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.

Despite the attention they have received, the telemorphs 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 Vibrissea have been reported to have aquatic anamorphs in the form-genera Ana- virga, Anguillospora, Articulospora, Helicodendron, and Tricladium (Abdullah et al., 1981; Fisher and Webster, 1983; Descals et al., 1984; Digby and Goos, 1987; Fisher and Spooner,
1987; Hamad and Webster, 1988; Webster et al., 1995; Mar- 
vanová 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 Heloti-ales also is 
uncertain. Because of the club-shaped apothecia, Mitrula spe-
cies have been accepted as members of the family Geoglos-
saeceae, 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 stud-
ies 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 Helotiiales in future clas-
sifications (Platt, 2000; Pfister and Kimbrough, 2001; Z. Wang 
et al., unpublished manuscript). Based on ultrastructure of the 
ascus (a sac-like cell containing ascospores) from selected taxa 
in the Helotiiales, Verkley (1994) concluded that Mitrula pal-
udosa Fr. is closely related to members of the Sclerotiniaeae, 
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 
(Sclerotiniaeae) (Gernandt et al., 1997). The family Heloti-
aceae 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 

Fifty-five species names have been recorded under the ge-
irus Mitrula (http://www.indexfungorum.org). Some species 
described in Mitrula have been transferred to other genera, 
such as Bryoglossum, Scleromitrula (= Verpatinia), and Hey-
deria (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 Mi-
trula is a monotypic genus, with M. paludosa as the sole spe-
cies.

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. Red-
head, M. lunulatospora S.A. Redhead, and one European spe-
cies M. paludosa, which may be referred to Asian material as 
well (Redhead, 1977). Mitrula borealis has a boreal and sub-
alpine 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 hy-
menophores, M. multiforme (E. Henning) Massae and M. om-
phalostoma E.-H. Benedix, were accepted in Europe by Benk-
ert (1983). The genus Bryoglossum was erected based on the 
bryophilous (moss-inhabiting) species Mitrula gracilis Kar-
sten. 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 re-
leasing a yellow to reddish brown pigment in 10% KOH so-
lution (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 Helotiiales 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 Helo-
tiales.

**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, Japa-
n). 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 col-
onies 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. At-
tached 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 Na2HPO4, 1.8 mM 
KHzPO4). The cells were then resuspended in DAPI solution (1 : 10000 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-isomyl alcohol (25 : 24 : 1), and precipitated with 95% ethanol and 3 mM 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 JLS1 and LRS5 (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 sequenc-
eses were downloaded from GenBank (Appendix 1).

Thirteen isolates of Mitrula were included in molecular studies, represent-
ing M. lunulatospora, M. borealis, and M. elegans from North America, M.
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 Neolecuta irregularris (Perk) Korf & J.K. Rogers (Landvik, 1996). Sequencing reactions were purified using Peqlab and 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 Neolecuta irregularris (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 Ruststroemia 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 with no sequences with 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 + 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, 0.6613; A-T, 0.3287; C-G, 0.1342; G-T, 0.1736) was used for the likelihood analysis. The PHUMCOSS model in MrBayes 3.0 was used for the Bayesian analyses. A likelihood command block was copied from MrModeltest 2.0. A likelihood command block was copied from 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 Ruststroemia 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 (MCMC) under the GTR + I + model in MrBayes 3.0 by running four chains with 500 000 generations using the default program 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)—Ascomata solitary, 14–32 mm althum. Ascogonea forma cylindracea, sub-albea vel lutea, 4–12 × 2–4.5 mm. Stipes 0.8–1 mm crassus. Hyphens 97–102 μm crassus. Asci cylindraceo-clavati, apicitermi attenuati, jodo abturatum minutissimo caeruleuscentes, 4–9.6 μm crassus. Paraphyses filiformes, 2.4–2.6 μm crassus. Sporangia 8: elliptica vel subfusoideae, 2.4–3.1 5.5–10.1 μm.

Holotype: 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).

Ascosporangia, sub-albea vel lutea, 4–12 × 2–4.5 mm. Ascoporene forma cylindracea, sub-albea vel lutea, 4–12 × 2–4.5 mm. Stipes 0.8–1 mm crassus. Hyphens 97–102 μm crassus. Asci cylindraceo-clavati, apicitermi attenuati, jodo abturatum minutissimo caeruleuscentes, 4–9.6 μm crassus. Paraphyses filiformes, 2.4–2.6 μm crassus. Sporangia 8: elliptica vel subfusoideae, 2.4–3.1 5.5–10.1 μm.

Holotype: 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).
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-
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, bicuspid, 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 = 11383.9154). 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
**BSA Clade**

Discomycetes with small or tiny apothecia, mostly bright-color, saprotrophic, aero-aquatic or prefer a habitat with high humidity.

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**Fig. 4.** Higher-level phylogenetic analyses of *Mitrula*. Aeo-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 microconidia synanamorph are followed by an asterisk. Bootstrap values greater than 50% are indicated along nodes. Branches not present in MP tree are marked with an asterisk.

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(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 (\(-\ln L = 11383.91954\)). Group frequencies greater than 50% are indicated as posterior probability (%) along nodes.
Dosa is monophyletic with moderate support (BP 94%), including six isolates of *M. elegans* G47 from eastern USA formed two independent lineages in the strict consensus tree are marked with an asterisk above the branch. Eight isolates formed a monophyletic group (BP 50%). More than six lineages were detected among rDNA regions (lsu and ITS). The combined data had an aligned length of 1390 base pairs with 143 uninformative variable positions. 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.

### 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, 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.

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, conidia. Plasmogamy and dikaryotic hyphae have not been observed, and no apothecia have been produced from the *Mitrula* cultures. Hyphae from the multisporic 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 *Vibrissa 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 *Vibrissa flavovirens* (Pers.) Korf & J.R. Dixon, *Mollisia gigantea* P.J. Fisher & J. Webster, and *Hydrocina chaetocladioida* Scheurer, 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 Helotiales. The precise placement of *Mitrula* requires a reclassification of the Helotiales and the Helotiales, which needs to include many more taxa.

Both parsimony and likelihood analyses suggested that the Helotiales-Rhytismatales clade 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 *Vibrissa*, *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, Vibrisea and Hydrocina. Vibrisea 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 chaetocladium produces minute, cup-shaped fruitbodies on submerged twigs and produces conidia of Tricladium chaetocladium and a macroconidial 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, Hemiphaciaceae, 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 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 bicalicular 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 habitat.

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Hyaloscypha daedalae Velen.; ZW-G138-CLARK; AY789414; AY789415; AY789416.
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Mitrula borealis Redhead G122; UWH-LLN930920–1; —; AY789404; AY789405.
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