal transcribed spacers 1 and 2 and the 5.8S rDNA (nuc-ITS rDNA) bounded by primers ITS1F and ITS4 (White et al., 1990), from 47 isolates of 31 genera. Sequences generated in this study were submitted to GenBank (accession numbers AY789276-AY789434). Twenty-nine additional sequences were downloaded from GenBank (Appendix 1).

Thirteen isolates of *Mitrula* were included in molecular studies, representing *M. lunulatospora*, *M. borealis*, and *M. elegans* from North America, *M.*

TABLE 1. Shimodaira-Hasegawa test was applied to determine whether the difference between the log-likelihood score (−lnL) of the best unconstrained maximum parsimonious tree and the −lnL of the best constrained maximum parsimonious trees (Diff −lnL) was statistically significant. The results rejected the alternative hypotheses of positioning *Mitrula* in either the Sclerotiniaceae or in the Geoglossaceae.

Tree	–lnL	Diff –lnL	*P* value
1. Nonconstrained tree as Fig. 4a	11583.95262	Best	
2. Force *Mitrula* in Sclerotiniaceae	11708.90576	124.95314	<0.001
3. Force *Mitrula* in Geoglossaceae	11736.24114	152.28853	<0.001
4. Force *Mitrula* in Geoglossaceae	11732.54783	148.59521	<0.001
5. Force *Mitrula* in Geoglossaceae	11729.76845	145.81583	<0.001

paludosa from Europe, and a subalpine collection from China that is described here as *M. brevispora* sp. nov. Of the 15 families accepted in the recent Helotiales classification (Kirk et al., 2001), 11 families, including Bulgariaceae, Leotiaceae, Cudoniaceae, Geoglossaceae, Hemiphacidiaceae, Rutstroemiaceae, Sclerotiniaceae, Helotiaceae, Hyaloscyphaceae, Vibrisseaceae, and Dermateaceae were sampled. Species of aquatic genera such as *Vibrissea*, *Hydrocina*, *Cudoniella*, and *Ombrophila* also were included, as were species with morphological similarity to *Mitrula*, such as *Bryoglossum gracile*, *Heyderia abietis* (Fr.) Link, and *Scleromitrula shiraiana* (Henn.) S. Imai. Species from Rhytismatales, Dothideales, Mycocaliciales, and Pezizales were sampled as well.

PCR reaction mixes (Promega Corp., Madison, Wisconsin, USA) contained 2.5 μL 10× PCR buffer, 5 μM dNTP, 12.5 pM of each PCR primer, and 5 μL DNA in 25 μL. The amplification program included 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. PCR products were purified using Pellet Paint (Novagen, Madison, Wisconsin, USA) and sequenced using the ABI Prism BigDye-terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's protocols. Primers used for sequencing were PNS1, NS19bc, NS19b, NS41, JS-1, LR3, LR3R, LR5, ITS1F, and ITS4 (Vilgalys and Hester, 1990; White et al., 1990; Landvik, 1996). Sequencing reactions were purified using Pellet Paint and were run on an Applied Biosystems 377XL automated DNA sequencer. Sequences were edited with Sequencher version 3.1 (GeneCodes Corp., Ann Arbor, Michigan).

Phylogenetic analyses—Two data sets were prepared, one for higher-level analyses (HLA) and one for lower-level analyses (LLA). The data set for HLA included sequences of 36 isolates from three genes, nuc-ssu rDNA, nuc-lsu rDNA and 5.8S rDNA. The HLAs were intended to resolve the placement of *Mitrula* in the Helotiales. The HLA data set was rooted using *Neolecta irregularis* (Perk) Korf & J.K. Rogers (Landvik, 1996). The nuc-ssu rDNA sequences of *Ciboria* sp. G36, *Bisporella* sp. G39, and *Scleromitrula shiraiana* G123 were about 360–560 base pairs (bp) shorter than sequences of the other taxa. The nuc-lsu rDNA sequence of *Rutstroemia bolaris* (Batsch) Rehm was about 527 bp shorter than sequences of other taxa.

The data set for the LLA included sequences of nuc-lsu rDNA and ITS from 24 isolates. LLA were intended to resolve the relationships among *Mitrula* species. *Mitrula* lsu-rDNA and ITS sequences were used as BLAST queries of the GenBank database, but no sequences with high similarity (>95%) were found. The LLA data set was rooted using *Heyderia abietis* based on the results of the HLA.

Sequences were aligned with ClustalX (Thompson et al., 1997) and adjusted by eye in the data editor of PAUP* 4.0b (Swofford, 2002). Alignments are available at TreeBASE (accession nos. M2285 and M2286). Both data sets were analyzed in PAUP* 4.0b (Swofford, 2002) and MrBayes 3.0 (Huelsenbeck and Ronquist, 2001), with gaps treated as missing data. Ambiguously aligned positions were excluded from the data sets before performing the analyses.

Parsimony analyses were performed using equal weighting of characters and transformations. Heuristic searches were performed with 1000 replicate searches, each with a random taxon addition sequence, MAXTREES set to autoincrease, and tree-bisection-reconnection (TBR) branch swapping. A bootstrap analysis was performed with 1000 replicates, each with 10 random taxon addition sequences, MAXTREES set to autoincrease, and TBR branch swapping.

Likelihood analyses were performed using a model and model parameters estimated with Modeltest version 3.5 (Posada and Crandall, 1998) and MrModeltest 2.0 (Nylander, 2004). A likelihood command block was copied from MrModeltest 2.0, which configures PAUP* to search for a maximum likelihood (ML) tree under the GTR + Γ + I model with 100 heuristic search replicates, each with a random taxon addition sequence, MAXTREES set to autoincrease, and TBR branch swapping. The rate matrix (A-C, 0.722900; A-G, 2.077300; A-T, 0.939200; C-G, 0.801700; C-T, 5.015800; G-T, 1.000000), assumed nucleotide frequencies (A = 0.26370, C = 0.22000, G = 0.27150, T = 0.24480), assumed proportion of invariable sites (=0.424) and gamma distribution (shape parameter alpha = 0.5056) were estimated by hLRT in MrModeltest 2.0.

Constrained analyses were performed using trees constructed with constraint command in PAUP* to test two alternative hypotheses: (1) *Mitrula* species are members of Sclerotiniaceae. In this case, a group including two *Mitrula* species, *Ciboria*, *Sclerotinia*, *Monilinia*, *Scleromitrula*, and *Rutstroemia* was forced to be monophyletic. (2) *Mitrula* belongs to the family Geoglossaceae. A group including *Mitrula*, *Geoglossum* and *Trichoglossum* was forced to be monophyletic. Tree searching in the constrained analyses used the same parsimony analyses settings described previously. Equally parsimonious trees from unconstrained and constrained analyses were compared using the SH test in PAUP* (Shimodaira and Hasegawa, 1999; Goldman et al., 2000). The SH test was performed for 1000 replicate bootstrap data sets by using full likelihood optimizations. Based on the overall likelihood value, trees significantly different ($P < 0.05$) from the best tree were rejected (Table 1).

Bayesian posterior probabilities were computed using the Metropolis-coupled Markov chain Monte Carlo method (MCMCMC) under the GTR + Γ + I model in MrBayes 3.0 by running four chains with 500 000 generations using the program default priors on model parameters. Trees were sampled every 100 generations. Likelihoods converged to a stable value after 10 000 generations, and all trees prior to this convergence were discarded as the ''burn-in'' phase before computing a majority rule consensus tree in PAUP*.

The same analytical settings of parsimony analyses as those for the HLA were applied to the LLA for combined nuc-lsu rDNA and ITS data.

RESULTS

Mitrula brevispora Zheng Wang sp. nov. (Fig. 1)—Ascoma solitaria, 14–32 mm altum. Ascogenea forma cylindracea, subalbea vel lutea, 4–12 × 2–4.5 mm. Stipes 0.8–1 mm crassus. Hymenium 97–102 μm crassus. Asci cylindraceo-clavati, apice attenuati, jodo abturaculo minutissimo caerulescentes, 4.9–6.7 μm crassus. Paraphyses filiformes, 2.4–2.6 μm crassus. Sporae 8: ellipticae vel subfusoideae, 2.4–3.1 5.5–10.1 μm.

Holotypus: On mossy soil and dead foliage in coniferous Forests. Satan forest farm (2500 m a.s.l.), Bailongjiang River, Gansu, China, 9 July 2002; leg. Zheng Wang No. ZW02-012 (Clark University)

Ascocarps solitary, flesh, 14–32 mm high. Ascogenous part cylindrical to slightly clavate, smooth, beige to yellow, 4–12

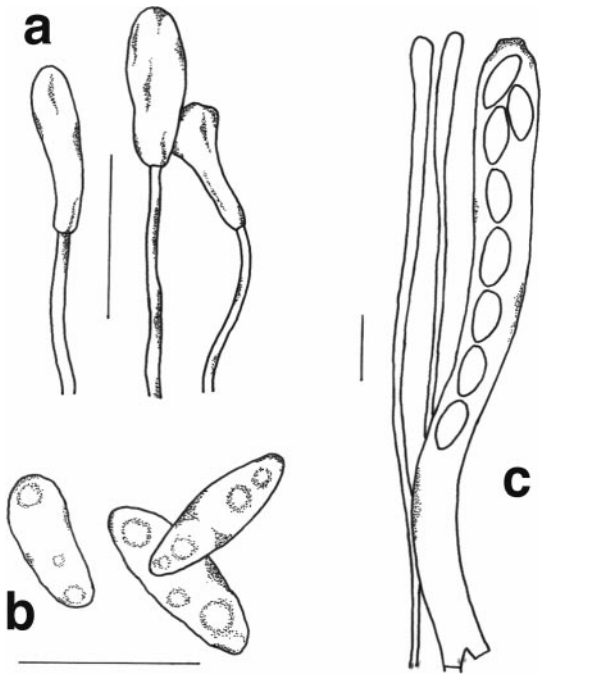

Fig. 1. Illustration of *Mitrula brevispora* based on specimen ZW02-012. (a) Ascocarps. (b) Ascospores. (c) Ascus and paraphyses. Scale bars: a = 10 mm; b, c = 10 μm.

× 2–4.5 mm. Stipe unbranched, 0.8–1 mm wide, hyaline to white. Hymenium about 97–102 μm thick. Asci eight-spored, elongate-clavate, 4.9–6.7 μm wide, croziers present, apical pores amyloid. Paraphyses filiform, slightly enlarged above, 2.4–2.6 μm wide. Ascospores elliptical-fusoid to broadly cylindrical, one-celled, bi-guttulate to multi-guttulate, rarely with a gelatinous sheath, 2.4–3.1 × 5.5–10.1 μm. Subhymenium not distinct. Clavula medulla hyphae 4.8–17 μm wide. Stipe medulla hyphae 14–24 μm wide, stipe inner cortical hyphae 3.5–7.5 μm wide. On mossy soil and dead foliage in coniferous forests in alpine or subalpine areas.

Additional specimens examined: On duff in conifer forests. Satan Forest farm (3300 m a.s.l.), Bailongjiang River, Gansu, China, 10 July 2002; leg. Zheng Wang No. ZW02-020 (Clark University).

Pure cultures and conidia of Mitrula elegans—Ascospores from two isolates, G45 from Massachusetts and G146 from West Virginia, germinated on 2% malt extract agar (MEA) plates in 24 h with germ tubes extending from both ends of the ascospores. All single spore isolates grew slowly, and colonies from single spores grew poorly even after transfer onto a new MEA plate. Multispore cultures reached 10 mm in diameter in 3 to 5 days at 16–20°C and became orange to brownish-orange because of brown incrustations produced by some cells. The function of brown incrustations and brown-walled cells is unknown. Brown pigments were detected in the medium as well. Hyphae became dark colored when the media became dry. Some cells became swollen to form chains of chlamydospore-like structures, and hyphae were found to grow out from those chlamydospore-like cells with or without rehydrating the media (Fig. 2a). A single nucleus was revealed in each cell by DAPI (Fig. 3).

Mycelia from multispore cultures that were transferred onto the submerged leaf litter in the flasks, colonized the substrate and formed a thick layer of white hyphae on the water–air interface within a week. Brown pigments were detected in the water, but no hyphae with brown incrustations were observed. No conidia were detected from the water or the foam created by shaking the flasks, although conidia of aquatic hyphomycetes are frequently collected from foam in the field. After 2 to 3 weeks, hyphae of *Mitrula elegans* covered the substrate. Club-shaped, brown conidia chains, from which mature bicel-

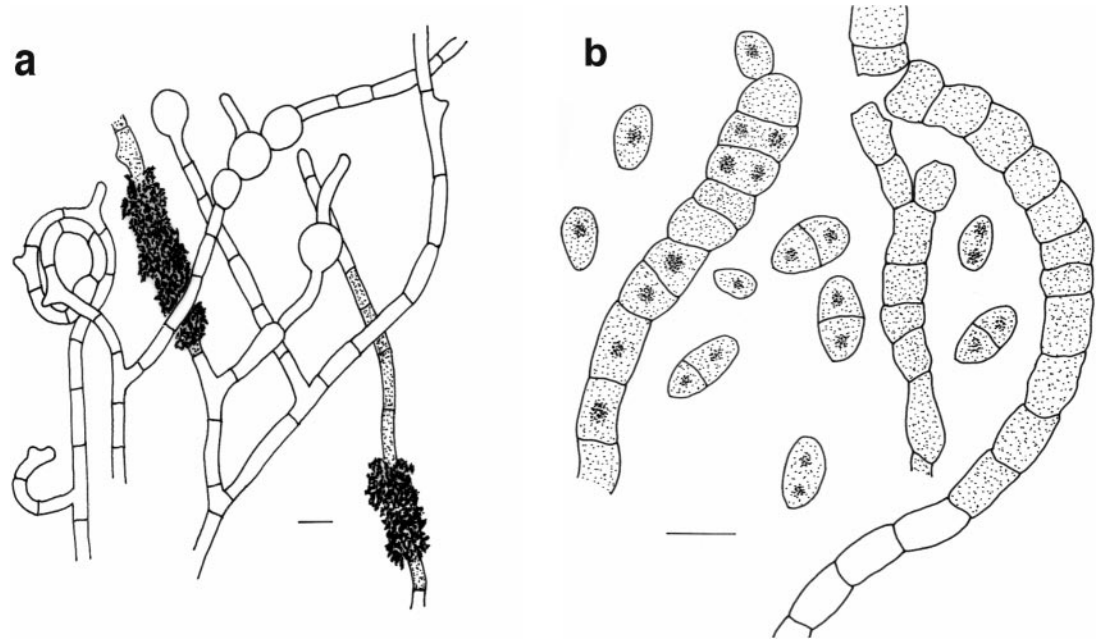

Fig. 2. Illustration of the anamorph of *Mitrula elegans*. (a) Somatic hyphae. (b). Conidia with some cells showing a darker spot inside. Scale bars = 10 μm.

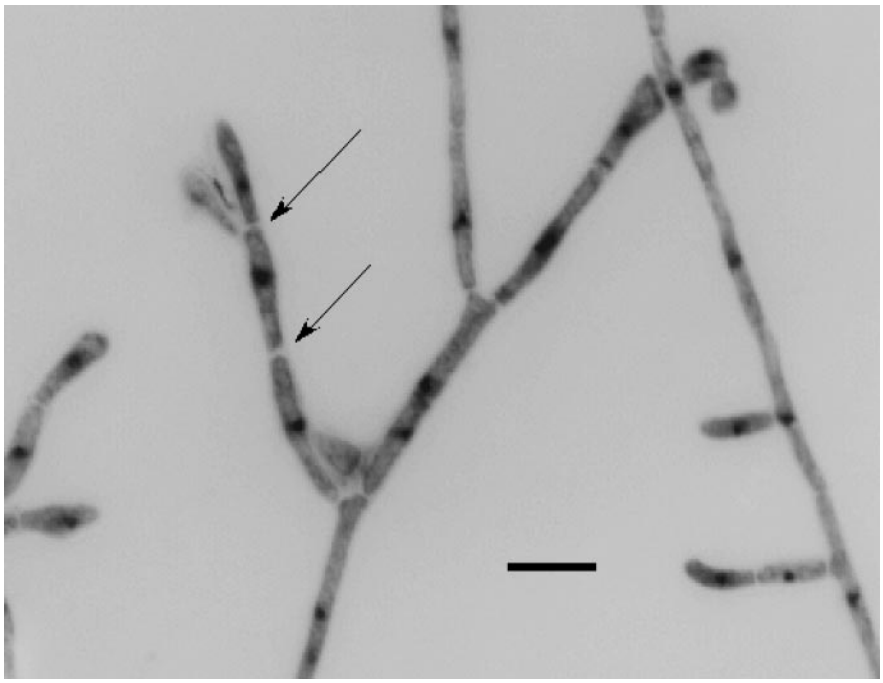

Fig. 3. Light micrograph after staining with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) shows monokaryotic hyphae obtained from culture of *Mitrula elegans*. Two septa are indicated with arrows. Scale bar = 10 μm.

lular conidia were produced, appeared in the leaf tissue and on the surface of the substrates a month after inoculation (Fig. 2b). Hyphae transferred from the flasks to sterilized filter paper in a moist petri dish produced the same anamorphic stage. ITS sequences of mycelia samples drawn from each step during the culturing confirmed the identity of *M. elegans*.

Anamorph of *Mitrula elegans*: Aquatic. Conidiophores not distinguishable from the vegetative hyphae. Conidiogenous cells terminal, hyaline, integrated. Conidia in early stage solitary, dark brown, filiform, septate, sometimes dichotomously branched, often of highly variable length, 10–20 μm wide. Mature conidia may be released by disarticulation of a septum or may simply be cleaved from the conidiogenous cell, brown to dark brown, ellipsoid to ovoid, bicellular, septa becoming distinct during development, 12–17 × 7.0–7.5 μm. No germination of the conidia was observed.

Phylogenetic inference from the HLA data set—The placement of *Mitrula* was estimated using three rDNA regions (lsu + ssu + 5.8S; Fig. 4). The combined genes had an aligned length of 2024 base pairs (22 positions were excluded from the analyses) with 220 uninformative variable positions and 395 parsimony-informative positions.

Equally weighted parsimony analysis based on combined rDNA genes generated a single maximum parsimony tree of 1688 steps and consistency index CI = 0.511 (Fig. 4a). *Mitrula*, represented by the boreal species *M. brevispora* and the aquatic *M. paludosa*, was strongly supported as monophyletic (bootstrap [BP] = 100%). A clade including three aquatic genera *Mitrula*, *Vibrissea* (Vibrisseaceae), and *Hydrocina* was resolved, but without strong support (BP < 50%). *Bryoglossum* was placed as the sister group of *Lachnum* (Hyaloscyphaceae) with strong support (BP = 92%). The family Helotiaceae was not monophyletic, and members of the family were present in 5 clades. The BSA clade (bright-colored, saprotrophic and aquatic), including species of *Bisporella*, *Chloroscypha*, *Ascocoryne*, *Mitrula*, and *Vibrissea*, was not supported by the bootstrap value (<50%), but it can be characterized by the bright hymenophore color (usually yellow to orange yellow), saprotrophic nutrition, and hygrophilous that are common in aquatic or highly humid habitats. The family Sclerotiniaceae was monophyletic (BP = 71%) and was nested in a clade with Rutstroemiaceae, Hemiphacidiaceae, and *Heyderia abietis* with 90% bootstrap support. The family Geoglossaceae was placed as a basal branch of the inoperculate ascomycetes and is not closely related to any of the other families in the Helotiales.

Maximum likelihood analysis based on the HLA data set under the GTR + Γ + I model generated two trees of equal likelihood value (−lnL = 11383.91954). The majority rule consensus tree of 4900 Bayesian trees under the same model supported the same tree topology as the maximum likelihood analysis, which differed from the MP tree mainly by the placement of *Mitrula* and the Rhytismatales (Fig. 4b). However, the conflict between MP and ML trees concerning the placement of *Mitrula* was not supported by high bootstrap or posterior probability (PP) values. In the ML tree (Fig. 4b), the genus *Mitrula* was supported as monophyletic (PP = 1.0) and was weakly supported (PP = 0.68) as the sister group of a clade including *Ascocoryne* and *Chloroscypha*. Members of the family Helotiaceae were present in four clades in the ML tree. The families Sclerotiniaceae, Rutstroemiaceae, and Hemiphacidiaceae and *Heyderia abietis* formed a well supported clade (PP = 1.0). In agreement with the result of MP analyses, the Geoglossaceae formed a basal branch of the inoperculate ascomycetes in the ML tree.

Constrained analyses under the assumption that *Mitrula* is a member of the Sclerotiniaceae generated a single most parsimonious tree. Constrained analyses under the assumption that *Mitrula* is a member of the Geoglossaceae generated three

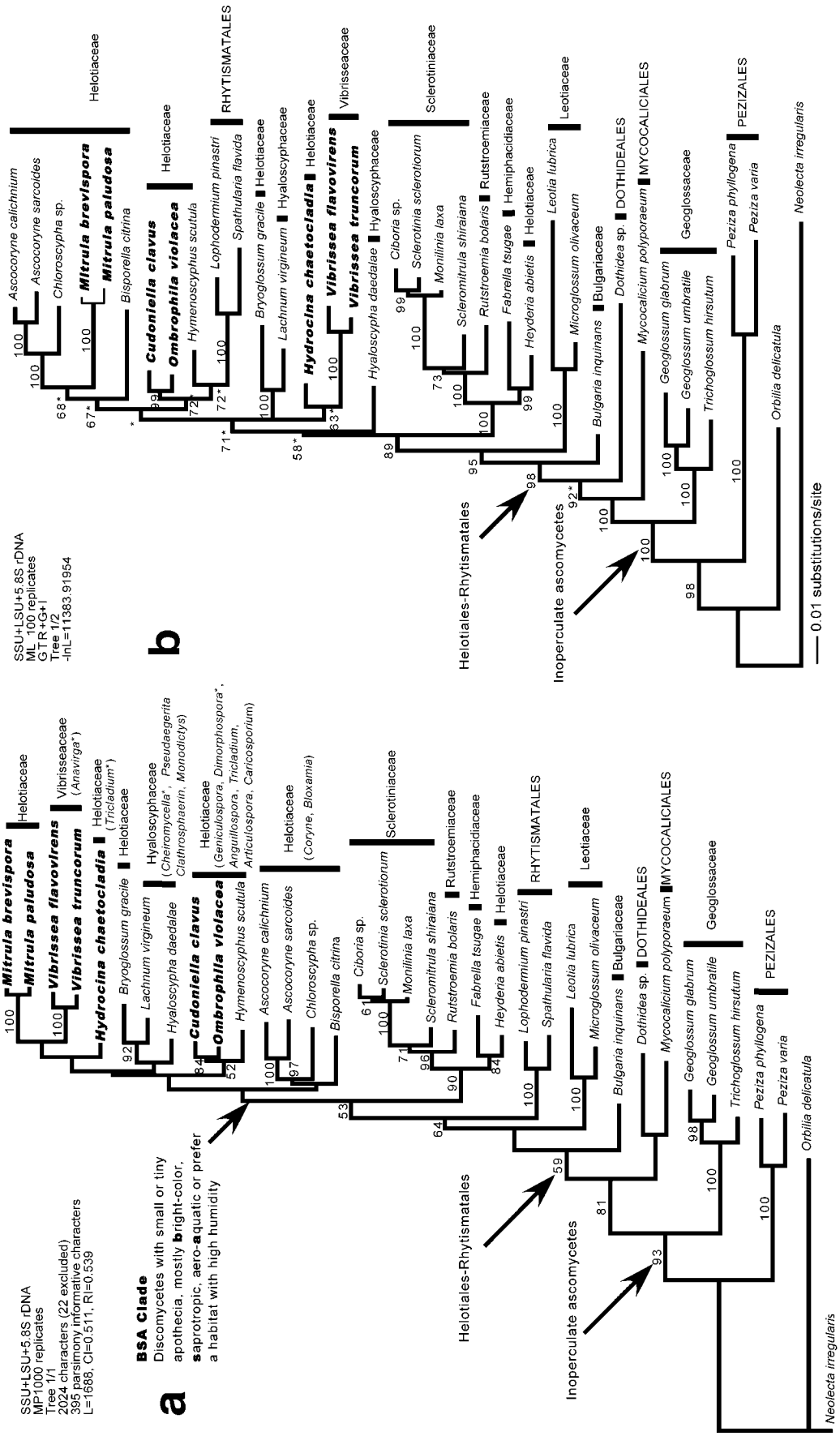

Fig. 4. Higher-level phylogenetic analyses of *Mitrula*. Aero-aquatic species are in bold type. Family level classifications of the clades are based on *The Dictionary of the Fungi*, vol. 9 (Kirk et al., 2001). (a) Higher-level phylogenetic relationships of *Mitrula* inferred with three rDNA regions parsimony analysis. The most parsimonious tree (length = 1688, CI = 0.511, RI = 0.539). Known anamorphs in the BSA clade (bright-colored, saprotrophic, aquatic) are listed next to the related teleomorph clades. Anamorphs with a microconidial synanamorph are followed by an asterisk. Bootstrap values greater than 50% are indicated along nodes. (b) Higher-level phylogenetic relationships of *Mitrula* inferred from three rDNA regions using maximum likelihood and Bayesian approaches. The majority rule consensus of 4900 the Metropolis-coupled Markov chain Monte Carlo method (MCMCMC) sampled trees supports the topology of the maximum likelihood tree (−lnL = 11383.91954). Group frequencies greater than 50% are indicated as posterior probability (%) along nodes. Branches not present in MP tree are marked with an asterisk.

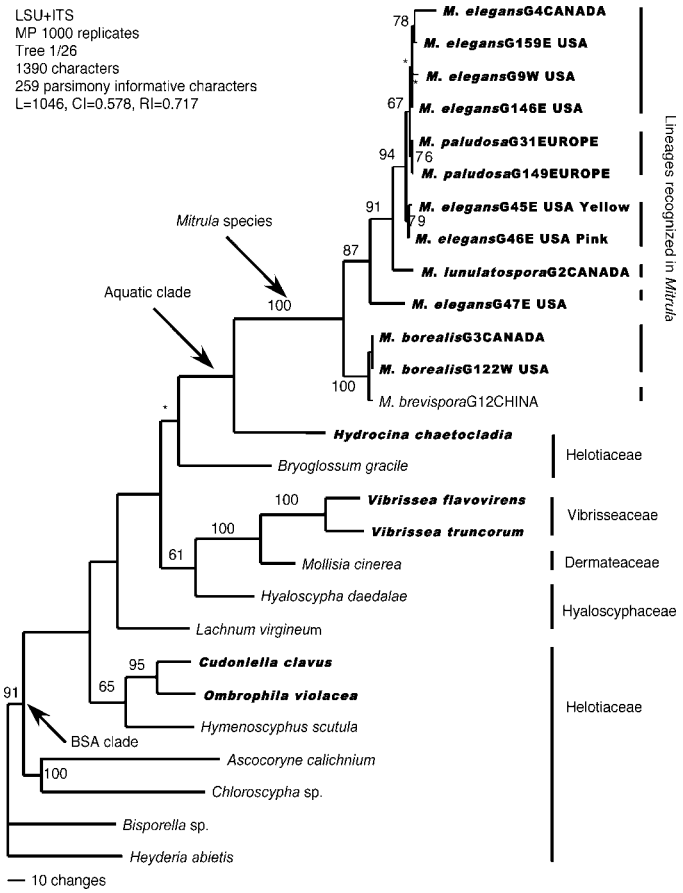

Fig. 5. Lower-level phylogenetic relationships of *Mitrula* inferred from lsu-rDNA and ITS data using parsimony analysis. One of the 26 equally parsimonious trees (length = 1046, CI = 0.578, RI = 0.717). Bootstrap values greater than 50% are indicated along nodes, and nodes that collapse in the strict consensus tree are marked with an asterisk above the branch.

equally parsimonious trees. Compared with the maximum parsimony tree generated from the unconstrained analyses, trees under both assumptions were rejected by the S-H test (Table 1).

Phylogenetic inference from the LLA data set—The relationships among *Mitrula* species were inferred using two rDNA regions (lsu + ITS). The combined data had an aligned length of 1390 base pairs with 143 uninformative variable positions and 259 parsimony-informative positions. Equally weighted parsimony analysis based on this data set generated 26 equally parsimonious trees of 1046 steps and consistency index (CI) = 0.578 (Fig. 5). All *Mitrula* isolates formed a clade (BP = 100%), and the aquatic species *Hydrocina chaetocladia* was the sister group to *Mitrula* species, but with weak support (BP < 50%). More than six lineages were detected among *Mitrula* species. Isolates of *M. borealis* from North America differed by 4 bp in the ITS region with *M. brevispora* from China, and these three subalpine species were well supported as a clade (BP = 100%). *Mitrula lunulatospora* and *M. elegans* G47 from eastern USA formed two independent lineages. Eight isolates formed a monophyletic group (BP = 94%), including six isolates of *M. elegans* from North America and two isolates of *M. paludosa* from Europe. *Mitrula paludosa* is monophyletic with moderate support (BP = 76%). Except for *M. elegans* G4 collected from Canada, isolates in the *M. elegans-M. paludosa* clade differed by less than 2 bp. Two isolates of *M. elegans* with different hymenophore colors, G45 (yellow) and G46 (pink), were from the same locality and only 1 bp differed.

DISCUSSION

Life history of Mitrula—The asexual state of *Mitrula elegans* has been successfully maintained in vitro, and an asexual sporulating aquatic stage has been observed to produce bicellular, brown conidia. Plasmogamy and dikaryotic hyphae have not been observed, and no apothecia have been produced from the *Mitrula* cultures. Hyphae from the multispore colonies of *Mitrula* are uninucleate and are probably haploid. However, the possibility that they are diploid cannot be ruled out, although this would be very unusual for ascomycetes. The bicellular, brown conidia that are released from club-shaped conidia chains in *Mitrula elegans* are unknown in other aquatic hyphomycetes in the Helotiales. Dark aerial and septate conidia (*Anavirga dendromorpha* Descals & Sutton) were reported for *Vibrissea flavovirens* (Hamad and Webster, 1988). Conidia from aquatic species *Hymenoscyphus malawiensis* P.J. Fisher & Spooner are somewhat similar to that of *M. elegans*, but conidia of *H. malawiensis* are hyaline and multicellular (Fisher and Spooner, 1987).

Spores of some aquatic hyphomycetes are usually branched or sigmoid, which is presumably an adaptation to aquatic habitats in flowing water. The conidia of *M. elegans* do not appear to be able to float in the water and may be an adaptation to aquatic habitats with standing or slow-moving water, such as vernal pools. Vernal pools provide unique systems for the wetland species, particularly aquatic plants (Keeley and Zedler, 1998; Weyembergh et al., 2004). Because the major carbon source for streams and forested pools is not in-pool photosynthesis but leaf litter from the surrounding forest (Allan, 1995). Thus, aero-aquatic fungi such as *Mitrula* species are important in carbon flow in vernal pools and similar habitats.

Teleomorphs in the Helotiales, such as *Vibrissea flavovirens* (Pers.) Korf & J.R. Dixon, *Mollisia gigantea* P.J. Fisher & J. Webster, and *Hydrocina chaetocladia* Scheuer, were found in association with phialophora-like anamorphs (microconidial synanamorph), which may have a spermatial function and can fertilize monokaryotic or dikaryotic hyphae produced by other conidia or sclerotia (Fisher and Webster, 1983; Hamad and Webster, 1988; Webster et al., 1991; Harrington and McNew, 2003). No microconidial synanamorph was observed from the cultures of *M. elegans*.

Position of Mitrula in the Helotiales—Phylogenetic analyses indicate that *Mitrula* is a member of the Helotiales-Rhytismatales clade and may be closely related to the small, brightly colored, saprotrophic, and aquatic or hygrophilous members in the Helotiaceae. The precise placement of *Mitrula* requires a reclassification of the Helotiaceae and the Helotiales, which needs to include many more taxa.

Both parsimony and likelihood analyses suggested that the Helotiales-Rhytismatales is monophyletic and that there were multiple origins of aero-aquatic habitat in the Helotiales (Fig. 4). The BSA clade, which is recognized without strong support, includes aero-aquatic species of *Vibrissea*, *Hydrocina*, *Cudoniella*, and *Ombrophila* and, in addition, *Ascocoryne*, *Bisporella*, *Hyaloscypha*, and *Hymenoscyphus* species, which

are associated with highly humid habitats. Various anamorphs are known in the BSA clade, but the anamorphs are not consistent with the phylogeny of related teleomorphs (Marvanová, 1997). More taxa and molecular data from additional loci are necessary to fully understand the evolution of these fungi.

In the HLA parsimony tree, *Mitrula* species share a clade with other two aquatic genera, *Vibrissea* and *Hydrocina*. *Vibrissea* species are found on substrate submerged in quickly moving streams, and produce filiform (thread-like) ascospores up to 470 μm in length (Sánchez and Korf, 1966; Korf, 1990; Iturriaga, 1997). *Hydrocina chaetocladia* produces minute, cup-shaped fruitbodies on submerged twigs and produces conidia of *Tricladium chaetocladium* and a microconidial synanamorph (Webster et al., 1991).

The Helotiaceae is not a monophyletic group based on our results. Presently, the Helotiaceae is a large family with about 100 genera, more taxa should be included for clarifying the phylogenetic structure in this family, which should be restricted to a much narrower sense as suggested by Kirk et al. (2001). Previous hypotheses of relationships between *Mitrula* species and either members of the Geoglossaceae or species of the Sclerotiniaceae (Korf, 1973; Verkley, 1994) were rejected. The Geoglossaceae occurs as a basal branch of inoperculate ascomycetes, and it should be excluded from the Helotiales. The Sclerotiniaceae, Rutstroemiaceae, Hemiphacidiaceae, and *Heyderia* are closely related, and there are some *Mitrula*-like fungi in this clade. Bryophilous *Bryoglossum* is closely related to *Lachnum* rather than *Mitrula*. No data of the aquatic family Loramycetaceae in the Helotiales were available.

Species of Mitrula—This study partially supports the morphological species delimitation of *Mitrula* in North America by Redhead (1977). The isolates representing *M. borealis*, *M. lunulatospora*, and *M. elegans*, *M. brevispora* from Asia, and *M. paludosa* from Europe, represented more than six lineages, whereas Redhead (1977) divided *Mitrula* into four species.

Two isolates of *Mitrula borealis* from North America (including the isotype) and the new species *M. brevispora* from China, formed a basal clade among *Mitrula* species. *Mitrula borealis* has also been reported from Europe (Redhead, 1977), but no recent collections are available. The Chinese collection is the first record of a subalpine species of *Mitrula* in Asia. Despite the small genetic divergence (4 bp) between *M. borealis* and *M. brevispora* based on partial lsu-rDNA and ITS sequences, the latter is significantly different morphologically from *M. borealis*. *Mitrula brevispora* occurs on mossy substrates, which are not submerged in water, and produces ascocarps that are more cylindrical rather than clavate, with much smaller ascospores than those of *M. borealis* (5.5–10.1 × 2.4–3.1 μm vs. 10.5–18 × 2.5–5 μm). Thus, the recognition of *M. brevispora* as a distinct species is warranted, although it is a close relative of *M. borealis*, based on rDNA sequences.

Mitrula elegans represented by seven isolates from eastern and western North America is not monophyletic based on our analyses. Isolate G47 collected from eastern North America fits the description of *M. elegans* morphology well, but it is not closely related to the other isolates in the clade that includes *M. elegans* isolates. Given the conservation of lsu-rDNA and ITS regions observed in the *M. borealis*–*M. brevispora* clade, the variation among *M. elegans* isolates is fairly high. Two European collections of *M. paludosa* showed a single base-pair difference to North American *M. elegans* in the ITS region. *Mitrula paludosa* has been reported from Asia as well (Imai, 1941; Teng, 1996), but no recent Asian collections are available to verify this occurrence with molecular methods. The close relationship between *M. elegans* in North America and *M. paludosa* in Europe suggested by previous morphological studies is supported by the molecular data.

Conclusions—In vitro cultural studies reveal that the life cycle of the aero-aquatic *Mitrula elegans* involves production of brown bicellular conidia, which may be an adaptation to habitats with standing or slow-moving water and vernal pools. Phylogenetic analyses based on three rDNA regions (lsu, ssu, and 5.8S) reject previously proposed relationships between *Mitrula* and Sclerotiniaceae or Geoglossaceae and imply multiple origins of the aquatic habitat in the Helotiales. More than six lineages can be recognized in *Mitrula* based on molecular data and morphological characters. Low variation is evident at both the ITS and lsu rDNA loci among species with significant morphological differences, but comparatively high molecular divergences are obvious at the same loci within the morphological species *M. elegans*. Further morphological, ecological and molecular studies are required to clarify the phylogenetic relationships both within *Mitrula* and within the Helotiales and to reconstruct the evolution of *Mitrula* in the aero-aquatic habit.

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APPENDIX 1. Taxa, vouchers, and GenBank accession numbers for all sequences analyzed with a dash (—) for missing information or unpublished data. Sequences of isolates (GenBank) were downloaded from GenBank. Sequences of isolates (AFTOL: Assembling Fungal Tree Of Life) were downloaded from the AFTOL database (http://aftol.biology.duke.edu/pub/dataIndex). After each *Mitrula* species is the tracking number (starting with the letter G) that is used in the lower-level analyses (Fig. 5).

Taxon; *voucher*; GenBank accession numbers: ssu-rDNA; lsu-rDNA; ITS.

Ascocoryne calichnium (Tul.) Korf; *PDD 75671*; AY789393; AY789394; AY789395.

Ascocoryne sarcoides (Jacq.) J.W. Groves & D.E. Wilson; *ZW-G92-CLARK*; AY789387; AY789388; AY781216.

Bisporella citrina (Batsch) Korf & S.E. Carp.; *ZW-G39-CLARK*; AY789324; AY789325; AY789326.

Bryoglossum gracile (P. Karst) Redhead; *MBH 52481*; AY789419; AY789420; AY789421.

Bulgaria inquinans (Pers.) Fr.; *ZW-G52-CLARK*; AY789343; AY789344; AY789345.

***Ciboria* sp.**; *WZ-JXD-10*; —; —; —.

***Chloroscypha* sp.**; *GenBank*; AY544700; AY544656; U92312.

Cudoniella clavus (Alb. & Schwein.) Dennis; *BM 18#13*; AY789372; AY789373; AY789374.

***Dothidea* sp.**; *GenBank*; AY016343; AY016360; AF027764.

Fabrella tsugae (Farl.) Kirschst.; *GenBank*; AF106015; AF356694; U92304.

Geoglossum glabrum Pers.; *OSC 60610*; AY789316; AY789317; AY789318.

Geoglossum umbratile Sacc.; *NIFG-mycorec 1840*; AY789302; AY789303; AY789304.
Heyderia abietis (Fr.) Link; *HMAS 71954*; AY789295; AY789296; AY789297.
Hyaloscypha daedalae Velen.; *ZW-G138-CLARK*; AY789414; AY789415; AY789416.
Hydrocina chaetocladia Scheuer; *HME 4375*; AY789411; AY789412; AY789413.
Hymenoscyphus scutula (Pers.) W. Phillips; *MBH 29259*; AY789430; AY789431; AY789432.
Lachnum virgineum (Batsch) P. Karst.; *GenBank*; AY544688; AY544646; AJ430221.
Leotia lubrica (Scop.) Pers.; *ZW-G59-CLARK*; AY789358; AY789359; AY789360.
Lophodermium pinastri (Schrad.) Chevall.; *GenBank*; AF106014; AY004334; AY775701.
Microglossum olivaceum (Pers.) Gillet; *FH-DSH97–103*; AY789396; AY789397; AY789398.
Mitrula borealis Redhead G3; *DAOM 160731*; —; AY789281; AY789282.
Mitrula borealis Redhead G122; *UWH-LLN930920–1*; —; AY789404; AY789405.
Mitrula elegans Berk. G4; *DAOM 166763*; —; AY789283; AY789284.
Mitrula elegans Berk. G9; *OSC 49859*; —; AY789286; AY789287.
Mitrula elegans Berk. G45; *ZW-G45-CLARK*; —; AY789330; AY789331.
Mitrula elegans Berk. G46; *ZW-G46-CLARK*; —; AY789332; AY789333.
Mitrula elegans Berk. G47; *ZW-G47-CLARK*; AY789334; AY789335; AY789336.
Mitrula elegans Berk. G146; *DMWV 04–3*; —; AY789417; AY789418.
Mitrula elegans Berk. G159; *DMWV 04–78*; —; AY789433; AY789434.
Mitrula lunulatospora Redhead G2; *DAOM 160732*; —; AY789279; AY789280.
Mitrula brevispora Zheng Wang G12; *ZW02–012-CLARK*; AY789292; AY789293; AY789294.
Mitrula paludosa Fr. G31; *NIFG 1113754*; —; AY789319; AY789320.
Mitrula paludosa Fr. G149; *MBH 50636*; AY789422; AY789423; AY789424.
Mollisia cinerea (Batsch) P. Karst.; *AFTOL*; —; —; —.
Monilinia laxa (Aderh. & Ruhland) Honey; *GenBank*; AY544714; AY544670; AY669337.
Mycocalicium polyporaeum (Nyl.) Vain.; *ZW-G60-CLARK*; AY789361; AY789362; AY789363.
Neolecta irregularis (Perk) Korf & J.K. Rogers; *ZW-G79-CLARK*; AY789379; AY789380; AY789381.
Ombrophila violacea P. Karst.; *FH-WZ0024*; AY789364; AY789365; AY789366.
Orbilia delicatula (P. Karst.) P. Karst.; *GenBank*; U72603; AY261178; U72595.
Peziza phyllogena Cooke; *ZW-G44-CLARK*; AY789327; AY789328; AY789329.
Peziza varia (Hedw.) Fr.; *ZW-G94-CLARK*; AY789390; AY789391; AY789392.
Rutstroemia bolaris (Batsch) Rehm; *GenBank*; Z81393; Z81419; Z80894.
Scleromitrula shiraiana (Henn.) S. Imai; *Hirayama062001*; AY789406; AY789407; AY789408.
Sclerotinia sclerotiorum (Lib.) de Bary; *FH-WZ0067*; AY789346; AY789347; AF455526.
Spathularia flavida Pers.; *GenBank*; Z30239; AF433145; AF433154.
Trichoglossum hirsutum (Pers.) Boud.; *OSC 61726*; AY789312; AY789313; AY789314.
Vibrissea flavovirens (Pers.) Korf & J.R. Dixon; *MBH 39316*; AY789425; AY789426; AY789427.
Vibrissea truncorum (Alb. & Schwein.) Fr.; *CUP 62562*; AY789401; AY789402; AY789403.