RESEARCH ARTICLE

Surprising spectra of root-associated fungi in submerged aquatic plants

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Abstract

Similarly to plants from terrestrial ecosystems, aquatic species harbour wide spectra of root-associated fungi (RAF). However, comparably less is known about fungal diversity in submerged roots. We assessed the incidence and diversity of RAF in submerged aquatic plants using microscopy, culture-dependent and culture-independent techniques. We studied RAF of five submerged isoetid species collected in four oligotrophic freshwater lakes in Norway. Levels of dark septate endophytes (DSE) colonization differed among the lakes and were positively related to the organic matter content and negatively related to pH. In total, we identified 41 fungal OTUs using culture-dependent and culture-independent techniques, belonging to *Mucoromycotina*, *Chytridiomycota*, *Glomeromycota*, *Ascomycota* as well as *Basidiomycota*. Sequences corresponding to aquatic hyphomycetes (e.g. *Nectria lugdunensis*, *Tetracladium furcatum* and *Varicosporium elodeae*) were obtained. Eight arbuscular mycorrhizal taxa belonging to the orders *Archaeosporales*, *Diversisporales* and *Glomerales* were also detected. However, the vast majority of the fungal species detected (e.g. *Ceratobasidium* sp., *Cryptosporiopsis rhizophila*, *Leptodontidium orchidicola*, and *Tuber* sp.) have previously been known only from roots of terrestrial plants. The abundance and phylogenetic distribution of mycorrhizal as well as nonmycorrhizal fungi in the roots of submerged plants have reshaped our views on the fungal diversity in aquatic environment.

Introduction

Plant roots are inhabited by a diverse spectrum of root-associated fungi (RAF; Vandenkoornhuyse *et al.*, 2002). Besides mycorrhizal fungi, the RAF include dark septate endophytes (DSE), aquatic hyphomycetes, hyaline fungal endophytes and other saprotrophic or pathogenic fungi (Vandenkoornhuyse *et al.*, 2002; Addy *et al.*, 2005). These fungi mostly belong to the phylum *Ascomycota* and *Basidiomycota*; however, also *Zygomycota* and *Chytridiomycota* are present (Vandenkoornhuyse *et al.*, 2002; Selosse *et al.*, 2009; Weiss *et al.*, 2011). In contrast to the vast literature on mycorrhizal fungi, particularly the most widespread arbuscular mycorrhizal fungi (AMF) from the phylum *Glomeromycota* (Schüssler *et al.*, 2001), our knowledge of the role of other RAF groups in plant ecophysiology and their importance in both terrestrial and aquatic ecosystem functioning is still very limited.

In aquatic environments, the first described RAF were the AMF (Søndergaard & Laegaard, 1977). Since then, arbuscular mycorrhizal symbioses have been observed in many species of aquatic plants in different sites around the world (Beck-Nielsen & Madsen, 2001; Radhika & Rodrigues, 2007; Baar *et al.*, 2011). In addition to AMF, roots of aquatic plants are frequently colonized by DSE (Kai & Zhao, 2006; Šraj-Kržič *et al.*, 2006; de Marins *et al.*, 2009). The DSE comprise a polyphyletic group of fungi that are known to colonize various mycorrhizal and nonmycorrhizal plant species (Jumpponen & Trappe, 1998) from different habitats across the world (Mandyam

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& Jumpponen, 2005). Although the DSE are common plant symbionts, their role in the nutrition and fitness of the host plants is still under debate, even in terrestrial ecosystems (Newsham, 2011). In aquatic environments, structures recognized as being produced by DSE in plant roots include melanized microsclerotia and dark septate hyphae, similarly to terrestrial plants (Kai & Zhao, 2006; Šraj-Kržič *et al.*, 2006). However, the identity and diversity of DSE colonizing the roots of aquatic plants have not been studied.

Besides the mycorrhizal fungi and the DSE, there are other groups of fungi capable of colonizing plant roots. One such group is aquatic hyphomycetes that are broadly defined as anamorphic fungi typically with relatively large branched (often stauroform) and scolecoform conidia occurring mainly in lotic waters. Their common substrate is submerged decaying tree leaves and woody debris (Webster & Benfield, 1986), but they also enter healthy tissues as endophytes and act even as necrotrophic plant pathogens (Fisher & Petrini, 1989; Sati & Belwal, 2005; Bärlocher, 2006). The same fungi were also found in permanently or intermittently humid layer of forest plant litter (Bandoni, 1972) or as endophytes of roots in nonaquatic habitats (Abadie *et al.*, 2006; Tedersoo *et al.*, 2007; Gao & Yang, 2010). The potential implications of the occurrence of aquatic hyphomycetes in roots of terrestrial plants for the fungal life cycle were discussed by Selosse *et al.* (2008).

In general, there is a lack of studies on the diversity of RAF and their role in lake ecosystems, and this topic deserves in-depth investigation (Shearer *et al.*, 2007; Wurzbacher *et al.*, 2010). To fill this gap, we initiated a study aimed at assessing the occurrence and taxonomic composition of RAF colonizing submerged aquatic plants in freshwater boreal lakes. The bottom vegetation of these habitats is often dominated by the so-called isoetids, that is, small, slow-growing, evergreen vascular plants sharing a similar morphology and anatomy (stiff leaves in basal rosettes, large root systems, extensive longitudinal lacunal system) but belonging to different taxonomic groups (Madsen *et al.*, 2002; Smolders *et al.*, 2002). Owing to a permeable root surface and high radial oxygen losses, isoetids are capable of oxygenating lake sediments (Sand-Jensen & Prahl, 1982), which might be an important prerequisite for the colonization of their roots by mycorrhizal and endophytic fungi.

Specifically, we studied fungal diversity in roots of five isoetid species belonging to four different plant families from four oligotrophic lakes in Norway. We aimed to (i) evaluate the frequency of mycorrhizal and nonmycorrhizal fungal endophytic structures in roots of isoetid plants, based on microscopic observations, (ii) identify RAF taxa using culture-independent technique and (iii) identify the non-AM RAF obtained using cultivation methods.

Materials and methods

Study sites and field sampling

RAF were investigated in isoetid vegetation inhabiting four freshwater oligotrophic lakes in southern and central Norway. Lake details and chemical sediment characteristics are presented in Table 1. Five isoetid species [*Isoëtes echinospora* Dur., *Isoëtes lacustris* L. (both *Isoëtaceae*), *Littorella uniflora* L. (*Plantaginaceae*), *Lobelia dortmanna* L. (*Campanulaceae*) and *Subularia aquatica* L. (*Brassicaceae*)] were sampled in August 2010 (Table 2). The former two species are heterosporous lycopods while the others are advanced dicotyledonous angiosperms. Entire root systems of the plants were collected, placed into sterile plastic bags and transferred to the laboratory. During transport, root samples were stored in darkness and cold (4 °C) and kept moist. In the laboratory, plant roots were thoroughly washed with tap water and large root systems were divided into two parts; one was used for microscopic examination while the other was surface sterilized (100% solution of household bleach for 30 s followed by three times washing in sterile distilled water) and used for the isolation of endophytic fungi. Smaller root systems (particularly those of *S. aquatica*) were only used for the isolation procedure. For the two lakes with the highest isoetid diversity (Ljøsvatn and Mjåvatn Lakes), a part of surface-sterilized roots (*c.* 50–100 mg) was frozen (−20 °C) for subsequent isolation of DNA and cloning, in an attempt to describe uncultured RAF communities and compare the results obtained using culture-dependent and culture-independent techniques.

Quantification of fungal structures in the roots

Roots of all species except *S. aquatica* were stained following the method of Koske & Gemma (1989) using 0.05% trypan blue in lactoglycerol. The presence of structures of AM fungi (hyphae, arbuscules and vesicles), DSE fungi (microsclerotia and melanized hyphae) or hyaline root endophytes was counted in 100 microscopic fields per each root system under a compound microscope equipped with DIC at 200× magnification according to McGonigle *et al.* (1990).

Isolation and characterization of endophytic fungi

Surface-sterilized root segments (2–3 cm in length) were aseptically plated on Petri dishes with agar media containing a low carbon level. Approximately nine root segments were placed in each Petri dish. Two different media were used: (i) modified Melin Norkrans medium

Table 1. Location of the lakes based on the European Environment Agency database together with the list of sampled plant species and chemical characteristics of the sediment samples collected from the lakes in August 2010

Lake	District (county)	Latitude (°N)	Longitude (°E)	Altitude (m a.s.l.)	Sampled plant species	Sediment analyses*					
						pH_{H2O}	pH_{KCl}	N-NH_4 (mg kg^{-1})	N-NO_3 (mg kg^{-1})	PO_4 (mg kg^{-1})	C_{org} (%)
Avsjøen	Central Norway (Oppland)	62.1747	9.4745	920	*ECHI*, *LAC*, *LIT*	6.2 ± 0.4	5.1 ± 0.3	2.2 ± 0.5	0.6 ± 0.4	9.4 ± 0.7	2.8 ± 1.3
Mjøsa	Central Norway (Buskerud)	60.4749	11.2109	120	*ECHI*, *LAC*, *SUB*	5.9 ± 0.5	5.3 ± 0.4	3.0 ± 2.5	2.5 ± 4.5	12.2 ± 1.8	3.7 ± 3.4
Ljøsvatn	South Norway (Rogaland)	58.4180	6.2110	150	*ECHI*, *LAC*, *LIT*, *LOB*	5.4 ± 0.1	4.4 ± 0.1	3.7 ± 0.4	0.4 ± 0.2	12.1 ± 4.6	13.3 ± 8.3
Mjåvatn	South Norway (Rogaland)	58.3814	6.1129	50	*ECHI*, *LAC*, *LIT*, *LOB*	5.3 ± 0.1	4.4 ± 0.1	7.4 ± 2.2	1.1 ± 1.3	10.4 ± 0.9	31.1 ± 3.2

ECHI, *Isoëtes echinospora*; LAC, *Isoëtes lacustris*; LIT, *Littorella uniflora*; LOB, *Lobelia dortmanna*; SUB, *Subularia aquatica*.
*The data represent means ± SE of the following number of replicates: Avsjøen and Mjøsa $n = 6$, Ljøsvatn $n = 5$, Mjåvatn $n = 8$.

Table 2. A summary of RAF obtained using culture-dependent and culture-independent techniques, respectively, from the roots of five submerged plant species. Root samples from all lakes were studied using the culture-dependent technique while only two lakes with the highest isoetid diversity (Mjåvatn/Ljøsvatn) were selected for the culture-independent DNA-based technique

Plant species	Culture-dependent technique				Culture-independent technique		
	Number of sampled plants	Number of sampled segments	Number of fungal isolates	Number of fungal OTUs	Number of sampled plants	Number of clones	Number of fungal OTUs
Littorella uniflora	11	227	109	7	4/–	41	6
Lobelia dortmanna	10	309	21	1	3/2	72	15
Subularia aquatica	2	63	6	5	–	–	–
Isoëtes echinospora	14	418	2	2	2/2	71	6
Isoëtes lacustris	17	568	1	1	2/2	50	8

with 1 g L^{-1} of glucose and 0.3 g L^{-1} of Malt extract and (ii) potato dextrose agar (PDA) medium with 4 g L^{-1} of commercial PDA (HIMEDIA, India) and additional agar (9 g L^{-1}) to solidify the medium. To suppress bacterial growth, 50 mg L^{-1} of Novobiocine was added to both media. The dishes were incubated in the dark at 20 °C and examined every other day for 9 weeks. Whole colonies of all rapidly growing fungi were removed to allow for the isolation of slow-growing endophytic fungi. Individual colonies of slow-growing hyphae were transferred to separate Petri dishes containing PDA.

Fungal isolates obtained from the roots were grown on the full strength PDA medium in the dark at 20 °C. Growth rates, colony pigmentation and the presence of aerial hyphae were determined by macroscopic observation during the 9-week period. These characteristics were used to assign the isolates to different morphotypes. Five samples of each morphotype were chosen for molecular identification.

Molecular analyses of the isolates

DNA was extracted from 55 isolates using either the SIGMA Extract-N-Amp™ Plant Kit (Sigma-Aldrich) following the manufacturer's instructions, or the DNeasy Plant Mini extraction kit (Qiagen GmbH, Hilden, Germany) if the DNA isolation using the SIGMA kit failed. The ITS region of the rDNA was amplified by the universal eukaryotic primer pair ITS1 and ITS4 (White *et al.*, 1990). If the amount of obtained DNA after the first PCR was not sufficient for direct sequencing, we used a semi-nested PCR. The first PCR was performed using the fungal-specific primer ITS1F (Gardes & Bruns, 1993) in combination with ITS4. The obtained PCR products were diluted 1 : 100 with sterile distilled deionized water (ddH_2O) and used as a template in the second PCR with the universal primer pair ITS1–ITS4. For the DNA templates obtained by the SIGMA isolation kit, the SIGMA REDEX PCR kit was used for the PCR amplification

according to the manufacturer's instructions. The PCR mix of all nested PCR reactions included 2.5 μL of 10× Taq buffer with KCl/without $MgCl_2$, 2.5 μL of dNTPs mixture (2 mM), 2 μL $MgCl_2$ (25 mM), 0.5 μL of each primer (10 μM), 1 U of *Taq* DNA polymerase (Fermentas International Inc., Burlington, Canada), 15.8 μL of sterile ddH_2O and 1 μL of the template in a final volume of 25 μL. Identical thermal cycling parameters as in Kohout *et al.* (2011) were used.

Culture-independent screening of RAF

To compare the culture-dependent technique of RAF isolation from root segments with the culture-independent technique, we also isolated DNA directly from surface-sterilized roots. Those parts of the root systems that were close to the segments used for the isolation of fungal colonies were selected for DNA isolation.

Total DNA was extracted using the DNeasy Plant Mini extraction kit (Qiagen GmbH) according to the manufacturer's instructions. Isolated DNA was 10 times diluted with ddH_2O and used as a template for subsequent PCR reactions. We ran three independent PCR reactions from each sample to avoid PCR bias. The fungal-specific primer combination ITS1F–ITS4 was used to amplify the fungal ITS region of rDNA. The same PCR mix and conditions were used as described earlier. The only difference concerned the number of cycles is as follows: we determined the lowest number of necessary PCR cycles for each sample (which ranged from 32 to 42), when the PCR product was already useful for cloning, to avoid possible co-amplification of contaminants in late PCR cycles. For samples with weak bands after the first PCR, a semi-nested PCR with primer combination ITS1–ITS4 was used (products after the first PCR were 1 : 100 diluted with ddH_2O). All positive PCR products from each plant species and lake were pooled before the cloning step, which resulted in seven samples. Unfortunately, no PCR products were obtained from the roots of *Lit. uniflora* collected in Lake Ljøsvatn. Amplification products were cloned using the pGEM-T cloning kit (Promega, Leiden, the Netherlands) according to the manufacturer's instructions. Clones were re-amplified using the primers ITS1/ITS4, and 30–40 positive clones from each sample were selected for sequencing with ITS1 in Macrogen (Seoul, South Korea).

Sequence and phylogenetic analyses

All obtained high-quality fungal sequences were edited with FinchTV 1.4.0 (Geospiza Inc.) and used for the taxonomic identification and delimitation of operational taxonomic units (OTUs) using TOPALi 2 (http://www.topali.org/), with 97% and 99% similarities for *Basidiomycota* (Nilsson *et al.*, 2008) and *Ascomycota*, respectively. A higher threshold for *Ascomycota* was selected because the vast majority of endophytic ascomycetes belong to the order *Helotiales*, for which a 99% similarity threshold was shown to be more appropriate (Hambleton & Sigler, 2005; Grünig *et al.*, 2009; Tedersoo *et al.*, 2009). A conservative approach was adopted for AMF known to exhibit a high level of intraspecies variability of the ITS region (Nilsson *et al.*, 2008). The OTUs were delimited on the basis of NJ/Bayesian phylogenetic analyses considering well-supported monophyletic groups (NJ bootstrap support higher than 75) and confirmed by distinct sequence signatures in alignments. Two or more representative sequences from each OTU were deposited in the INSD (accession numbers HQ623444–63, HQ691250–54, JN581944–81 and JN569101–37). Preliminary identification of all OTUs was carried out using a BLASTN search against the INSD or PlutoF (Abarenkov *et al.*, 2010) public sequence databases. The putative ecology of the OTUs was inferred from the known ecology of the phylogenetically closest species.

Sequences chosen for phylogenetic analyses were aligned together with the downloaded database sequences using MAFFT 6.6 (http://mafft.cbrc.jp/alignment/server/), followed by a manual correction. Phylogenetic trees of the non-*Glomeromycota* sequences were obtained primarily by the neighbour-joining analysis in MEGA5 (Tamura *et al.*, 2011), employing the LogDet (Tamura–Kumar) model using all sites in the alignments. Posterior probabilities were estimated with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) using the same model (parameters: lset nst = 6, rates = invgamma). An MCMC analysis was performed, initiated with random starting trees, and run for 10 000 000 generations. Every 100th generation was sampled, and the first 10 000 trees were discarded as burn-in.

Two alignments of the *Glomeromycota* sequences were prepared: (i) *Archaeosporales* and (ii) *Glomerales* and *Gigasporaceae*. Poorly aligned positions and divergent regions were removed from the alignments using Gblocks ver. 0.91b (Castresana, 2000), under the less stringent conditions. Phylogenetic trees were at first obtained using the neighbour-joining algorithm in MEGA5 (Tamura *et al.*, 2011), with 1000 bootstrap replicates, the Kimura two-parameter model, and a gamma shape parameter = 0.5. Results were verified by Bayesian analyses, using the same settings as described earlier.

Statistical analyses

Data on the presence of fungal structures in plant roots were analysed using STATISTICA 9 software (StatSoft Inc.).

First, the data were checked for normal distribution and homogeneity of variance. As they were not normally distributed even after transformation, the effects of host plant species and lake were evaluated using the nonparametric Kruskal–Wallis test; means were compared at a significance level of $P < 0.05$.

To summarize the major patterns in variation among the lake sediment samples (5–8 samples per lake, each characterized by five chemical parameters; Table 1), principal component analysis (PCA) was performed on untransformed and centred data using CANOCO ver. 4.56 (ter Braak & Šmilauer, 2002).

To study the differences in accumulating species richness among plant species, we calculated rarefaction curves using EstimateS 8.20 (Colwell, 2006). The calculation was based on the sequences obtained by culture-independent approach.

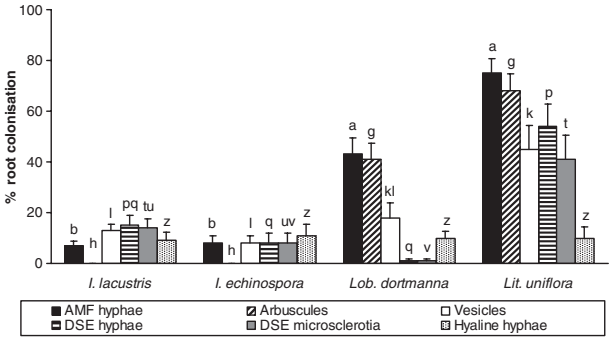


Fig. 1. Percentage of root length colonized by arbuscular mycorrhizal (AM) and non-AM RAF in four plant species (*Isoëtes lacustris*, $n = 16$; *Isoëtes echinospora*, $n = 12$; *Lobelia dortmanna*, $n = 10$; and *Littorella uniflora*, $n = 9$) from four Norwegian lakes. The columns marked with different letters are significantly different between different plant species at $P < 0.05$ according to nonparametric Kruskal–Wallis test.

Results

All of the plant species subjected to microscopic investigation were colonized by hyaline hyphae of endophytic fungi (*c.* 10% of root lengths in any of the host plants; see the column 'Hyaline hyphae' in Fig. 1). DSE colonization was significantly higher in roots of *Lit. uniflora* (*c.* 50%) than in roots of *Lob. dortmanna* (*c.* 1%) and *I. echinospora* (*c.* 8%). *Isoëtes lacustris* showed significantly higher DSE colonization (*c.* 15%) than *Lob. dortmanna* but did not differ from the other plant species. The frequency of AMF hyphae was significantly higher in angiosperms (*Lit. uniflora*, *c.* 72%; *Lob. dortmanna*, *c.* 43%) than in both *Isoëtes* species. The same pattern was observed in the abundance of arbuscules (*Lit. uniflora*, *c.* 70%; *Lob. dortmanna*, *c.* 40% vs. no arbuscules in either *Isoëtes* spp.). Unlike arbuscular colonization, vesicles were found in all plant species. Fungal colonization of the roots of the same plant species did not differ significantly among lakes (data not shown), except for a significantly ($P = 0.043$) higher DSE colonization in the roots of *Lit. uniflora* from Lake Mjåvatn (*c.* 78%) than from Avsjøen (*c.* 29%). Plants of *Lit. uniflora* from Lake Ljøsvatn had intermediate DSE colonization levels (*c.* 56%). Representative pictures of observed fungal structures are presented in Fig. 2.

Figure 3 shows the relationships between organic carbon, pH, and inorganic N and P in sediments of the four lakes studied; mean values for each lake are presented in Table 1. The content of organic carbon was negatively correlated with pH, and these two factors were strongly correlated with the first ordination axis, which explained a substantial amount of the variation (92.8%). According to the PCA results, the abiotic conditions of the lake Mjåvatn differed from those of the Mjøsa and Avsjøen lakes while the Ljøsvatn lake occupied the intermediate position. The level of organic carbon was lowest at Lakes Avsjøen and Mjøsa, intermediate at Lake Ljøsvatn and highest at Lake Mjåvatn. Whereas Lake Mjøsa showed high within-site variation with respect to nutrient availability (NO_3^- and PO_4^{3-}), the other lakes were more uniform in this parameter.

A total of 139 isolates belonging to 14 OTUs of RAF were obtained from 1585 root segments. The highest number of isolates per root segment was obtained from *Lit. uniflora* (Table 2) whereas both *Isoëtes* spp. yielded the fewest fungal colonies. The BLASTN search resulted in the identification of six OTUs (Table 3). The identity of the remaining eight OTUs, which belonged to the order *Helotiales*, was determined by means of BLASTN search and phylogenetic analysis.

The most commonly isolated fungal taxa were: (i) an undetermined member of the phylum *Ascomycota*, tentatively named *Ascomycota* sp. 2 (from *Lit. uniflora*), (ii) isolates closely related to *Cryptosporiopsis rhizophila* (from *Lit. uniflora* and *Lob. dortmanna*) and (iii) isolates highly similar to *Leptodontidium orchidicola* (from *Lit. uniflora*). Additionally, fungi closely related to known species of aquatic hyphomycetes were isolated, including *Nectria lugdunensis*, *Tetracladium* sp. 1 (both from *Lit. uniflora*), *Tetracladium furcatum* and *Tricladium* sp. 1 (both from *S. aquatica*). Only two fungal taxa were isolated from the roots of *Isoëtes* spp.: *Leohumicola minima* (from *I. echinospora*) and *Ascomycota* sp. 1 (from both *Isoëtes* species). The numbers of isolates per fungal OTU obtained from each of the five studied host plants are summarized in Fig. 4a.

The culture-independent approach yielded 234 high-quality sequences of RAF, which clustered into 29 OTUs. These OTUs were largely different from those obtained

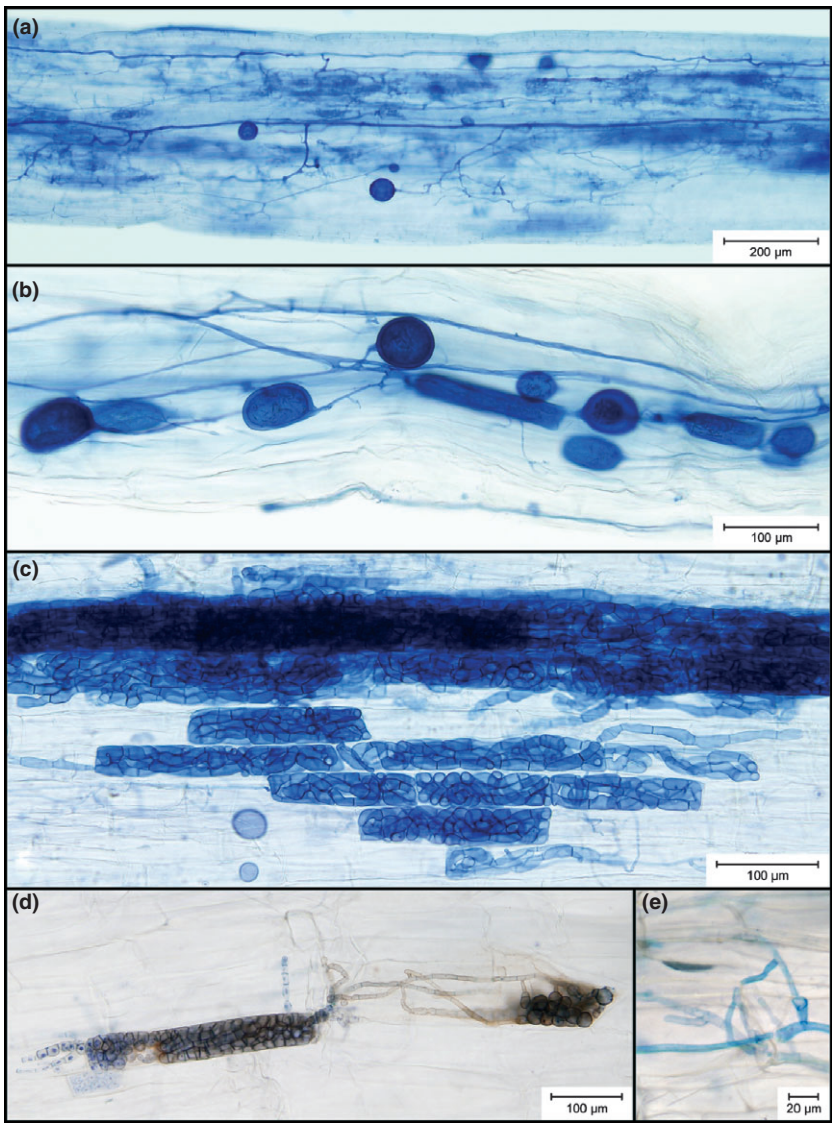


Fig. 2. Fungal structures in the roots of submerged aquatic plants. (a) Arbuscular mycorrhizal (AM) colonization with intraradical hyphae, vesicles and arbuscules (*Lobelia dortmanna*); (b) vesicles of AM fungi (*Isoëtes lacustris*); (c) unusual intracellular colonization in roots of *Isoëtes echinospora* – hyphal complexes fill some of the cortical cells. It is noted that colonization is most dense around the central cylinder and fungal hyphae are stained with trypan blue, but lack dark pigmentation; (d) a dark septate mycelium colonizes cortical cells forming an intracellular microsclerotium (left) and producing intracellular conidia (right) (*I. lacustris*); (e) intracellular hyaline hyphae (*Littorella uniflora*).

by the isolation procedure, with only two being in common (OTUs 10 and 19). The most diverse community of uncultured RAF was found in the roots of *Lob. dortmanna* (Fig. 4b). The identity of nine OTUs (Table 3) was determined by the BLASTN search while eight OTUs by phylogenetic analyses. Both BLASTN search and phylogenetic analyses were employed to identify the remaining 12 OTUs. Species accumulation curves, based on the culture-independent results, were almost linear for *Lit. uniflora* and *Lob. dortmanna* compared with relatively well saturated curves for *I. echinospora* and *I. lacustris* (Fig. 5).

Eight OTUs of AMF – four from the order *Glomerales*, one from the family *Gigasporaceae* (Fig. 6) and three from the order *Archaeosporales* (Fig. 7) – were detected in the root systems of *Lit. uniflora* and *Lob. dortmanna*. There was a little overlap in taxonomic composition of AMF between the studied Lakes Ljøsvatn and Mjåvatn (only two OTUs of *Archaeosporales*) as well as between both plant species from the latter lake (three shared OTUs). No AMF sequences were recovered from either *Isoëtes* species (they also showed much lower proportion of root length colonized by AMF structures).

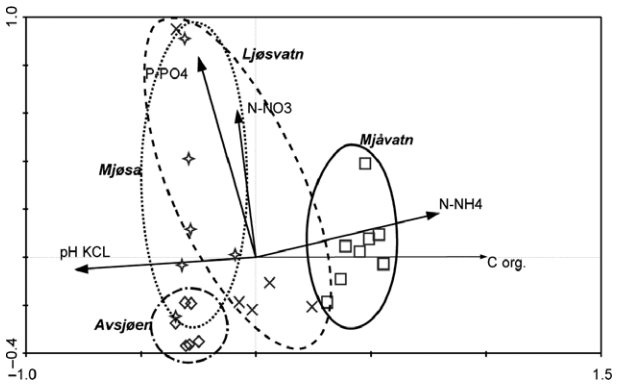


Fig. 3. PCA biplot showing lake sediment samples collected in four lakes and measured sediment characteristics. Diamonds represent samples from the lake Avsjøen, X-marks from Ljøsvatn, squares from Mjåvatn and stars from Mjøsa. Samples from each lake were enclosed by an ellipse for a better visualization. The first and second axes explain 92.8% and 3.2% of the total variation, respectively. Positively correlated variables have acute angles between their biplot arrows whereas negatively correlated variables have arrows pointing in opposite directions; perpendicular arrows indicate no correlation between the respective variables. The length of the arrow describes the relative importance of the variable for the displayed ordination.

Similarly to the results obtained from isolations, *Helotiales* constituted an abundant and diverse group of nonmycorrhizal RAF in culture-independent screening (Table 3, Fig. 8). However, there was a relatively low overlap in species assemblages detected using both techniques. The dominant sequences with affinities to the *Helotiales* matched well with known aquatic hyphomycetes (OTUs 2, 4, 5, 8 and 9; Fig. 8). In addition to *Ascomycota*, several basidiomycetous fungi were also recorded (Table 3), including the two OTUs (nos. 20 and 21) from both *Isoëtes* species with high similarities to *Cantharellales*. In phylogenetic analysis, these sequences clustered together with *Ceratobasidium* spp. from orchid or ericaceous roots (Fig. 9). Additional components of the community of uncultured RAF were *Chytridiales* (OTUs 34, 35, 36), *Endogonales* (OTU 28), *Rhizophydiales* (OTU 29) and *Monoblepharidales* (OTU 30). Sequences of 5.8S rRNA genes of those taxa were phylogenetically analysed with similar sequences from the INSD (Fig. 10). The remaining uncultured RAF are summarized in Table 3.

Discussion

Many studies investigated the occurrence of RAF in various terrestrial ecosystems (Mandyam & Jumpponen, 2005; Öpik *et al.*, 2006; Kivlin *et al.*, 2011), while the studies on RAF in aquatic plants are rare and usually based only on microscopic observation (Kai & Zhao, 2006; Šraj-Kržič *et al.*, 2006; de Marins *et al.*, 2009). The last decade has seen some research on the diversity of AMF colonizing roots of aquatic plants (Nielsen *et al.*, 2004; Baar *et al.*, 2011), but the identity of non-AM RAF in aquatic environments has been completely neglected. Our study aims to fill this significant gap and addresses the diversity and community assemblages of RAF in isoetid aquatic plants using both culture-dependent and culture-independent techniques.

In general, the diversity of nonmycorrhizal RAF is quite high, even within a single plant species (e.g. Neubert *et al.*, 2006; Tedersoo *et al.*, 2009). Because of their worldwide distribution and very high abundance, it has been predicted that endophytic fungi comprise a substantial component of global fungal diversity (Arnold *et al.*, 2000). There is a general consensus among researchers that a large proportion of fungal species still awaits description. Hyde & Soytong (2007) envisaged that investigations into new habitats or host species will likely result in a discovery of new species and/or lineages of RAF. Recent publications focusing on fungal diversity in freshwater or marine environments confirmed their assumption and described a number of uncultured taxa, especially from the phylum *Chytridiomycota* and some basal fungal lineages (Mohamed & Martiny, 2011; Monchy *et al.*, 2011). This study adds to this knowledge and demonstrates that the roots of aquatic plants are inhabited by a diverse spectrum of RAF, including previously unknown lineages of uncultured fungi and fungal species similar to those found in terrestrial plants.

Microscopic investigation

Both arbuscules and vesicles of AM fungi were observed in the roots of *Lit. uniflora* and *Lob. dortmanna* whereas only vesicles occurred in the roots of *I. echinospora* and *I. lacustris*. This pattern accords with our previous results obtained from two glacial lakes in South Bohemia (Central Europe), where none and only a few arbuscules were found in the roots of *I. lacustris* and *I. echinospora*, respectively (Sudová *et al.*, 2011).

Large inter-specific differences were also recorded in the level of root colonization by DSE. The roots of *Lob. dortmanna* showed the lowest colonization by DSE hyphae and microsclerotia (*c.* 1%) as compared to the other species (*c.* 8–50%). It is plausible that the low colonization in *Lob. dortmanna* is related to its aquatic lifestyle because several terrestrial species belonging to the same plant family *Campanulaceae* were previously shown to be highly colonized by DSE (Read & Haselwandter, 1981; Väre *et al.*, 1992). The results of the isolation procedure generally agreed with our microscopic observations, as no DSE isolates were obtained from the

Table 3. Identification of obtained OTUs by culture-dependent and culture-independent techniques, based on the BLASTN search of the INSD and the MASSBLASTER in the PLUTOF database

OTU	Identified*	Detected†	Species	Order	Putative ecology	Best matches	Similarity (%)	MisM
1	B + P	D	*Cryptosporiopsis rhizophila*	*Helotiales*	RAF	*Calluna vulgaris* root-associated FM172838	100.0	0
						C. rhizophila AY176761	100.0	0
2	B + P	D	*Tricladium* sp. 1	*Helotiales*	RAF	Uncultured saccharomyceta HQ212134	98.99	4
						Zalerion varium AJ608987	98.99	4
3	B + P	D	*Leohumicola minima*	*Helotiales*	RAF/ErM	Epacrid root endophyte sp. AY279184	99.80	1
						L. minima AY706329	99.58	2
4	B + P	D	*Tetracladium furcatum*	*Helotiales*	RAF/Aquatic	*T. furcatum* FJ000375	100.0	0
5	B + P	D	*Tetracladium* sp. 1	*Helotiales*	RAF/Aquatic	Uncultured ectomycorrhizal AB219857	99.79	1
						Tetracladium setigerum FJ000374	99.52	2
6	B + P	D	*Leptodontidium orchidicola*	*Helotiales*	RAF	Uncultured *Ascomycota* EU003069	100.0	0
						L. orchidicola AF486133	99.65	2
7	B	D	*Helotiales* sp. 1	*Helotiales*	Unknown	Uncultured fungus EU516819	97.81	9
						Meliniomyces bicolor EF517302	89.92	36
8	B + P	I	*Varicosporium elodeae*	*Helotiales*	RAF/Aquatic	*Varicosporium elodeae* GQ411275	99.81	1
9	B + P	I	*Articulospora tetracladia*	*Helotiales*	RAF/Aquatic	*Articulospora tetracladia* FJ000393	99.22	4
10	B + P	D + I	*Helotiales* sp. 2	*Helotiales*	RAF	Uncultured *Helotiales* FJ827196	99.81	1
						Fontanospora fusiramosa GQ411266	97.65	6
11	B + P	D	*Helotiales* sp. 3	*Helotiales*	Unknown	Uncultured saccharomyceta HQ212292	98.33	4
						Varicosporium elodeae GQ152149	94.80	23
12	B + P	I	*Helotiales* sp. 4	*Helotiales*	Unknown	Uncultured *Ascomycota* HQ211907	94.19	24
						Varicosporium elodeae GQ152148	93.17	26
13	B	D	*Nectria lugdunensis*	*Hypocreales*	Aquatic	*Nectria lugdunensis* FJ000394	98.09	9
14	B	I	*Tuber dryophilum*	*Pezizales*	EcM/OrM	Uncultured *Tuber* AY634173	99.35	4
						Tuber dryophilum EU784424	99.18	5
15	B	I	*Aspergillus versicolor*	*Eurotiales*	Saprotrophic	*Aspergillus versicolor* FJ878627	100.0	0
16	B	I	*Davidiella tassiana*	*Capnodiales*	RAF	*Davidiella tassiana* GU566258	100.0	0
18	B	D	*Ascomycota* sp. 1	Unknown	Unknown	*Alatospora acuminata* AY204589	87.02	29
19	B	D + I	*Ascomycota* sp. 2	Unknown	Unknown	Uncultured *Ascomycota* GQ268553	94.60	19
						Uncultured *Ascomycota* AJ810900	88.32	43
20	B + P	I	*Ceratobasidium* sp. 1	*Cantharellales*	RAF/OrM	Fungal endophyte sp. AP117 FM200484	99.24	3
						Ceratobasidiaceae sp. M327 HM141014	91.00	36
21	B + P	I	*Ceratobasidium* sp. 2	*Cantharellales*	Unknown	Uncultured *Ceratobasidium* FM866376	93.94	17
						Ceratobasidium sp. AG-Ba AB286930	86.80	42

Table 3. Continued

OTU	Identified*	Detected†	Species	Order	Putative ecology	Best matches	Similarity (%)	MisM
22	B	I	*Clavulinopsis* sp. 1	*Agaricales*	Unknown	*Clavulinopsis helvola* UDB001534	99.29	3
24	B	I	*Cryptococcus victoriae*	*Tremellales*	RAF/ Saprotrophic	*Cryptococcus victoriae* AF444645	99.78	1
26	B	I	*Clavispora lusitaniae*	*Saccharomycetales*	Unknown	*Clavispora lusitaniae* FJ183442	99.69	1
27	B + P	I	*Chytridiomycota* sp. 1	Unknown	Unknown	*Spizellomyces* sp. AD 020 FJ827727	93.87‡	8
						Chytriomyces hyalinus DQ536499	93.87‡	8
28	B + P	I	*Endogonales* sp. 1	*Endogonales*	Unknown	*Endogone lactiflua* AY997045	96.72‡	3
29	B + P	I	*Rhizophydiales* sp. 1	*Rhizophydiales*	Unknown	*Rhizophydium globosum* DQ485616	96.13‡	5
30	B + P	I	*Monoblepharidales* sp. 1	*Monoblepharidales*	Unknown	*Monoblepharella mexicana* AY997061	98.38‡	1
31	B	D	*Pleosporales* sp. 1	*Pleosporales*	Unknown	Uncultured fungus HQ436095	85.51	42
						Alternaria brassicicola HQ377367	92.56‡	7
32	B	D	*Spirosphaera* sp. 1	Unknown	Unknown	*Spirosphaera cupreorufescens* AY616232	93.85	18
33	B	I	Fungus sp. 1	Unknown	Unknown	Uncultured fungus AM999749	86.68	54
						Uncultured fungus AM999754	86.55	55
34	B + P	I	*Chytridiales* sp. 1	*Chytridiales*	Unknown	Uncultured *Chytridiomycota* GU327529	100.0‡	0
						Synchytrium macrosporum AY997095	93.24‡	8
35	B + P	I	*Chytridiales* sp. 2	*Chytridiales*	Unknown	Uncultured *Chytridiomycota* GU327529	100.0‡	0
						Synchytrium macrosporum AY997095	92.62‡	9
36	B	I	*Chytridiales* sp. 3	*Chytridiales*	Unknown	Uncultured *Chytridiomycota* GU327529	98.18‡	2
						Synchytrium macrosporum AY997095	93.29‡	8
37	P	I	*Glomerales* sp. 1	*Glomerales*	AM			
38	P	I	*Glomerales* sp. 2	*Glomerales*	AM			
39	P	I	*Glomerales* sp. 3	*Glomerales*	AM			
40	P	I	*Glomerales* sp. 4	*Glomerales*	AM			
41	P	I	*Gigasporaceae* sp. 1	*Diversisporales*	AM			
42	P	I	*Archaeosporales* sp. 1	*Archaeosporales*	AM			
43	P	I	*Archaeosporales* sp. 2	*Archaeosporales*	AM			
44	P	I	*Archaeosporales* sp. 3	*Archaeosporales*	AM			

AM, arbuscular mycorrhizal; EcM, ectomycorrhizal; ErM, ericoid mycorrhizal; OrM, orchid mycorrhizal; MisM, the number of mismatches over the BLAST alignment.

*Species identified using BLASTN search (B), phylogenetic analyses (P) or both approaches (B + P).

†OTU detected using culture-independent (I) or culture-dependent (D) techniques.

‡Similarity is based only on sequences of 5.8S region of rDNA.

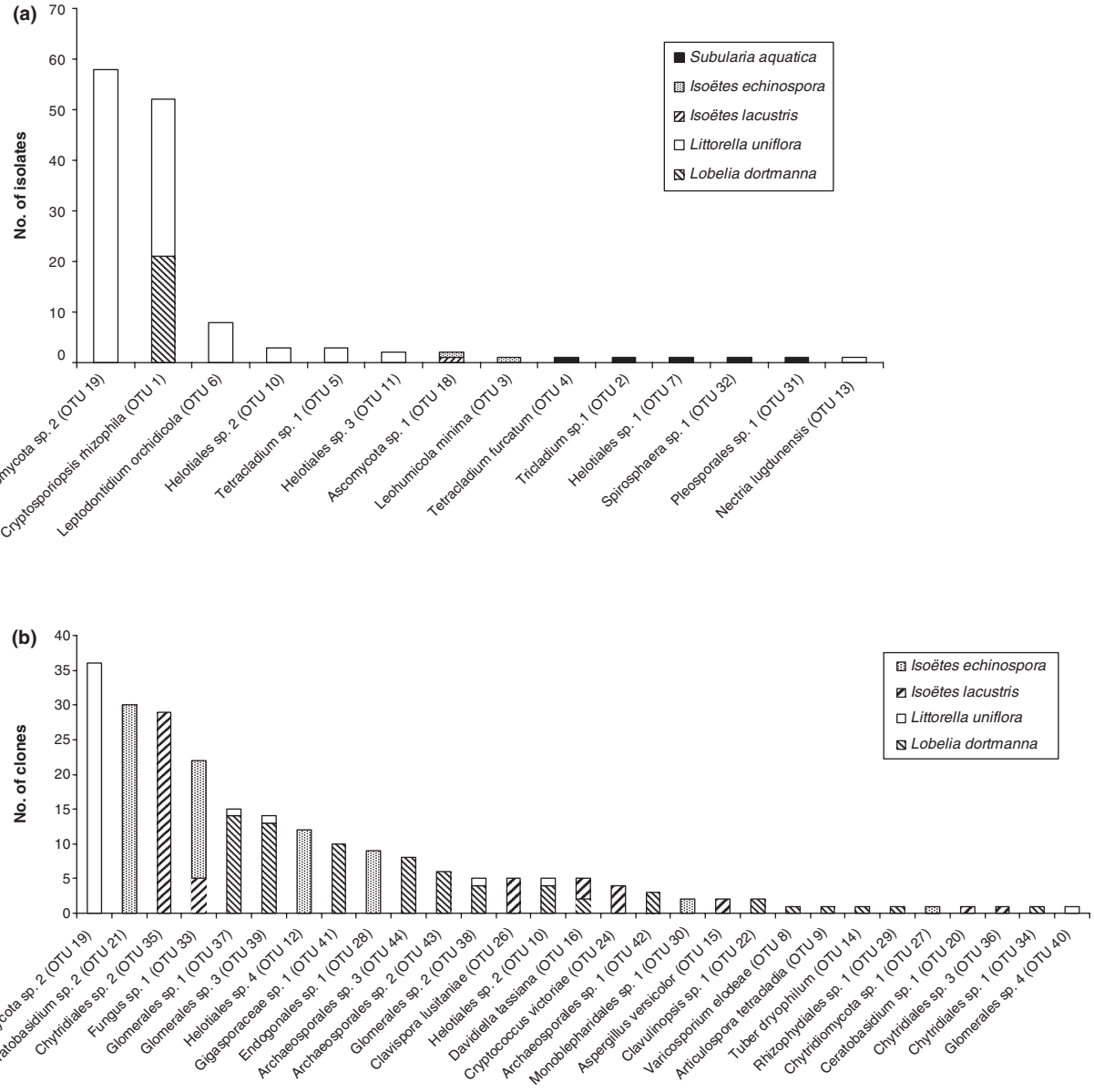


Fig. 4. Number of isolates (a) or sequences (b) of the RAF OTUs obtained from the roots of studied plant species using culture-dependent (a) and culture-independent (b) techniques.

roots of *Lob. dortmanna*. Levels of DSE colonization in *Lit. uniflora* (i.e. the species with the highest colonization values) differed among the lakes sampled, likely as a consequence of different sediment chemistry; DSE colonization was positively related to organic matter content and negatively related to pH.

A microscopic evidence for the presence of hyaline hyphae of endophytic fungi in all studied plant species was also confirmed by the isolation procedure. However, only three fungal isolates were obtained from the roots of *Isoëtes* spp., in contrast to our microscopic observations. The disagreement between the two methods might have stemmed from the harmful effect of surface sterilization of *Isoëtes* roots with specific anatomy, that is, bleach might have infiltrated large root cavities and damaged endophytic fungal structures.

Diversity and ecology of non-AM RAF

The only DSE fungus obtained in our study was *L. orchidicola*, which was repeatedly isolated from *Lit. uniflora* roots. *Leptodontidium orchidicola* is a root endophyte

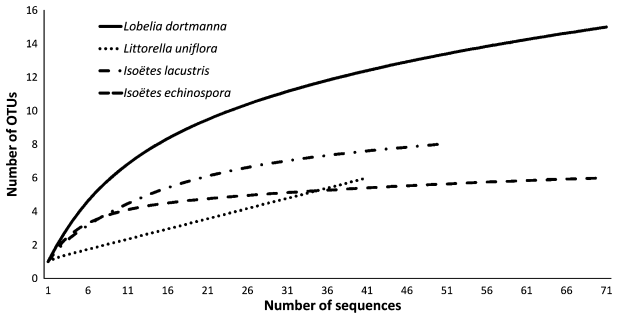


Fig. 5. Species accumulation curves of RAF colonizing isoetids from Mjåvatn and Ljøsvatn lakes. Curves were calculated based on the number of sequences obtained using the culture-independent method.

of many plant species (Jumpponen & Trappe, 1998); however, it has never been found in the aquatic environment. According to Fernando & Currah (1996), the effect of *L. orchidicola* on plant growth ranges from negative to positive, depending on the fungal strain. The ecological role of *L. orchidicola* in freshwater habitats remains open to question.

The most common RAF detected, using both isolation and cloning approach, in this study was an unknown species from the phylum *Ascomycota* (OTU 19), which exclusively associated with *Lit. uniflora*. There are only two similar sequences in public databases and, interestingly, both of them came from SE Asia. The first one originates from the roots of *Aphyllorchis caudata* from tropical forest of Thailand (Roy *et al.*, 2009), while the other was recently isolated from an ectomycorrhizal root tip in a dipterocarp rain forest in Malaysia (Peay *et al.*, 2010). These findings, considered together with our results, suggest an endophytic lifestyle of this group of *Ascomycota*. However, no similar fungi have been reported from temperate regions despite the fact that they are comparatively well researched. Further investigation may reveal whether geographical range of this group of fungi is disjunct and restricted to tropical and boreal zones or rather continuous.

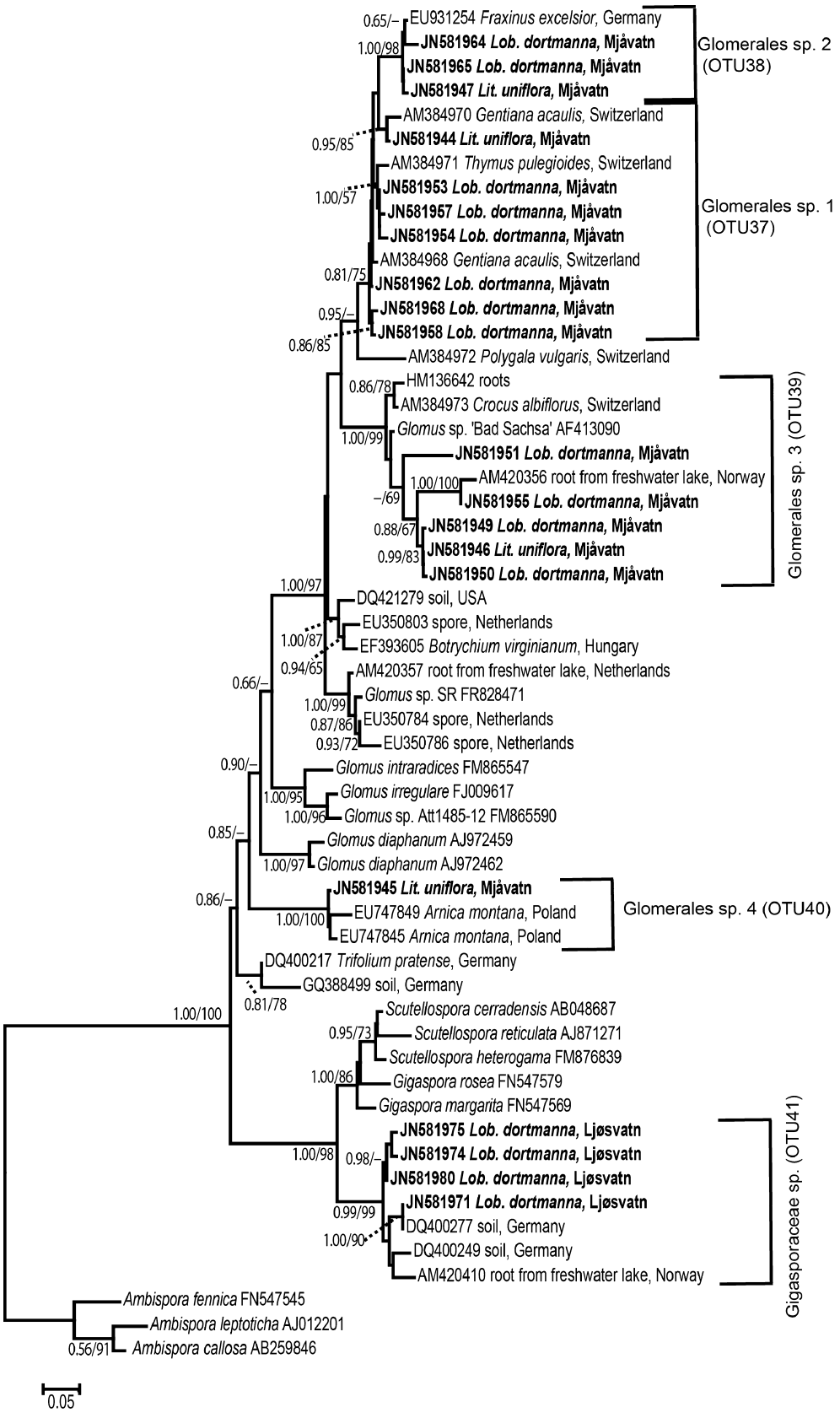
The second most common RAF obtained exclusively using culture-dependent technique, from the roots of *Lit. uniflora* and *Lob. dortmanna*, was *C. rhizophila*. This fungus was previously found as an endophyte from roots of four European species from the family *Ericaceae* (Verkley *et al.*, 2003) and several nonericaceous plants from Australia (Chambers *et al.*, 2008). Recently, we have also identified *C. rhizophila* as a common endophyte in *Pseudorchis albida*, a member of the family *Orchidaceae* (P. Kohout, unpublished data). Zijlstra *et al.* (2005) reported a positive effect of *C. rhizophila* on the growth and nitrogen uptake of some plant species. To the best of our knowledge, this study is the first to detect *C. rhizophila* in roots of aquatic plants and demonstrate that their terrestrial and aquatic strains are closely related (Fig. 8).

The cloning approach yielded sequences similar to *Endogone lactiflua* (*Mucoromycotina*) from the roots of *I. echinospora*. These fungi are known as ectomycorrhizal on the roots of conifers (Fassi *et al.*, 1969) and recently were also detected in the earliest branching lineage of liverworts (*Haplomitriopsida*) and simple thalloid liverworts (*Jungermanniopsida*) (Bidartondo *et al.*, 2011). The authors therefore hypothesized that *Mucoromycotina* may form symbioses with ancient plant lineages and might also have played a role in plant terrestrialization, because their origin predates that of *Glomeromycota* (James *et al.*, 2006). Their occurrence in the roots of submerged lycopsids, as observed in our study, partly supports both these hypotheses.

Another fungi obtained by cloning were two species of *Ceratobasidium* from the roots of both *Isoëtes* spp., which interestingly clustered with known RAF sequences from orchids. In general, *Ceratobasidium* spp. are well known and abundant orchid mycorrhizal fungi (Cameron *et al.*, 2006, 2008) with global geographical distribution (Dearnaley, 2007). Some strains have also been shown to be endophytes of *Pinus sylvestris* (Sen *et al.*, 1999), root-growth promoters (Grönberg *et al.*, 2006) and ectomycorrhizal (Bougoure *et al.*, 2009). Our study extends the ecological amplitude of the genus *Ceratobasidium* and provides the first evidence for its ability to colonize roots of submerged plants.

One of the most diverse groups of uncultured RAF detected in the roots of both *Isoëtes* spp. and *Lob. dortmanna* constitute *Chytridiomycota*. These fungi usually act as saprotrophs or parasites on a wide range of hosts (Shearer *et al.*, 2007). Although they occur in different environments, they are more common in aquatic habitats. However, Freeman *et al.* (2009) recently reported heretofore unseen diversity and abundance of chytrids from high-elevation soils, well-watered from the melting snow

Fig. 6. Phylogenetic tree of a part of the *Glomerales* and *Gigasporaceae* based on a neighbour-joining analysis of ITS1, 5.8S rDNA and part of the ITS2 sequences (328 characters). The numbers above or below branches denote probability from Bayesian analyses/neighbour-joining bootstrap values from 1000 replications. The tree was rooted by *Ambispora fennica*, *Ambispora leptoticha* and *Ambispora callosa*. Sequences obtained in this study are shown in bold. They are labelled with the database accession number, host plant species and lake. The brackets show the delimitation of OTUs.



belts. Their results show that water content can play an important role in the occurrence of *Chytridiomycota* in microbial communities.

Our observation of *L. minima* in the roots of *I. echinospora* represents the first record from roots of freshwater submerged plants. Originally, this fungus was described from volcanic ash soil in Chile (Hambleton *et al.*, 2005), and similar fungal species were recently obtained from the roots of *Pyrola* spp. (Hynson & Bruns, 2009) and *Epacris microphylla* (A. F. Williams, S. M. Chamber, P. W. Davies, C. B. McLean and J. W. G. Cairney, unpublished data). All these observations together with the work of Hambleton *et al.* (2005) show that some species of the genus *Leohumicola* are able to form ericoid mycorrhiza while others likely have an endophytic lifestyle (Vrålstad *et al.*, 2002; Bergero *et al.*, 2003).

Nectria lugdunensis, a well-known aquatic hyphomycete, was recognized as another species with capability to colonize roots of submerged aquatic plants (*Lit. uniflora* in our case). This fungus is a common inhabitant of limnic waters in temperate regions (Gulis *et al.*, 2005). Pascoal *et al.* (2005) reported *N. lugdunensis* among the most abundant aquatic hyphomycetes in Portuguese rivers. Additionally, it was isolated from submerged wood from an estuary in Scotland (Pearman *et al.*, 2010) and from agricultural soils in Austria (Klaubauf *et al.*, 2010). Therefore, it is clear that *N. lugdunensis* is a widely distributed species with a broad ecological amplitude and capability to grow in terrestrial soils, freshwater and brackish habitats as well as in plant tissues as an endophyte. However, only two recent studies have investigated the effects of this fungus on host plant growth (Sati & Arya, 2010a, b).

Although *Tuber* species are generally considered as xerophilous fungi, we detected *Tuber dryophilum* in the roots of *I. echinospora*. Congruently, *T. dryophilum* fruiting has previously been reported from sites with standing water in Pennsylvania, USA (W. Sturgeon, pers. observation, 1986). Therefore, we assume that truffles are not restricted to xerophytic habitats and may also occur in aquatic environment. Our record of *T. dryophilum* is also supported by previous observation of this species in Norway (Biodiversity occurrence data published by: Natural History Museum, University of Oslo, Mycology herbarium; Accessed through GBIF Data Portal, www.data.gbif.org, 26 October 2008).

Together with some of the previously discussed fungi, we totally detected 12 OTUs with affinity to the order *Helotiales*. In general, members of the *Helotiales* have very heterogeneous ecology and lifestyle. They include plant pathogens (Queloz *et al.*, 2011), ectomycorrhizal (Vrålstad *et al.*, 2000) or ericoid mycorrhizal fungi (Grelet *et al.*, 2009; Tedersoo *et al.*, 2011) and occur in various environments as for example aquatic hyphomycetes (Shearer *et al.*, 2007) or soil saprobes (Piercey *et al.*, 2002). We showed that diverse species of the *Helotiales* can also be present in the roots of submerged plants. The recognized OTUs had close affinities to endophytic fungal sequences previously isolated from terrestrial habitats (OTUs 1, 3, 6, 7 and 10), as well as to fungal taxa belonging to a polyphyletic group of aquatic hyphomycetes (OTUs 2, 4, 5 and 8). In accordance with published studies (Selosse *et al.*, 2008), our phylogenetic analyses showed that all obtained isolates of aquatic hyphomycetes closely resembled those known from terrestrial habitats (Fig. 8). Interpreting these results in the light of the evolutionary history of host plants allows us to develop hypotheses about the evolution of some aquatic fungi. It is quite likely that terrestrial ancestors of the present-day aquatic plant species interacted with different groups of ubiquitous RAF, both mycorrhizal and nonmycorrhizal (Jumpponen & Trappe, 1998). The ecological transition from terrestrial to freshwater environments was connected with transition of the fungal partners, as documented for AMF (Søndergaard & Laegaard, 1977), and can be anticipated for their nonmycorrhizal counterparts. Some of the extant free-living aquatic hyphomycetes might have evolved from nonmycorrhizal RAF that once entered aquatic habitats together with their host plants. An indirect support for this hypothesis comes from the fact that some aquatic hyphomycetes still retain the ability to colonize roots of both submerged and terrestrial plants. The transition from terrestrial to aquatic environment was previously also reported for marine fungi (e.g. Spatafora *et al.*, 1998; Kohlmeyer *et al.*, 2000). However, further research is needed to adequately address our hypothesis.

Diversity and ecology of AMF

Although we did not focus primarily on the molecular diversity of AMF, we were able to detect eight AMF taxa belonging to the orders *Archaeosporales*, *Diversisporales* and *Glomerales* in the roots of *Lit. uniflora* and *Lob. dortmanna* from two Norwegian lakes. Our results thus confirm previous reports on a relatively high AMF diversity (Nielsen *et al.*, 2004) and the presence of ancestral AMF lineages in submerged aquatic plants (Baar *et al.*, 2011). Nevertheless, it is likely that the diversity of AMF in our study is underestimated because we employed fungal-specific (not AMF-specific) primers and therefore, only few Glomeromycotan sequences were among the analysed clones in *Lit. uniflora*.

Interestingly, none of the taxa recorded in our study was related to any morphologically described AMF species and the obtained sequences matched only to uncultured fungi from environmental samples. Notably, all three *Archaeosporales* taxa clustered with AMF sequences obtained

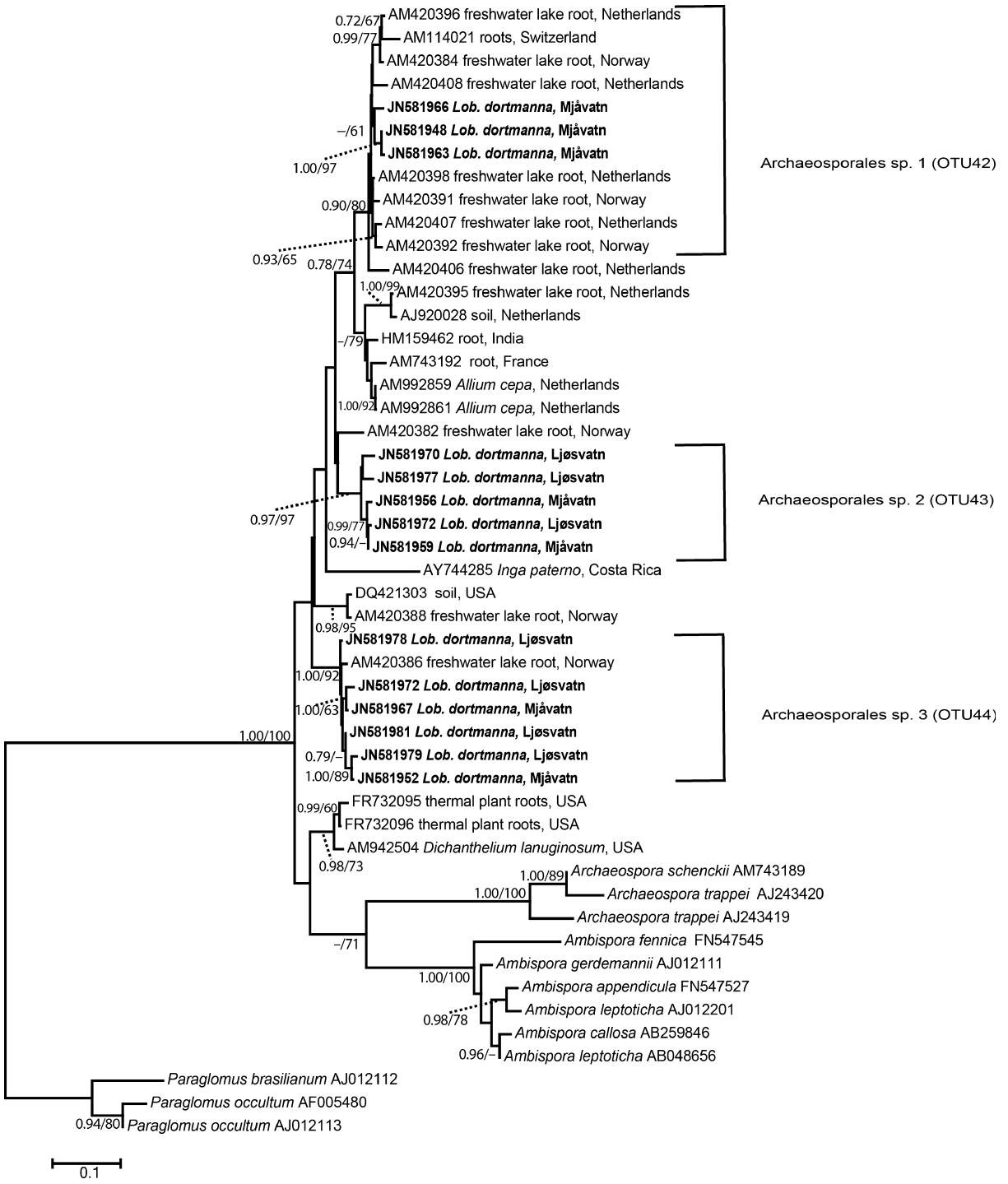


Fig. 7. Phylogenetic tree of *Archaeosporales* based on a neighbour-joining analysis of ITS1, 5.8S rDNA and part of the ITS2 sequences (331 characters). The numbers above or below branches denote probability from Bayesian analyses/neighbour-joining bootstrap values from 1000 replications. The tree was rooted by *Paraglomus brasilianum* and *Paraglomus occultum*. Sequences obtained in this study are shown in bold. They are labelled with the database accession number, host plant species and lake. The brackets show the delimitation of OTUs.

from the roots of submerged plants inhabiting freshwater lakes in the Netherlands and Norway (Baar *et al.*, 2011). In contrast to *Archaeosporales* spp. 3 and 2, *Archaeosporales* sp. 1 was also reported from terrestrial habitats (Hijri *et al.*, 2006). The sequences corresponding to *Glomerales* spp. 1, 2 and 4 have hitherto been reported

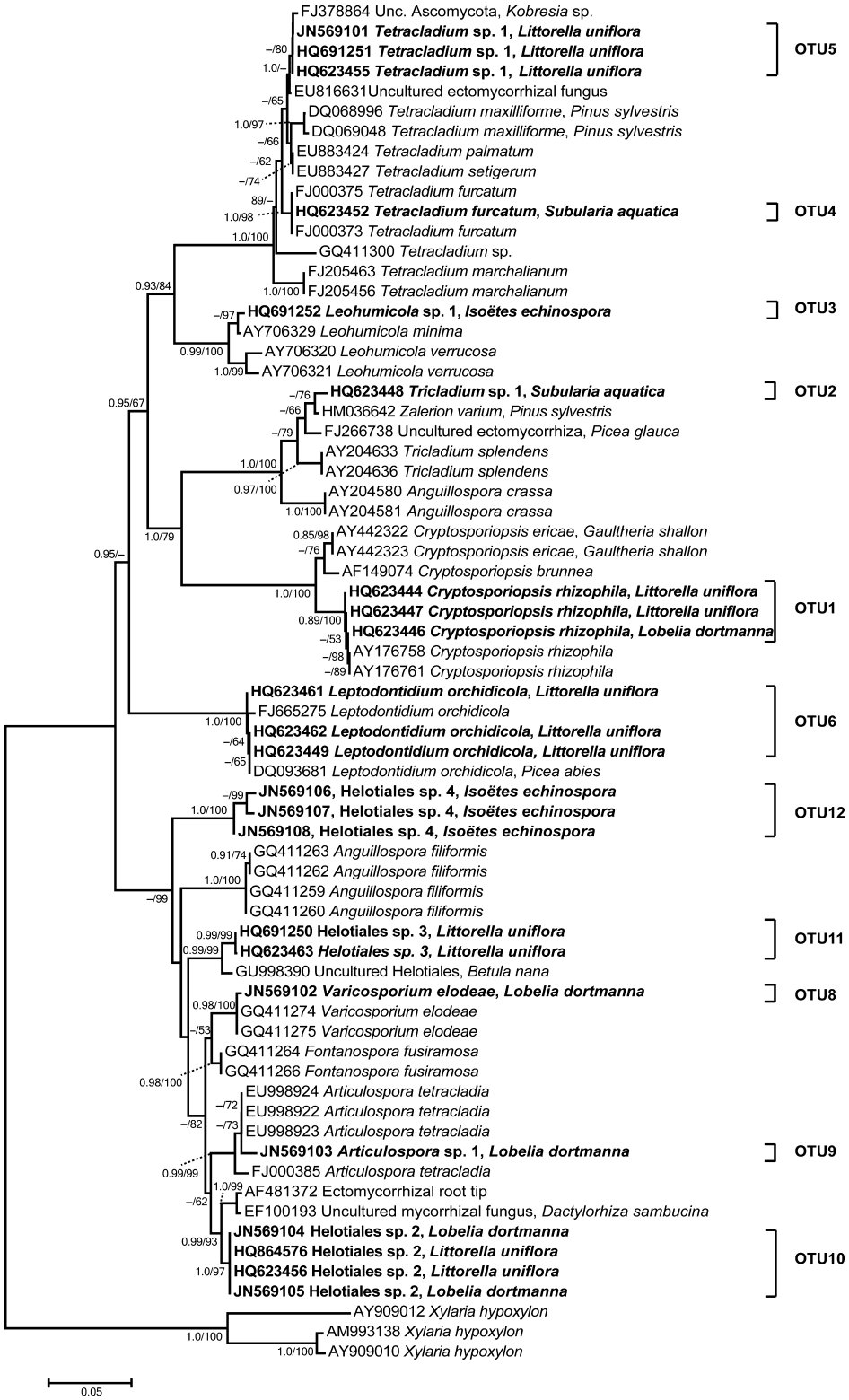


Fig. 8. Phylogenetic tree of a part of the order *Helotiales* based on a neighbour-joining analysis of ITS1, 5.8S rDNA and part of the ITS2 sequences. The numbers above or below branches denote probability from Bayesian analyses/neighbour-joining bootstrap analysis values. The tree was rooted using sequence of *Xylaria hypoxylon*. Sequences obtained in this study are shown in bold. They are labelled with the database accession number, name of fungal taxon and host plant species.

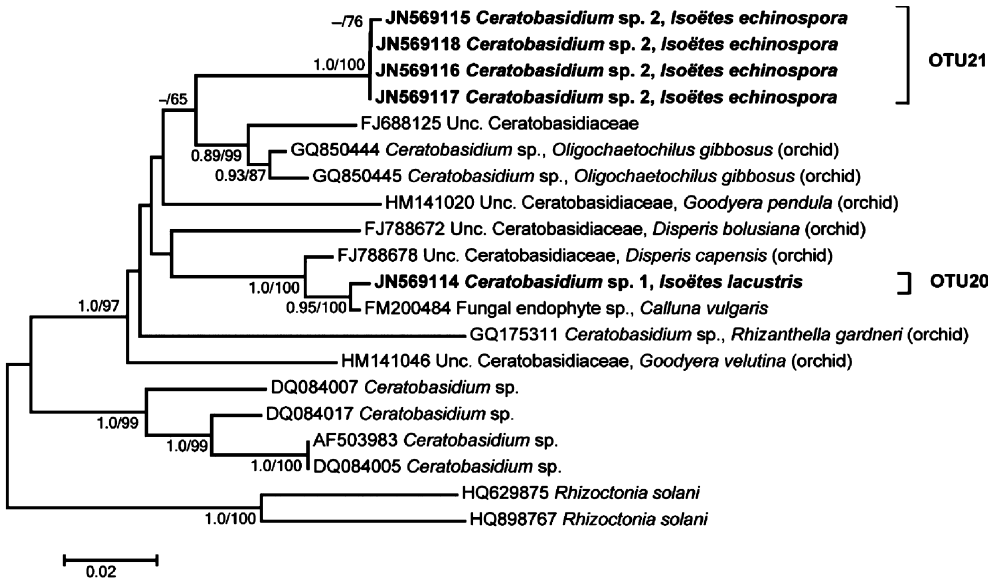


Fig. 9. Phylogenetic tree of a part of the genus *Ceratobasidium* based on a neighbour-joining analysis of ITS1, 5.8S rDNA and part of the ITS2 sequences. The numbers above or below branches denote probability from Bayesian analyses/neighbour-joining bootstrap analysis values. The tree was rooted using sequence of *Rhizoctonia solani*. Sequences are labelled as described in the legend of Fig. 8.

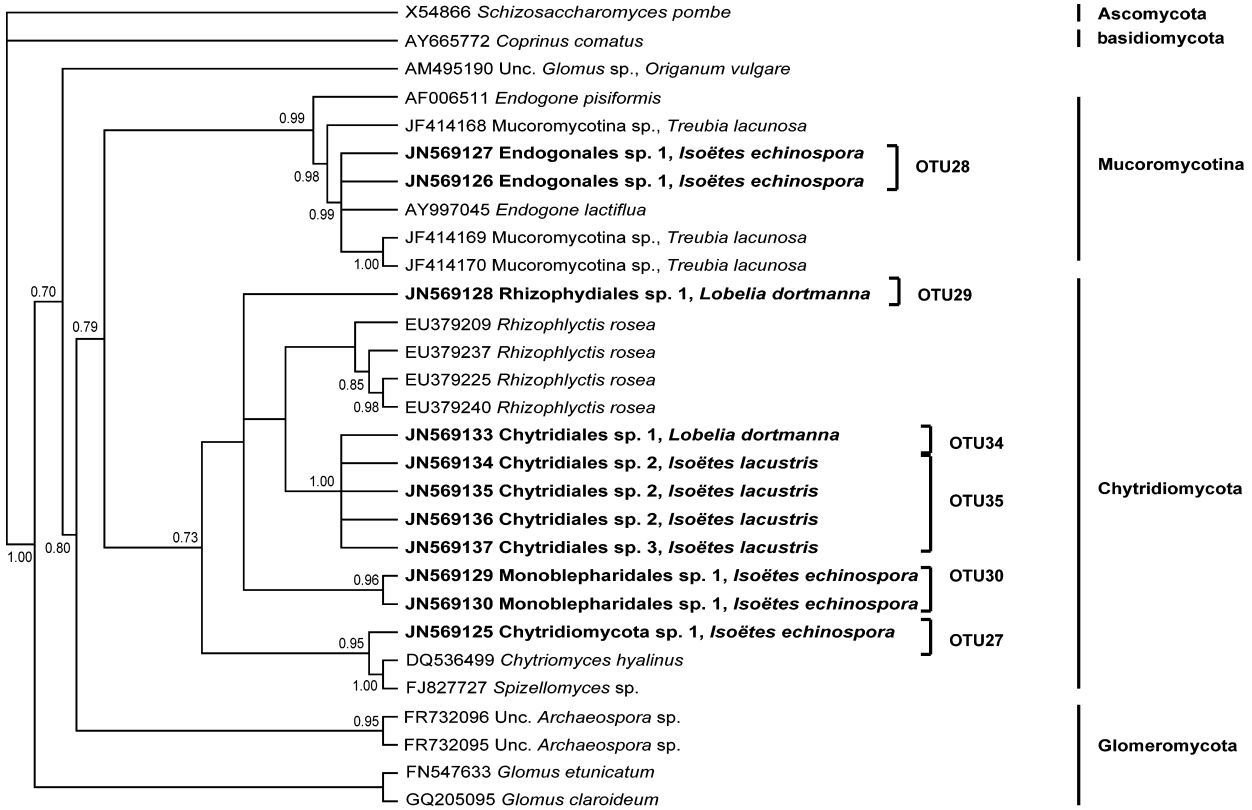


Fig. 10. Phylogenetic tree of a part of the Fungal kingdom based on a Bayesian analyses of 5.8S rDNA sequences. The numbers above or below branches denote probability from Bayesian analyses. Sequences are labelled as described in the legend of Fig. 8.

only from terrestrial ecosystems (Sýkorová *et al.*, 2007; Ryszka *et al.*, 2010; Lang *et al.*, 2011), whereas *Glomerales* sp. 3 and *Gigasporaceae* sp. 1 are known from both terrestrial (Landwehr *et al.*, 2002; Hempel *et al.*, 2007; Sýkorová *et al.*, 2007) and aquatic environments (Baar *et al.*, 2011). The AMF detected in our study actually represent a subset of taxa revealed by Baar *et al.* (2011) using AMF-specific primers in the same host plant species in other lakes in Norway and the Netherlands. This may indicate a preference of aquatic plants for specific AMF taxa. A marked underrepresentation of the AMF sequences from aquatic ecosystems in the INSD database, however, precludes any firm conclusion.

To conclude, we found that roots of submerged aquatic plants might be colonized by diverse spectrum of mycorrhizal as well as nonmycorrhizal RAF, belonging to *Mucoromycotina*, *Chytridiomycota*, *Glomeromycota*, *Ascomycota* as well as *Basidiomycota*. According to putative ecology of RAF, we observed species with very diverse ecology, including arbuscular mycorrhizal, ericoid mycorrhizal, orchid mycorrhizal, ectomycorrhizal, saprotrophs as well as endophytes.

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