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Colonisation and molecular diversity of arbuscular mycorrhizal fungi in the aquatic plants *Littorella uniflora* and *Lobelia dortmanna* in southern Sweden

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The colonisation intensity and composition of the mycorrhizal community in the aquatic plants *Lobelia dortmanna* and *Littorella uniflora* were studied. The mycorrhizal fungi were characterised by fungal specific nested PCR and sequencing using the 5'-end of the LSU rDNA as target. For this, primers for the clade of *Acaulospora*, the clade including *Glomus mosseae* and *G. intraradices* and the clade containing *G. etunicatum* and *G. claroideum* were used.

The nested PCR products were screened for different sequence types using single stranded conformation polymorphism (SSCP) and representatives for each type were sequenced. A phylogenetic analysis of the sequences showed two phylotypes of *Acaulospora*, one phylotype within the clade of *G. etunicatum*/*G. claroideum* and five within the *G. mosseae*/*G. intraradices* clade. The colonisation intensity was comparable to that seen in typical grassland vegetation. The neutral lipid fatty acid 16:1 ω 5 was seen to be indicative of mycorrhizal colonisation with concentrations up to 35 nmol mg⁻¹ root DW, which indicates that the fungi are active.

INTRODUCTION

The vegetation of oligotrophic, softwater lakes is dominated by the aquatic plants *Littorella uniflora*, *Lobelia dortmanna* and *Isoetes lacustris* (Sculthorpe 1967). They belong to the isoetids, which are characterised by a rosette growth form, a well-developed root system and an extensive aerenchyma. The occurrence of arbuscular mycorrhiza (AM) in *Lobelia* and *Littorella* was first reported in Danish oligotrophic, softwater lakes by Søndergaard & Lægaard (1977). Since then, the symbiosis has been found in aquatic and semi aquatic plants in many places in the world (Khan & Belik 1995), however only a minority of aquatic plant species have been reported to be colonised by AM fungi (Chaubal, Sharma & Mishra 1982, Nielsen, Thingstrup & Wigand 1999, Beck-Nielsen & Madsen 2001). The isoetids are adapted to nutrient uptake in the root, for example CO₂ is taken up from the interstitial water (Wium-Andersen 1971) where it is found in 20–200-fold higher concentrations than in the overlying water (Wium-Andersen & Andersen 1972). The permeable root surface results in oxygen leaking into

the sediment (Sand-Jensen & Prahl 1982), whereby the sediment is oxygenated and the sediment redox potential (E_h) is increased (Pedersen, Sand-Jensen & Revsbech 1995). Mycorrhizal fungi are believed to be dependent on oxic conditions for their growth (Tanner & Clayton 1985, Beck-Nielsen & Madsen 2001), and the extensive aerenchyma and the oxic rhizosphere may likely explain the well-developed mycorrhiza in isoetids.

From terrestrial studies it is known that the AM symbiosis can improve uptake of phosphorus (P) and micronutrients in plants (Smith & Read 1997). The nutritional role of mycorrhiza in aquatic plants is not completely clarified, but the extensive mycorrhizal colonisation tend to correlate both with low tissue P content of the aquatic plants (Christensen & Wigand 1998) as well as with low sediment nutrient concentration (Chaubal *et al.* 1982). Furthermore, the low P availability in the oligotrophic and mesotrophic softwater lakes indicates a potential benefit of mycorrhiza to the plants in this ecosystem.

Presently, not much is known about the fungi forming the symbiosis with isoetids in terms of diversity

Table 1. Sampling sites and degree of mycorrhizal colonisation of *Lobelia dortmanna*, *Littorella uniflora* and *Isoetes lacustris*. TRLC, total root length colonised; AC, arbuscular colonisation; VC, vesicular colonisation (according to McGonigle *et al.* 1990).

Lake	Lake location	Plant species	Water depth (m)	TRLC (%)	AC (%)	VC (%)
Norre sjö	56° 44' N, 13° 89' E	<i>L. dortmanna</i>	0.1–0.2	57	43	36
		<i>L. uniflora</i>	0.1–0.2	60	41	27
		<i>I. lacustris</i>	0.3	0	0	0
Vittsjön	56° 35' N, 13° 69' E	<i>L. dortmanna</i>	0.1–0.2	85	83	65
		<i>L. uniflora</i>	0.1–0.2	82	76	46
Skeingesjön	56° 39' N, 13° 90' E	<i>L. dortmanna</i>	0.1–0.2	57	51	43
Tydingen	56° 24' N, 14° 03' E	<i>L. dortmanna</i>	0.1–0.2	55	49	18
Svanshalssjön	56° 41' N, 13° 94' E	<i>L. dortmanna</i>	0.5–1.0	34	28	6
		<i>I. lacustris</i>	1.0	0	0	0
Osbysjön I	56° 34' N, 13° 99' E	<i>L. dortmanna</i>	0.1–0.2	78	75	53
Osbysjön II		<i>L. dortmanna</i>	0.1–0.2	66	57	50

and type of colonisation. A variety of glomalean fungi have been isolated from aquatic sediments as spores in earlier studies (e.g. Ragupathy, Mohankumar & Mahadevan 1990, Miller *et al.* 1999), but it is difficult to relate the presence of spores to colonisation of particular plants. PCR-based methods have a great potential for the study of diversity of AM fungi (AMF) in field-collected roots (Helgason *et al.* 1998, Kjølner & Rosendahl 2000), but so far these methods have not been used in aquatic plant communities. The aim of the present study was to describe the mycorrhizal community and the intensity of colonisation in six softwater lakes in southern Sweden and to see if the mycorrhizal flora in aquatic plants deviated from that found in terrestrial systems. Another aim was to investigate if the mycorrhizal flora varied between the studied plant species and between the different lakes. The large ribosomal subunit (LSU) gene was chosen as target for the PCR in this study, as this gene contains enough variation to distinguish species of AMF (Clapp, Rodriguez & Dodd 2001). The intensity of the AM colonisation was studied by use of signature fatty acids (Olsson 1999). The amount of phospholipid fatty acids (PLFA) in a given cell is well correlated to biomass (Tunlid & White 1992), whereas neutral lipid fatty acids (NLFA) are indicators of storage carbon. We used fatty acids to describe the AMF colonisation and to compare their biomass and carbon storage levels with terrestrial ecosystems.

MATERIALS AND METHODS

Collection and microscopy of plant material

Lobelia dortmanna (Campanulaceae) was collected from six lakes and *Littorella uniflora* (Plantaginaceae) and *Isoetes lacustris* (Isoetaceae) from two lakes in southern Sweden (Table 1). The lakes had varying nutrient concentrations (2–111 µg l⁻¹ NO₃-N, 371–927 µg l⁻¹ tot-N, <1–2 µg l⁻¹ PO₄-P, 24–34 µg l⁻¹ tot-P; data from the Swedish Agricultural University). Three to eight specimens of each species were collected from

each lake and stored in plastic bags until returned to the lab. Plant roots were pooled, cleaned and divided into three portions. Colonisation was measured by the microscopy method and by fatty acid analysis. Root samples for fatty acid analysis and molecular identification were stored at –80 °C until analysis. For microscopic analysis root samples were cleared in 10% KOH and stained with 0.05% aniline blue in lactoglycerol (Brundrett *et al.* 1996). The percentage of root length colonised and the occurrence of arbuscules and vesicles was assessed employing the method of McGonigle *et al.* (1990) using light microscopy at 200 times magnification. The presence of fine endophytic fungi (Thippayarugs, Bansal & Abbott 1999) was also recorded.

Analysis of fatty acid signatures

Phospholipid fatty acid (PLFA) composition varies between different groups of organisms, and a distinctive composition exists for the AMF. The concentration of fatty acids reflects the living biomass of fungi. The lipids from freeze-dried root samples (10–30 mg dw) were extracted according to Bligh & Dyer (1959). The lipids were separated into neutral lipids (NLFAs), glycolipids and phospholipids (PLFAs) on pre-packed silica columns (100 mg sorbent mass, Varian) by eluting with 1.5 ml chloroform, 6 ml acetone and 1.5 ml methanol, respectively. The fatty acid residues of NLFAs and PLFAs were transformed into free fatty acid methyl esters and analysed by gas chromatography using a 50 m HP5 capillary fused silica column (Hewlett Packard) with H₂ as carrier gas (Frostegård, Tunlid & Bååth 1993). The fatty acids were identified from their retention times in relation to the internal standard (FA methyl ester 19:0). These were compared to fatty acids identified using gas chromatography-mass spectrometry. We compared the NLFA/PLFA ratios for the dominant fatty acids in the roots in order to determine which were of AM fungal origin. It has earlier been shown that AM fungi have large amounts of neutral FA compared with bacteria and

Table 2. Primers used to amplify the LSU rDNA sequences from mycorrhizal roots.

LSU 0061 ^{a,b}	5' AGCATATCAATAAGCGGAGGA
NDL22 ^{a,b}	5'-TGGTCCGTGTTTCAAGACG
LSURK4f ^{c,d,e}	5'-GGGAGGTAAATTTCTCCTAAGGC
LSURK7mr ^{c,d,e}	5'-ATCGAAGCYACATTCTCC
LSUAca1f ^{d,e}	5'-GGGTGGTAAATTTACCTAAGGC
LSUAca3r ^{d,e}	5'-RRWCYCACATYYMYCARGG
LSUcla1f ^{d,e}	5'-GGGTGGTAAACATCACCTAAGGC
LSUcla2r ^{d,e}	5'-AAGGAGCCACATCCCTCCAG

R = A or G; W = A or T; Y = C or T; M = A or C.

^a Primers from van Tuinen *et al.* (1998b).

^b Primers used for primary PCR amplifications.

^c Primers from Kjølner & Rosendahl (2000).

^d Primers used for the second, nested PCR amplifications.

^e Primers used for sequencing.

roots and therefore the NLFA/PLFA ratio for AM fungal fatty acids is normally higher than 1 and for other fatty acids lower than 1 (Olsson & Johansen 2000). It has earlier been found that the amount of NLFA 16:1 ω 5 in non-mycorrhizal *Plantago lanceolata* (a plant in the same family as *L. uniflora*) from pot cultures was less than 0.1 nmol mg⁻¹ dry root (van Aarle & Olsson 2003). This value was used as an indication of the amount of this NLFA in roots that was of another origin than of AM fungi. Furthermore, we compared the fatty acid composition in the AM colonised plants with the composition in the non-mycorrhizal *Isoetes lacustris* to verify which fatty acids could be used as indicators for AM fungal biomass.

PCR

From each sampling site and from each of the mycorrhizal plant species 16–25 randomly chosen individual root pieces of 5 mm length were transferred to 1.5 ml Eppendorf tubes and DNA was extracted by the Chelex extraction method (van Tuinen *et al.* 1998a, Kjølner & Rosendahl 2000). The samples were spun at 13 000 rpm for 5 min. Primary PCR was performed with the eukaryote specific primers LSU 0061 (LR1)/NDL22 (van Tuinen, Zhao & Gianinazzi-Pearson 1998b) giving an approx. 750 bp product in the variable 5'-end of the LSU rDNA (Table 2). The PCR conditions were as described by Kjølner & Rosendahl (2000).

Amplification products were used as templates in a nested PCR. For this, two new sets of primers were constructed, LSUcla1f/LSUcla2r specific for the *Glomus claroideum*/*G. etunicatum* clade and LSUAca1f/LSUAca3r for the *Acaulospora* (*A. longula*, *A. laevis*, *A. tuberculata*, and *Entrophospora colombiana*) clade. A third primer set LSURK4f/LSURK7mr, modified after Kjølner & Rosendahl (2000), amplifying a lineage of *Glomus* (including *G. caledonium*, *G. mosseae*, *G. geosporum*) was also used (Table 2). The PCR conditions were as described by Kjølner & Rosendahl

(2000). The new primers were designed from sequences available from GenBank.

Single stranded conformation polymorphism

The nested PCR products were screened for sequence variation using single stranded confirmation polymorphism (SSCP). Nested PCR products were grouped according to SSCP pattern. Representatives of each SSCP type were sequenced at the MWG Biotech facility in Germany after purification using Qiagen's 'Qiaquick PCR purification kit' (Qiagen, Valencia, CA). The samples were prepared as described by Kjølner & Rosendahl (2000).

In the case where SSCP analysis indicated the presence of only one sequence the nested PCR product was sequenced directly. In the case where SSCP analysis yielded complex patterns indicating that several sequences were present in the nested PCR product gel bands were cut out, squashed in 10 ml TE-buffer (Kjølner & Rosendahl 2000), and used as template in a new PCR reaction with nested primers. If the resulting pattern was similar to the original pattern it was accepted as the pattern of one sequence due to alternative conformations of this sequence. If the pattern was less complex it was assumed that the first, more complex, pattern was due to more than one sequence in the PCR product and the rest of the bands from the original SSCP gel pattern were cut out and used as PCR templates to get all the potential sequences from the original PCR product. Sequences have been deposited in the EMBL database under accession nos AJ620952–AJ620980.

Analysis of molecular data

Sequencing traces were checked for base-calling errors using the program 'Chromas'. Sequences were compared to known sequences using the BLAST search option at NCBI (<http://www.ncbi.nih.gov/index.html>). Sequences that did not match with glomalean sequences were omitted from the following phylogenetic analysis.

The sequences were aligned using the computer program Clustal W, integrated in the BioEdit program package on the internet (Thompson *et al.* 1997) and then refined manually. The phylogenetic analysis was performed as a maximum parsimony (MP) analysis with the program PAUP (Swofford 1998), using heuristic search options with 10 000 bootstrap replications. The settings for the analysis were: branch swapping by tree-bisection-reconnection (TBR), branches able to collapse to yield polytomies and with parsimony uninformative characters excluded (Clapp *et al.* 2001). The phylogenetic trees were rooted with *Paraglomus occultum* as an out-group (Morton & Redecker 2002). Using the information in a strict consensus tree 'phylotypes' (Vandenkoornhuys *et al.* 2002) were assigned to clades of closely related

Table 3. The quantitatively most important 18C and 16C fatty acids identified in roots of the isoetids. Total amounts of phospholipid fatty acids (PLFA), neutral lipid fatty acids (NLFA) and the NLFA/PLFA ratio for each fatty acid are given. Significant differences in this ratio between mycorrhizal *Lobelia dortmanna* and *Littorella uniflora* compared with the non-mycorrhizal plant *Isoetes lacustris* were found in three cases (indicating that these NLFAs to a large part represent AM fungi). The amount of these specific NLFAs is therefore also presented and significant differences between mycorrhizal and non-mycorrhizal plant species is seen in all three cases. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	Fatty acid content nmol mg ⁻¹ root			Significance
	<i>L. uniflora</i> (n=2)	<i>L. dortmanna</i> (n=8)	<i>I. lacustris</i> (n=2)	
Total PLFA	15.9 ± 1.6	13.6 ± 1.2	14.9 ± 3.0	
Total NLFA	37.9 ± 9.7	19.5 ± 9.1	2.3 ± 0.4	*
NLFA/PLFA				
16:0	2.24 ± 0.30	1.31 ± 0.44	0.12 ± 0.01	*
16:1ω5	79.6 ± 13.3	25.8 ± 9.7	0.40 ± 0.01	**
16:1ω7	2.30 ± 0.54	2.38 ± 0.48	0.15 ± 0.01	***
18:0	0.69 ± 0.02	0.55 ± 0.10	0.39 ± 0.11	
18:1ω7	0.35 ± 0.05	0.88 ± 0.21	0.59	
18:2ω6,9	0.31 ± 0.05	0.65 ± 0.16	0.17 ± 0.03	
NLFA				
16:0	12.3 ± 3.8	5.8 ± 2.5	0.78 ± 0.16	*
16:1ω5	21.9 ± 5.1	7.5 ± 4.8	0.04 ± 0.03	**
16:1ω7	0.28 ± 0.04	0.57 ± 0.19	0.05 ± 0.01	*

sequences that had high statistical support by bootstrap analysis.

RESULTS

AM fungal colonisation and signature fatty acids

The AMF colonisation of *Lobelia* and *Littorella* were from 34–85% of the total root length, the range of arbuscular colonisation was 28–83%, and vesicular colonisation 6–65% (Table 1). The lowest colonisation was found in plants from Lake Svanshalssjön where plants were sampled at the largest depth. *Isoetes* was not colonised by AM fungi (Table 1). Intraradical spores were recorded in *Lobelia* collected in Lake Osbysjön (site II). Colonisation by fine endophytic fungi was recorded in *Lobelia* in two of the lakes (Lake Norre sjö and Lake Skeingesjön). *Ranunculus reptans* and *Hydrocotyle vulgaris* were found in semiaquatic environments in some of the lakes and were also found to be AM colonised. *Hydrocotyle* showed Paris-type of colonisation, while the other sampled species had colonisation of Arum-type (Smith & Smith 1997).

The most common 18C and 16C fatty acids identified in roots of the collected aquatic plants were 16:0, 16:1ω5, 16:1ω7, 18:0, 18:1ω7, and 18:2ω6,9. There was no difference in total PLFA content between the different plants, while the two AM species had significantly higher NLFA contents than the non-mycorrhizal *Isoetes* (Table 3). AM fungi have a high NLFA to PLFA ratio and this ratio for different fatty acids may thus indicate if they are AM fungal signatures. Fatty acids 16:0, 16:1ω5, and 16:1ω7 had NLFA/PLFA ratios higher than one, while the other fatty acids had a ratio lower than one. All fatty acids had a ratio lower than one in the non-mycorrhizal *Isoetes*. The content of the signature NLFAs was in

general higher in *Littorella* than in *Lobelia*. The NLFA concentrations for the signature fatty acids in *Lobelia* and *Littorella* were many times higher than in the non-mycorrhizal *Isoetes*, and also several orders of magnitude higher than what have been reported for non-mycorrhizal control plants in pot experiments (van Aarle & Olsson 2003). The content of NLFA 16:1ω5 in *Lobelia* was highest in Lake Osbysjön (35 nmol mg⁻¹), whereas the lowest content was found in Lake Tydingen (0.15 nmol mg⁻¹).

Fungal diversity

DNA was extracted from 202 root pieces of which 181 (90%) gave a primary PCR product. Of these, 117 (64%) gave a nested PCR product with one or more primer sets (Table 4). In all, 50 nested PCR products from *Littorella* and 104 from *Lobelia* were subject to SSCP analysis. PCR products with the LSU RK4f/RK7mr primers yielded 9 different SSCP patterns indicating potentially 9 different sequences. Three different patterns were identified with the primers LSU cl1f/cl2r and 4 different patterns with the LSU Aca1f/Aca3r primers, in all 16 potential patterns were distinguished. Representatives for each pattern were sequenced; in all 29 nested PCR products were sequenced. 16 sequences were most closely related to the clade containing *Glomus mosseae*, seven to the clade of *G. etunicatum*/*G. claroideum*, and six to the *Acaulospora* clade. The 29 root derived glomalean sequences were used in a maximum parsimony analysis comparing them to earlier obtained spore and root-derived sequences in the Department of Mycology (University of Copenhagen) and from the NCBI-GenBank database (Fig. 1). The phylogenetic analysis grouped the sequences into eight different phylotypes, meaning that the root systems analysed potentially contained eight

Table 4. Number of root pieces (RP) analysed, number of RP yielding primary PCR product, success rate of nested PCR, number of sequences with LSU4f/7mr primers, LSU1f/2r primers, LSU1f/3r primers, total number of sequences examined, number of AM LSU gene sequences found, listed for each lake studied.

Plant species, Lake	RP	1° PCR	Success rate (%)	LSURK4f/7m	LSU1f/2r	LSU1f/3r	AM sequences
<i>L. dortmanna</i> , Norre sjö	16	10	40	3	2	3	7
<i>L. dortmanna</i> , Vittsjön	20	16	50	5	0	8	13
<i>L. dortmanna</i> , Skeingesjön	17	17	59	7	4	8	15
<i>L. dortmanna</i> , Tydingen	21	18	33	6	1	4	7
<i>L. dortmanna</i> , Svanshallssjön	44	42	31	23	2	3	14
<i>L. dortmanna</i> , Osbysjön I	22	21	67	13	6	9	27
<i>L. dortmanna</i> , Osbysjön II	22	18	78	9	5	7	21
<i>L. uniflora</i> , Norre sjö	20	19	68	9	8	6	23
<i>L. uniflora</i> , Vittsjön	20	20	60	11	8	8	27

taxa within the families studied. Eleven sequences fell in two closely related clusters between *G. intraradices* and the clade containing *G. caledonium* and *G. mosseae*. These 2 clades were named phylotype 1 and 2 (Fig. 1). Five of the sequences clustered with root derived *Glomus* sp. sequences found by S. R. in *Hieracium pilosella* (data not shown). These were named phylotype 3, 4, and 5. Seven sequences clustered to form a clade with some sequences from *G. claroideum* isolates and were distinguishable from the clade containing isolate BEG88. This clade was termed phylotype 6. Six sequences clustered within the *Acaulospora* clade, five in one distinct clade close to *A. longula*, that we named phylotype 7; the sixth sequence was named phylotype 8.

Both *Lobelia* and *Littorella* harboured sequences targeted with all three primer-sets. Phylotypes 1, 6, and 7 were found in high numbers in both plant species, phylotype 5 was only found in *Littorella*, phylotypes 2, 3, and 8 were only found in *Lobelia* (Fig. 2). The number of phylotypes encountered for one plant species in one lake varied from 2–6. Looking at the distribution of phylotypes in different lakes (Fig. 2) phylotypes 1, 6, and 7 were in general present at high frequencies. Three lakes were atypical in that they lacked one of these common phylotypes. In Lake Svanshallssjön no *Acaulospora* sequences were seen (out of 14 sequences). In Lake Vittsjön no sequences from the clade of *G. etunicatum*/*G. claroideum* were seen (out of 13 sequences). In Lake Skeingesjön the most dominant phylotype in the clade containing *G. mosseae* and *G. intraradices*, phylotype 1, was not found. Only one sequence from this clade was encountered in this lake (phylotype 4, accounting for 7% of the found sequences). For the one lake where we sampled at two places (100 m apart), Lake Osbysjön, a comparable phylotype composition was seen at both places.

DISCUSSION

The dense vegetation of isoetids often found in temperate, oligotrophic, softwater lakes plays an important role in keeping the lakes in an oligotrophic state

(e.g. Andersen & Olsen 1994, Olsen & Andersen 1994). The isoetid vegetation has been dramatically reduced in lakes in Northern Europe and Northern America during the last century (Arts 2002). A better understanding of the ecology of isoetids is therefore needed. The heavy mycorrhizal colonisation found in some isoetids indicates that this symbiosis may be important for many members of this plant group. There are indications of the importance of mycorrhiza for the phosphorus supply of isoetids (Christensen & Wigand 1998). However, there is a big gap in our knowledge regarding the influence of diversity in AMF, studied as molecular variation, on the function of these in the aquatic plants. This subject is important for understanding the ecology of isoetids.

The phylotype composition of the six lakes studied was quite similar although the lakes were situated up to 25 km apart and had a rather broad span of nutrient concentrations. For some of the lakes we had a relatively low success rate of the nested PCR (Table 4), which was independent of the degree of colonisation. It is likely that not all fungi observed by microscopy were viable or that the observed fungi were for some part of other families than the ones targeted with our primers, which would explain this observation. The number of phylotypes found varied among lakes from two (*Lobelia* in Lake Vittsjön) to six (*Lobelia* at Lake Osbysjön II) and was not dependent on the number of sequences found. In Lake Tydingen we found five different phylotypes with only seven sequences, and in Lake Vittsjön only two phylotypes were found although thirteen sequences were obtained.

A comparison of the phylogeny presented by Schwarzott, Walker & Schüssler (2001) based on the SSU with the one presented here (Fig. 1) shows that both clearly differentiate between the groups containing *Glomus mosseae*, with *G. intraradices*, and *G. claroideum*/*G. etunicatum*. This shows that the two datasets are comparable. The 5' LSU region is more variable than the SSU region (van Tuinen *et al.* 1998a), and using the LSU region may identify additional phylotypes than the SSU region would have identified from the same material. Bearing this in mind we compared the diversity of RFLP types in woodland and farmland

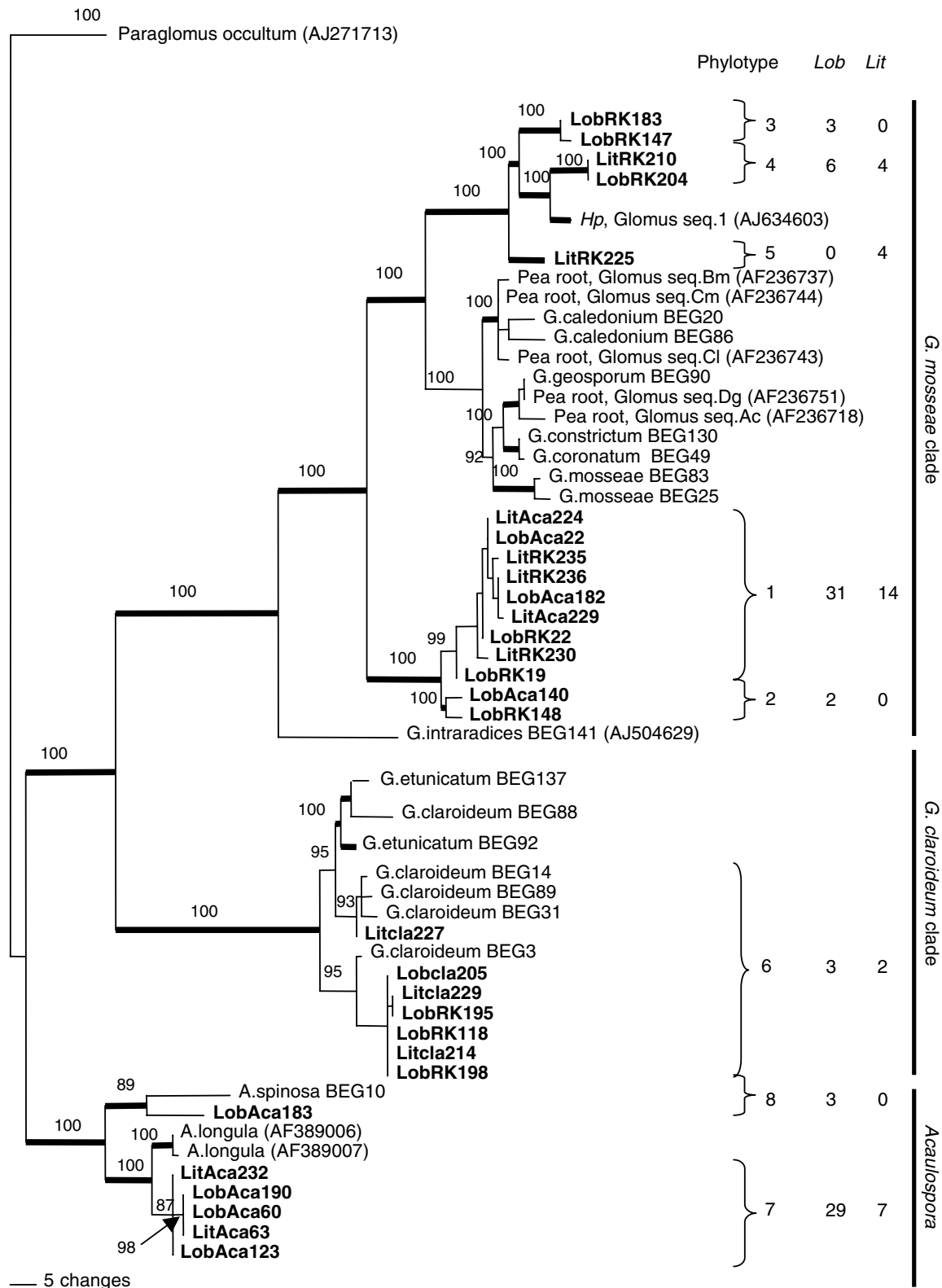


Fig. 1. A maximum parsimony phylogenetic tree showing AM fungal sequences isolated from roots of *Lobelia dortmanna* (*Lob*) and *Littorella uniflora* (*Lit*) using the LSURK4f/RK7mr (*Glomus mosseae* clade), LSUcla1f/cia2r (*G. claroideum*/ *etunicatum* clade), and LSUAca1f/Aca3r (*Acaulospora*) primers. Bootstrap values are from the majority rule consensus tree (10 000 replicates). Values are shown where they exceed 80%. Branches present in a strict consensus tree are drawn as fat. Sequences from this study are in bold and the names are composed of abbreviations of plant species name (*Lob* = *Lobelia*, *Lit* = *Littorella*), primer (*Glo* = LSURK4f/RK7mr, *cla* = LSUcla1f/cia2r, *Aca* = LSUAca1f/Aca3r) and root piece number extracted. Other names are *A* = *Acaulospora*; *G* = *Glomus*, followed by strain name, BEG code or accession no. from NCBI. Pea root derived sequences are from Kjøller & Rosendahl (2001). The sequences named *Hp* are from colonised roots of *Hieracium pilosella* from an ongoing field study in Denmark.

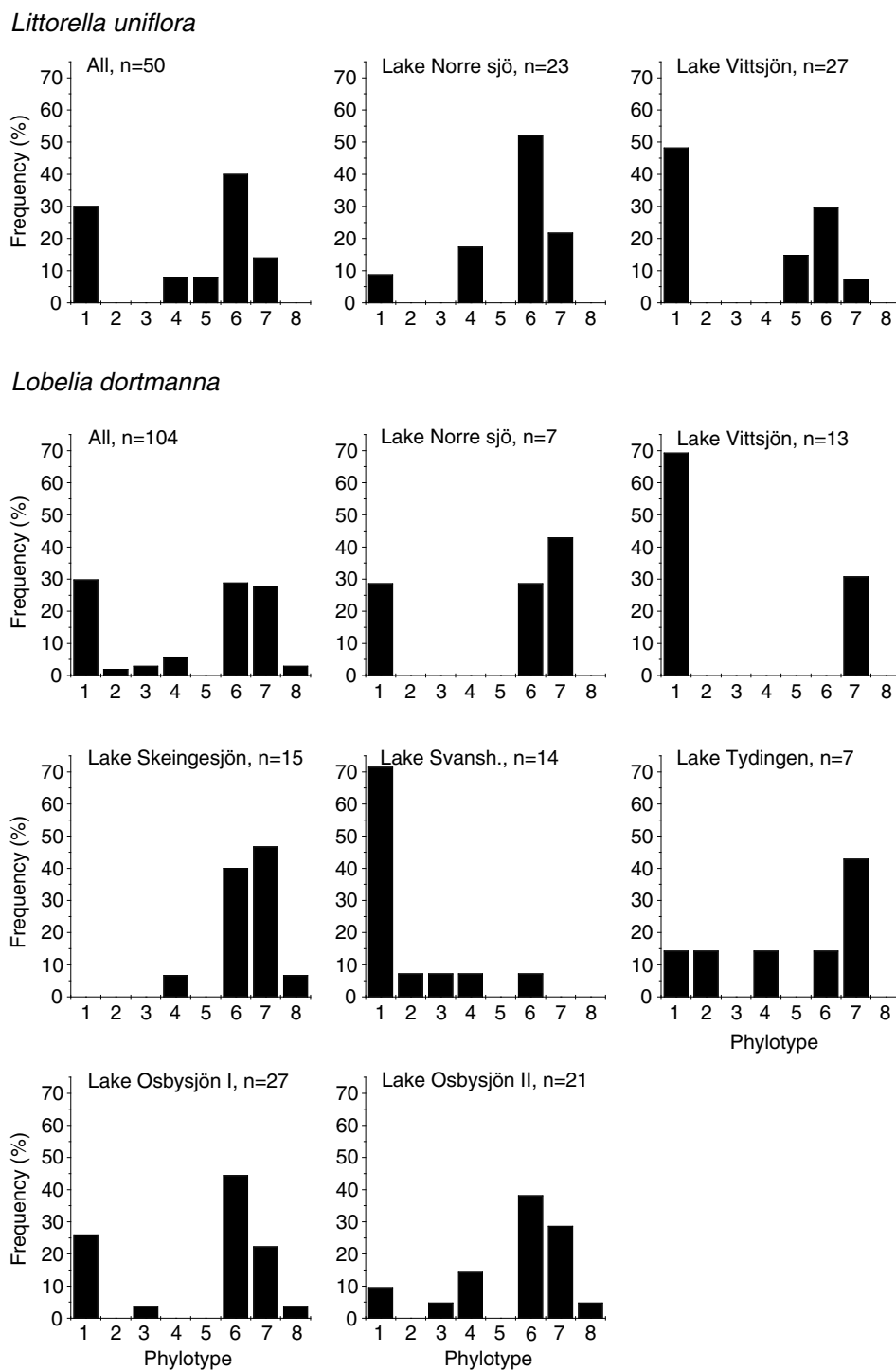


Fig. 2. The frequency of AM fungal phylotypes in *Littorella uniflora* and *Lobelia dortmanna* both as a total of all lakes sampled and for each of the seven studied locations in southern Sweden. 'n' denotes the number of sequences used for each graph.

(Helgason *et al.* 1998) in Yorkshire (determined from the SSU gene) to the diversity of phylotypes in the present study (determined from the LSU gene). It seems that the lakes contained a higher diversity than what was found for farmland where the sequence type called Glo1 (containing sequences of *G. mosseae*) was dominant, with only one *Scutellospora* and one *Acaulospora* sequence found out of 100 sequences.

The diversity of phylotypes we saw in the lakes was more similar to the woodland, which had a higher diversity than the farmland, with a higher number of sequences in several different clades. In comparison to the studies by Vandenkoornhuysen *et al.* (2002) in permanent grassland in Scotland, both the lake, woodland and farmland sites mentioned above seem to have a rather low diversity even when disregarding

Scutellospora sequences, a clade not studied in our work.

Within the clades investigated we saw that the fungi forming symbiosis with *Lobelia* and *Littorella* in lakes in southern Sweden are not so different from already known sequences of glomalean fungi from terrestrial plants (Fig. 1). It is accepted that aquatic angiosperms have evolved from terrestrial angiosperms (Cook 1990) and it is likely that the fungi and plants recolonised the aquatic habitat together. From the present data we neither prove nor disprove that some of the identified phylotypes are specific to the aquatic environment. For this, reciprocal tests of AMF cultures originating from terrestrial and from the aquatic environment with plants from both environments are needed.

The variation of AMF sequences found in *Lobelia* and *Littorella* are similar (Fig. 2). The phylotypes only encountered in *Lobelia* (nos 2–3 in the *G. mosseae* clade, and 8 in *Acaulospora*) were found in lakes where *Littorella* was not sampled (Fig. 2). We can therefore not conclude that these are specific for *Lobelia*. Phylotype 5 was only found in *Littorella* in Lake Vittsjön.

None of the phylotypes found in the *G. mosseae*/*G. intraradices* clade clustered closely with known *Glomus* species. The sequences constituting phylotype 1 and 2 were positioned between *G. intraradices* and the clade of *G. caledonium*, *G. mosseae*, *G. constrictum*, *G. coronatum*, and *G. geosporum* (Fig. 1). Phylotypes 3, 4, and 5 clustered with root-derived sequences from field samples of *Hieracium pilosella* L. (abbreviated *Hp* in Fig. 1) from eastern Denmark (S.R. unpubl.). The lack of named sequences in some clades could be due to incomplete sequencing of known *Glomus* species; these clades could represent non-described *Glomus* species or even non-sporulating and therefore also non-described *Glomus* species. Our sequences in phylotype 6 clustered closely with *G. claroideum* strain BEG3 and the sequences could not be differentiated from *G. claroideum* strain BEG14, BEG89, and V284 in the strict consensus tree and are thus identified as *G. claroideum* sequences. Phylotype 7, in the *Acaulospora* clade, is very close to *Acaulospora longula* (accession nos AF389006, and AF389007 at NCBI, Fig. 1). Phylotype 8 (LobAca183, Fig. 1) groups with an *A. spinosa* sequence. Large ribosomal subunit gene sequences are not well represented in the NCBI database as yet and the designation of species to the rest of the phylotypes must await the sequencing of more named isolates for comparison.

The high AM colonisation in *Lobelia* and *Littorella* is similar to the intensity found in typical P limited terrestrial grasslands. For example *Plantago lanceolata*, which also belongs to the family *Plantaginaceae*, had 60% of the root length colonised in limestone habitats in southern Sweden but only 20% in acid rock habitats where the availability of P is much higher than in limestone habitats (van Aarle, Söderström & Olsson

2003). *Isoetes lacustris*, a seedless vascular plant belonging to the division Microphylophyta, did not show any sign of AM colonisation but has earlier been reported both as non-mycorrhizal (Søndergaard & Lægaard 1977) and mycorrhizal (Farmer 1988, Sharma 1998, Wigand *et al.* 1998) in different studies. None of these studies, however, have reported the occurrence of arbuscules. Because no mycorrhizal structures were observed in *Isoetes* roots by microscopic examination in this study, we used the *Isoetes* plants as non-mycorrhizal reference in the fatty acid analysis together with data from earlier studies on the concentration in non-mycorrhizal roots.

AM fungi have a high NLFA to PLFA ratio and this ratio for different fatty acids may thus indicate if they are AM fungal signatures. That the fatty acids 16:0, 16:1 ω 5, and 16:1 ω 7 had NLFA/PLFA ratios higher than one, while the other fatty acids had a ratio lower than one, together with the fact that all fatty acids had a ratio lower than one in the non-mycorrhizal *Isoetes*, shows that these three fatty acids do indicate mycorrhizal colonisation.

The results in this study, together with earlier studies (Olsson 1999) shows that NLFA 16:1 ω 5 is indicative of mycorrhizal colonisation and therefore we used it as indicator of AM fungal storage lipids. It showed that the amount of AM fungal storage lipids was in some cases higher than what has earlier been obtained using *Glomus* in greenhouse studies. Cucumber roots colonised with *Glomus invermaium* had 15 nmol NLFA 16:1 ω 5 mg⁻¹ (Olsson *et al.* 1995), while up to 52 nmol NLFA 16:1 ω 5 g⁻¹ was found in an experiment with *Plantago lanceolata* (van Aarle & Olsson 2003). In field grown linseed with around 40% of the root length colonised, the amount was only 1 nmol 16:1 ω 5 mg⁻¹ (Olsson *et al.* 1999). This shows that the AM fungal colonisation in several of the lakes was vital in the sense that the carbon nutrition of the AM fungi was at least as successful as in many terrestrial systems. Earlier work by Olsson and others has shown that hyphal biomass correlates with 16:1 ω 5 (Olsson 1999). There was, however, no correlation observed between colonisation intensity and the NLFA 16:1 ω 5 in this study (results not shown). It should be noted that the staining for microscopic examination stains both living and dead fungi, whereas the fatty acids represents living biomass. This is illustrated by the fact that the biomass indicators (NLFA) correlated with the number of sequences found for a root sample ($R^2=0.6$).

This study represents the first step towards describing the diversity of AMF found in aquatic plants. The data show that both *Littorella* and *Lobelia* have similar dominating AM fungi throughout the range of the six lakes studied. We found that the AM fungal community in the aquatic environment was not composed of a low number of specific fungi. Instead, the diversity was just as high as in terrestrial communities so far investigated.

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REFERENCES

- Andersen, F. Ø. & Olsen, K. R. (1994) Nutrient cycling in shallow, oligotrophic Lake Kvie, Denmark II: effects of isoetids on the exchange of phosphorus between sediment and water. *Hydrobiologia* **275/276**: 267–276.
- Arts, G. H. P. (2002) Deterioration of Atlantic soft water macrophyte communities by acidification, eutrophication and alkalisation. *Aquatic Botany* **73**: 373–393.
- Beck-Nielsen, D. & Madsen, T. V. (2001) Occurrence of vesicular–arbuscular mycorrhiza in aquatic macrophytes from lakes and streams. *Aquatic Botany* **71**: 141–148.
- Bligh, E. G. & Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**: 911–917.
- Brundrett, M., Bougher, N., Dell, B., Grave, T. & Malejczuk, N. (1996) *Working with Mycorrhizas in Forestry and Agriculture*. Australian Centre for International Agricultural Research, Canberra.
- Chaubal, R., Sharma, G. D. & Mishra, R. R. (1982) Vesicular arbuscular mycorrhiza in subtropic aquatic and marshy plant communities. *Proceedings of the Indian Academy of Sciences, Plant Sciences* **91**: 69–77.
- Christensen, K. K. & Wigand, C. (1998) Formation of root plaques and their influence on tissue phosphorus content in *Lobelia dortmanna*. *Aquatic Botany* **61**: 111–122.
- Clapp, J. P., Rodriguez, A. & Dodd, J. C. (2001) Inter- and intrasolate rRNA large subunit variation in *Glomus coronatum* spores. *New Phytologist* **149**: 539–554.
- Cook, D. K. (1990) *Aquatic Plant Book*. SPB Academic Publishing, The Hague.
- Farmer, A. M. (1988) Vesicular–arbuscular mycorrhiza in submerged *Isoetes*. *The Mycologist* **2**: 74.
- Frostegård, Å., Tunlid, A. & Bååth, E. (1993) Phospholipid fatty-acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Applied and Environmental Microbiology* **59**: 3605–3617.
- Helgason, T., Daniell, T. J., Husband, R., Fitter, A. & Young, J. P. W. (1998) Ploughing up the woodwide web? *Nature* **394**: 431.
- Khan, A. G. & Belik, M. (1995) Occurrence and ecological significance of mycorrhizal symbiosis in aquatic plants. In *Mycorrhiza: structure, function, molecular biology and biotechnology* (A. Varma & B. Hock, eds): 627–666. Springer-Verlag, Berlin.
- Kjøller, R. & Rosendahl, S. (2000) Detection of arbuscular mycorrhizal fungi (*Glomales*) in roots by nested PCR and SSCP (Single Stranded Conformation Polymorphism). *Plant and Soil* **226**: 189–196.
- Kjøller, R. & Rosendahl, S. (2001) Molecular diversity of glomalean (arbuscular mycorrhizal) fungi determined as distinct *Glomus* specific DNA sequences from roots of field grown peas. *Mycological Research* **105**: 1027–1032.
- McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L. & Swan, J. A. (1990) A new method which gives an objective measure of colonization of roots by vesicular–arbuscular mycorrhizal fungi. *New Phytologist* **115**: 495–501.
- Miller, R. M., Smith, C. I., Jastrow, J. D. & Bever, J. D. (1999) Mycorrhizal status of the genus *Carex* (*Cyperaceae*). *American Journal of Botany* **86**: 547–553.
- Morton, J. B. & Redecker, D. (2002) Two new families of *Glomales*, *Archaeosporaceae* and *Paraglomaceae*, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. *Mycologia* **93**: 181–195.
- Nielsen, S. L., Thingstrup, I. & Wigand, C. (1999) Apparent lack of vesicular–arbuscular mycorrhiza (VAM) in the seagrasses *Zostera marina* L. and *Thalassia testudinum* Banks ex König. *Aquatic Botany* **63**: 261–266.
- Olsen, K. R. & Andersen, F. Ø. (1994) Nutrient cycling in shallow, oligotrophic Lake Kvie, Denmark. *Hydrobiologia* **275/276**: 255–265.
- Olsson, P. A. (1999) Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. *FEMS Microbiology Ecology* **29**: 303–310.
- Olsson, P. A., Bååth, E., Jakobsen, I. & Söderström, B. (1995) The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. *Mycological Research* **99**: 623–629.
- Olsson, P. A. & Johansen, A. (2000) Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. *Mycological Research* **104**: 429–434.
- Olsson, P. A., Thingstrup, I., Jakobsen, I. & Bååth, E. (1999) Estimation of the biomass of arbuscular mycorrhizal fungi in a linseed field. *Soil Biology & Biochemistry* **31**: 1879–1887.
- Pedersen, O., Sand-Jensen, K. & Revsbech, N. P. (1995) Diel pulses of O₂ and CO₂ in sandy lake sediments inhabited by *Lobelia dortmanna*. *Ecology* **76**: 1536–1545.
- Ragupathy, S., Mohankumar, V. & Mahadevan, A. (1990) Occurrence of vesicular–arbuscular mycorrhizae in tropical hydrophytes. *Aquatic Botany* **36**: 287–291.
- Sand-Jensen, K. & Prahl, C. (1982) Oxygen exchange with the lacunae and across leaves and roots of the submerged vascular macrophyte, *Lobelia dortmanna* L. *New Phytologist* **91**: 103–120.
- Schwarzott, D., Walker, C. & Schüssler, A. (2001) *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (*Glomales*), is non-monophyletic. *Molecular Phylogenetics and Evolution* **21**: 190–197.
- Sculthorpe, C. D. (1967) *The Biology of Aquatic Vascular Plants*. Edward Arnold, London.
- Sharma, B. D. (1998) Fungal associations with *Isoetes* species. *American Fern Journal* **88**: 138–142.
- Smith, F. A. & Smith, S. E. (1997) Structural diversity in (vesicular–arbuscular) mycorrhizal symbioses. *New Phytologist* **137**: 373–388.
- Smith, S. E. & Read, D. J. (1997) *Mycorrhizal Symbiosis*. 2nd edn. Academic Press, London.
- Swofford, D. L. (1998) *PAUP*: phylogenetic analysis using parsimony and other methods*. Sinauer Associates, Sunderland, MA.
- Søndergaard, M. & Lægaard, S. (1977) Vesicular–arbuscular mycorrhiza in some aquatic vascular plants. *Nature* **268**: 232–233.
- Tanner, C. C. & Clayton, J. S. (1985) Effects of vesicular–arbuscular mycorrhizas on growth and nutrition of a submerged aquatic plant. *Aquatic Botany* **22**: 377–386.
- Thippayarugs, S., Bansal, M. & Abbott, L. K. (1999) Morphology and infectivity of fine endophyte in a mediterranean environment. *Mycological Research* **103**: 1369–1379.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.
- Tunlid, A. & White, D. C. (1992) Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. In *Soil Biochemistry* (G. Stotzky & J.-M. Bollag, eds): 229–262. Marcel Dekker, New York.
- van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A. & Gianinazzi-Pearson, V. (1998a) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. *Molecular Ecology* **7**: 879–887.
- van Tuinen, D., Zhao, B. & Gianinazzi-Pearson, V. (1998b) PCR in studies of AM fungi: from primer to application. In *Mycorrhiza Manual* (A. Varma, ed.): 387–399. Springer Verlag, Heidelberg.
- van Aarle, I. M. & Olsson, P. A. (2003) Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi. *Applied and Environmental Microbiology* **69**: 6762–6767.

- van Aarle, I. M., Söderström, B. & Olsson, P. A. (2003) Growth and interactions of arbuscular mycorrhizal fungi in soils from limestone and acid rock habitats. *Soil Biology & Biochemistry* **35**: 1557–1564.
- Vandenkoornhuyse, P., Husband, R., Daniell, T. J., Watson, I. J., Duck, J. M., Fitter, A. H. & Young, J. P. W. (2002) Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. *Molecular Ecology* **11**: 1555–1564.
- Wigand, C., Andersen, F. Ø., Christensen, K. K., Holmer, M. & Jensen, H. S. (1998) Endomycorrhizae of isoetids along a biogeochemical gradient. *Limnology and Oceanography* **43**: 508–515.
- Wium-Andersen, S. (1971) Photosynthetic uptake of free CO₂ by the roots of *Lobelia dortmanna*. *Physiologia Plantarum* **25**: 245–248.
- Wium-Andersen, S. & Andersen, J. M. (1972) Carbon dioxide content of the interstitial water in the sediment of Grane Langsø, a Danish *Lobelia* lake. *Limnology and Oceanography* **17**: 943–947.

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