Journal of Applied Botany and Food Quality 83, 28 - 36 (2009)

[1]Eötvös Loránd University, Department of Plant Physiology and Molecular Plant Biology,
[2]Corvinus University of Budapest, Department of Plant Biology and Plant Biochemistry

Changes in the diversity of the mycorrhizal fungi of orchids as a function of the water supply of the habitat

Zoltán Illyés[1], Krisztián Halász[2], Szabolcs Rudnóy[1], Noémi Ouanphanivanh[1], Tamás Garay[1], Zoltán Bratek[1]

(Received May 18, 2009)

Summary

Studies were made on the symbiotic associations of orchids in four habitats with diverse water regimes in order to determine whether there was any difference in the diversity of the symbiotic fungi of orchids in the various habitats. The habitats were classed along an environmental gradient based on the water supplies as follows: 'floating': an extremely wet floating mat, 'wet': terrestrial fens, 'variable': wet habitats that dry out periodically, and 'dry': drier steppe areas in the vicinity of wet habitats. Nine photosynthesising orchid species were included in the study, some of which were habitat-specific (*Liparis loeselii, Hammarbya paludosa*), while others had a broader range of habitats and were found on several of the habitats examined (*Orchis laxiflora* ssp. *palustris, Dactylorhiza incarnata, Epipactis palustris*). A total of 94 fungal strains were isolated from the orchid roots and seedlings and were identified using nrITS sequence analysis. Representatives of four widely occurring groups of orchid mycobionts were identified, but they were present in different ratios in each habitat. Opposing habitat preferences were observed for two groups of the anamorphic fungus genus *Epulorhiza*, which are frequent orchid symbionts. The first group (*Epulorhiza* 1), which includes the fungal partner of *Liparis loeselii*, was dominant in the floating habitats, where no members of the *Epulorhiza* 2 group were found, while the latter were more typical of drier habitats, where they were dominant. The Ceratobasidiaceae fungi, also isolated in considerable numbers, were more dominant in habitats with moderate water supplies, which were also home to representatives of the Sebacinaceae, though these were isolated in smaller numbers. The great variability in the composition of the symbiotic fungi of orchids in the different habitats suggests that efforts to preserve orchids require an accurate knowledge of orchid habitats, including data on potential fungal partners.

Introduction

Research has sparsely been carried out on orchid mycorrhiza than on other widespread mycorrhiza types (ectomycorrhiza, arbuscular mycorrhiza) (DEARNALEY, 2007). An increasing body of information is available on the fungal partners of various orchid species, but reports on the specificity of these relationships and on the incidence of the fungal partners are contradictory (BONNARDEAUX et al., 2007; FEUERHERDT et al., 2005).

The strength of the symbiotic relationship and the extent of dependence have resulted in the development of special orchid-fungus partnerships and determine the specificity of the relationship. Among the fungal taxa involved in symbiosis, some can be detected from many groups of orchids, and were originally classed together in the form genus *Rhizoctonia* (WARCUP, 1981). CURRAH et al. (1997) designated the anamorphic genera *Ceratorhiza* and *Epulorhiza* as the most frequent mycorrhizal fungi of orchids. More detailed morphological and molecular analyses gradually identified the teleomorphic equivalents of these anamorphic genera (*Ceratobasidium, Thanatephorus, Tulasnella*), though teleomorphic equivalents have not yet been found for all the anamorphic genera. MA et al. (2003) reported that of the two *Epulorhiza* groups, which have very different ITS sequences, only one (group 1) proved to be *Tulasnella*, while no teleomorphic equivalent of group 2 could be found on the basis of its sequences.

In mycoheterotrophic orchid taxa a high degree of symbiont specificity is generally observed (TAYLOR et al., 2003), manifested in the fact that the fungal partners are not those usually associated with orchids. In the case of partially mycoheterotrophic orchid species the degree of specificity varies, but preference for a particular fungal taxon may still be observed (GIRLANDA et al., 2006). Narrow specificity has been detected even for some photosynthesising orchids (MCCORMICK et al., 2004; SHEFFERSON et al., 2005; IERWIN et al., 2007). For *Liparis liliifolia* a single taxon belonging to the *Tulasnella* genus was isolated from the plants (MCCORMICK et al., 2004). In this case the strength of the specificity was given not by the preference of the orchid for an unusual fungus taxon, but by the exclusive relationship with a fungus taxon which is generally widespread among orchid species.

The specificity demonstrated by investigations in natural habitats is remarkably influenced, however, by the heterogeneity of the habitats and by the symbiont diversity of the orchids, leading to the concept of ecological specificity (MASUHARA and KATSUYA, 1994). Not all the potential symbiotic fungal partners are present in any given habitat, so the number of compatible relationships identified in the laboratory (potential specificity) is usually greater than that detected in natural habitats (ecological specificity). The specificity data obtained in habitat studies should thus be treated with caution. A good example of this is the species *Goodyera repens*, from which a single fungus species could be isolated. According to HADLEY (1970), this can be attributed to the fact that, due to the low pH of the given habitat, the number of available symbiotic fungi was reduced to one.

The DNA-based identification of the symbiotic fungal partner has become common practice, and one of the most frequently used markers is the ITS region of the nuclear 18-26S ribosomal RNA genes (nrITS) (TAYLOR and BRUNS, 1997; BONNARDEAUX et al., 2007). Recent results indicate that nrITS is especially suitable for the determination of differences between fungi at the species level (FROSLEV et al., 2007), and thus for the identification of the fungal partners of orchids and for revealing the degree of relationship between groups of fungal symbionts.

A complex analysis of orchid habitats is required if their potential to support orchids is to be correctly judged. Many treeless habitats in Europe provide homes for orchids, and among these the aqueous habitats are particularly important for many species. Among the taxa investigated in the present work, large populations of *Dactylorhiza incarnata, Orchis laxiflora* ssp. *palustris* and *Epipactis palustris* are to be found in aqueous habitats in lower-lying parts of the Carpathian Basin, while the mass occurrence of *Gymnadenia conopsea, Orchis militaris, Ophrys scolopax* and *Ophrys sphegodes* is characteristic of higher-lying areas which are only flooded for short periods, if at all.

Apart from botanical knowledge, however, little is known about the fungal diversity of orchid-symbiont associations in peat habitats. MARKUS et al. (2007) summarised the fungal flora of peat habitats, but of the 601 taxa cited, only one was a potential orchid symbiont, a *Rhizoctonia* sp. isolated from the roots of *Salix*. These authors re-

ported that Ascomycetes were dominant in peat habitats (46%), but Ascomycetes are less significant as orchid symbionts. Although *Tuber* species have been detected on the roots of forest orchids (SELOSSE et al., 2004; BIDARTONDO et al., 2004; OUANPHANIVANH et al., 2008), they belonged to the *Epipactis* and *Cephalanthera* genera. In addition, due to their ectomycorrhiza formation, *Tuber* species only occur in wooded habitats. When examining the roots of reeds, one of the most frequent plants in aqueous habitats, NEUBERT et al. (2006) detected *Sebacina vermifera* and *Tuber maculatum*, both of which are potential orchid symbionts. The saturation of the soil or peat with water has a great influence on the fungal diversity of the habitat, as oxygen is essential for fungal growth. This is also true for mycorrhizal fungi. There is a much smaller proportion of mycorrhizal plants in peatlands, and the majority of the fungi inhabit the surface layers (RYDIN and JEGLUM, 2006). This is particularly true for the wettest fen habitats, the floating mats, various types, ages and sizes of which are found in the Carpathian Basin, whose fungal flora is now under investigation (ALBERT et al., 2004; BRATEK and ZÖLD-BALOGH, 2002; ZÖLD-BALOGH et al, 2002). Little information is available, however, on orchid symbionts (ILLYÉS et al., 2005). Despite the fact that the conditions are not favourable for fungi, aqueous habitats are rich in orchid species and a certain distinction can be made between the orchid species of individual types of aqueous habitats. Some species are found over a wide range of habitats (e.g. *Dactylorhiza incarnata, Epipactis palustris*), while others are associated with specific habitat types. *Liparis loeselii* (fen orchid) is found almost exclusively on extremely wet floating mats in the Carpathian-Pannonian region (ILLYÉS, 2006). Although almost all fen types may develop on floating mats (BALOGH, 2000), fen orchids do not appear in large numbers on other fen habitats on the flat areas of the Carpathian Basin, suggesting that the floating mat environment is special for fen orchids even if it cannot always be distinguished botanically from its terrestrial variants. In addition to *Liparis loeselii*, which behaves as a floating mat specialist in the Carpathian Basin, another fen specialist, *Hammarbya paludosa* is also found on the area investigated (KRÖEL-DULAY et al., 1995). Unlike the former, this taxon inhabits acid transition mires or raised bogs (MOLNÁR, 1999) and no reference to its fungal partner was found in previous papers.

The present work aimed to investigate the orchid-symbiont diversity of aqueous habitats, the preference of individual fungus taxa for particular habitat types, and the fungus and habitat specificity of the orchid species found in these habitats.

Materials and methods

Orchid species

No orchid species that had fully or partially lost the ability to photosynthesise were found in the habitats examined, so the study included only photosynthesising species, which generally have a wider range of symbiotic fungal partners (RASMUSSEN, 2002). Nine species belonging to seven orchid genera were investigated (the abbreviations used in the paper are given in brackets). For some of these species, in addition to fungus isolation from the roots of adult plants, fungal strains were also isolated from protocorms germinated *in situ*. Two of the orchid species examined were habitat-specific. These were the calciphilous species *Liparis loeselii* (LL), found on floating mats, and the acidophilous species *Hammarbya paludosa* (HP), found on transition mires and raised bogs. The other orchid taxa had a wider range of habitats. Three were found on both floating mats and terrestrial habitats: *Orchis laxiflora* ssp. *palustris* (OL), *Dactylorhiza incarnata* (DI) and *Epipactis palustris* (EP), while the remaining four species were only observed on terrestrial habitats: *Gymnadenia conopsea* (GC), *Ophrys scolopax* (OpX), *O. sphegodes* (OpS) and *Orchis militaris* (OMt).

Habitats

Four habitats with diverse water regimes were investigated, three of them wet and one somewhat drier, but contiguous with wet habitats. The wet habitats were graded according to the water regime. The first category (floating) was an extremely wet habitat, formed of a floating mat of peat. Although this type of habitat is rare in Europe, it covers large areas of Lake Velence, with peat mats of various thickness and age, providing a home for the largest *L. loeselii* population in the Carpathian Basin. Younger floating mats were investigated on regulated stretches of the Danube and were also found to include *L. loeselii* (ILLYÉS, 2006). The only known occurrence of *Hammarbya paludosa* in Hungary was noted in Gelénes in 1994 (KRÖEL-DULAY et al., 1995) on a floating transition mire. Although the coenological and hydrochemical parameters of this habitat differed substantially from those of the *L. loeselii* habitat, they were classified in the same category due to the similarity in water regime. The second category (wet) included terrestrial fen habitats which had constant water supplies. The third category (variable) was coenologically extremely variable, as it included mostly low-lying areas which occasionally dried up. The area of this habitat type depends greatly on the rainfall quantity, and it is these areas that are most affected by global drying processes (drainage, climate drying). The fourth habitat category (dry) consisted of drier areas in the vicinity of wetter habitats, where the groundwater does not reach the upper soil layers during the greater part of the year, so there is no peat formation.

The habitat types were also characterised from the coenological point of view. Vegetation cover data were recorded on 2×2 m quadrats for rich fens, marshes and steppes, and on 5×5 quadrats for reedbeds. The relative ecological indicator scale (BORHIDI, 1995) was then used to characterise the four habitat types based on the relative ground water or soil moisture (WB index). The WB index lists the following categories: 1. Plants of extremely dry habitats or bare rocks; 2. Xero-indicators on habitats with long dry periods; 3. Xero-tolerants, sometimes occurring on fresh soils; 4. Plants of semi-dry habitats; 5. Plants of semi-humid habitats, under intermediate conditions; 6. Plants of fresh soils; 7. Plants of moist soils, not drying out and well aerated; 8. Plants of moist soils tolerating short floods; 9. Plants of wet, poorly aerated soils; 10. Plants of frequently flooded soils; 11. Water plants with floating or partly emergent leaves; 12. Water plants almost wholly submersed in water. A detailed description of the habitats is given in Tab. 1.

Isolation of fungal symbionts

In order to determine the characteristics of the peloton-forming fungi found on the seedlings and roots of orchids, the symbionts were isolated aseptically and grown on potato dextrose agar (PDA) medium (HADLEY, 1970). The seedlings and roots were surface-sterilised by immersion in 0.1% $AgNO_3$ solution for 1-3 min, then rinsed in distilled water and placed on the agar.

Isolation was also carried out by removing pelotons directly from the cortex cells of cut roots using an insect pin and then placing them on 1 ml liquid maize starch solution.

Fungi were also isolated from seedlings developing from seeds placed *in situ* at the habitat to trap potential orchid symbionts occurring in the given habitat. Fungus isolation was then carried out as described for the roots (ILLYÉS et al., 2006).

DNA extraction, PCR, phylogenetic tree construction

DNA was extracted from fungal cultures according to GARDES et al. (1991). PCR amplification was carried out on a Techne TC312 thermal cycler with the following profile: 4.5 min/94°C preliminary denaturation, followed by 33 three-step cycles of 30s/94°C, 30s/51°C and

45s/72°C. The universal ITS1 and ITS4 primers (WHITE et al., 1990) were used for the PCR. A BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) was used to sequence the purified PCR products with the same primers as used for the PCR amplification. Capillary electrophoresis was carried out using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems), according to the manufacturer's instructions. The BlastN 2.2.2. program (ALTSCHUL et al., 1997) was used to search for published data similar to the monitored sequences in the international database (GenBank-EMBL). The exact alignment of the sequences was executed using the ClustalW algorithm (THOMPSON et al., 1994) of the MEGA 4 program package (TAMURA et al., 2007). Programs from the MEGA 4 program package (TAMURA et al., 2007) were also used for phylogenetic analysis and tree drawing.

Tab. 1: Description of the four habitat types investigated. The WB analyses give the minimum and maximum values for each habitat, and the WB values recorded for the majority of species. The list of frequent species includes plant species with a cover of over 10% for the first three habitat types, and over 7% for the dry habitat type, due to the low cover values. Except for a *L. loeselii* habitat in the Czech Republic, all the locations studied were in Hungary.

1st type of habitat	Floating		
Brief description of habitat	Floating reeds and rushes, floating transition mires		
Results of WB analysis	**WB min.**	**WB max.**	**WB index for the majority of species**
for each location:	7-8	10	9
Orchid species and locations investigated:			
Dactylorhiza incarnata	Dunaharaszti, Szigetcsép		
Epipactis palustris	Dunaharaszti		
Hammarbya paludosa	Gelénes		
Liparis loeseli	Dunaharaszti, Pákozd		
Orchis laxiflora ssp. *palustris*	Dunaharaszti		
Species with the largest cover (max. cover %, WB):			
Thelypteris palustris (95, 9), *Salix cinerea* (70, 9), *Typha angustifolia* (70, 10), *Phragmites australis* (50, 10), *Carex acutiformis* (15, 9), *Eupatorium cannabinum* (30, 7), *Valeriana dioica* (10, 8)			

2nd type of habitat	Wet		
Brief description of habitat	Terrestrial reedbeds, fens		
Results of WB analysis	**WB min.**	**WB max.**	**WB index for the majority of species**
for each location:	2-7	10	7-10
Orchid species and locations investigated:			
Dactylorhiza incarnata	Aszód, Domonyvölgy, Dunaharaszti, Szabadszállás		
Epipactis palustris	Kistómalom		
Liparis loeselii	Ceska Lípa - Czech Republic		
Orchis laxiflora ssp. palustris	Balatonszentgyörgy, Kunpeszér, Szabadszállás		
Species with the largest cover (max. cover %, WB):			
Juncus effusus (90, 9), *Schoenus nigricans* (70, 9), *Carex flava* (60, 9), *Carex gracilis* (60, 9), *Carex elata* (40, 10), *Phragmites australis* (40, 10), *Carex panicea* (30, 8), *Salix cinerea* (30, 9), *Calystegia sepium* (20, 9), *Holoschoenus romanus* (20, 8), *Cirsium canum* (10, 8), *Eriophorum latifolium* (10, 9), *Festuca arundinacea* (10, 8), *Lysimachia vulgaris* (10, 8), *Poa pratensis* (10, 6), *Salix repens* ssp. *rosmarinifolia* (10, 7), *Valeriana dioica* (10, 8)			

3rd type of habitat	Variable		
Brief description of habitat	Marshes, meadows, rich fens subject to drying out, (marsh developed on the low-lying parts of an old sand quarry)		
Results of WB analysis	**WB min.**	**WB max.**	**WB index for the majority of species**
for each location:	2-4	9-10	4-8
Orchid species and locations investigated:			
Dactylorhiza incarnata	Kunpeszér, Ócsa, Tokod		
Epipactis palustris	Kunpeszér		
Gymnadenia conopsea	Kunpeszér, Ócsa		
Ophrys scolopax	Kunpeszér		
Ophrys sphegodes	Ócsa		
Orchis laxiflora ssp. *palustris*	Dinnyés, Kunpeszér, Ócsa, Pákozd		
Species with the largest cover (max. cover %, WB):			
Alopecurus pratensis (90, 6), *Festuca pratensis* (70, 6), *Briza media* (60, 6), *Poa pratensis* (60, 6), *Molinia coerulea* (50, 7), *Carex panicea* (35, 8), *Carex distans* (30, 7), *Phragmites australis* (30, 10), *Sanguisorba officinalis* (30, 7), *Carex acutiformis* (20, 9), *Equisetum x moorei* (20, 8), *Festuca rubra* (20, 5), *Galium mollugo* (20, 5), *Salix repens* ssp. *rosmarinifolia* (20, 7), *Centaurea jacea* (15, 5), *Colchicum autumnale* (15, 6), *Schoenus nigricans* (15, 9), *Dactylis glomerata* (13, 6), *Carex flacca* (10, 7), *Festuca rupicola* (10, 3), *Galium verum* (10, 4)			

4th type of habitat	Dry		
Brief description of habitat	Steppe meadows and hillsides		
Results of WB analysis	**WB min.**	**WB max.**	**WB index for the majority of species**
for each location:	2-3	5-8	3-4
Orchid species and locations investigated:			
Epipactis palustris	Mogyorósbánya		
Ophrys sphegodes	Ócsa		
Orchis militaris	Érd, Mogyorósbánya, Ócsa		
Species with the largest cover (max. cover %, WB):			
Festuca rupicola (70, 3), *Brachypodium pinnatum* (15, 4), *Carex caryophyllea* (10, 5), *Centaurea sadleriana* (8, 3), *Inula ensifolia* (8, 3), *Salvia pratensis* (8, 3), *Lotus corniculatus* (7, 4)			

The "Neighbor-Joining" application was used in the analysis, with the Maximum Composite Likelihood substitution model and the pairwise deletion of the missing data. The reliability of the phylogenetic analysis was tested with the bootstrap method, using 1000 replications.
The sequences obtained were deposited in the international EMBL database with the following accession numbers: AJ549124–AJ549126, AJ549128–AJ549133, AJ549180–AJ549182, AM040890, AM697888–AM697958, AM711614–AM711615, AM711617, AM711619–AM711620 and FM177768–FM177772.

Results

Among the cultured fungal strains, 94 were analysed using the molecular taxonomic method. The ITS sequences of several of the strains exhibited 100% identity. Of these, the ones which originated from the same orchid species and habitat type were treated as a single sequence in the phylogenetic tree (Fig. 1). A total of 43 different sequences were thus used to construct the tree. The various groups within the Basidiomycetes are taxonomically distant and their status is not always clear, so it was considered advisable to choose a member of the Ascomycetes (*Morchella* sp.), also isolated from the roots of an orchid species, *O. scolopax*, as the root of the tree.
On the basis of the relatedness of their entire ITS sequences, the strains could be divided into four orchid-symbiont fungus groups. The published sequences exhibiting the greatest similarity with the sequences of these groups are presented in Tab. 2. The four groups correspond to the symbiotic fungus groups frequently occurring on terrestrial orchids and belong to the Sebacinaceae and Ceratobasidiaceae families, and to two groups of the Tulasnellaceae family with extremely distinct DNAs.
The smallest group consists of only 5 sequences, shown by sequence similarity analysis to be related to the Sebacinaceae family. The ITS sequences exhibited very great variability within the group, with similarity values of 31-99%. The entire ITS region also contains the 5.8S rRNA gene, which is far more conserved than the non-coding parts. Great similarity was observed for this gene (97-100%) between the fungi in this group. All five fungal strains were isolated from the roots of *Dactylorhiza incarnata* growing in variable and wet habitats.
The second biggest group consisted of 30 strains, which exhibited similarity to the sequences of the Ceratobasidiaceae family. Variability within the group ranged from 80-100% for the ITS region and from 99-100% for the 5.8S gene. Although there was little variability within the group compared with the previous group (Sebacinaceae), the ITS sequences of the strains exhibited a similarity of above 90% with GenBank sequences of two different genera: *Ceratobasidium* and *Thanatephorus* (Tab. 2). The fungi in this group were detected on all the orchid genera examined with the exception of the *Hammarbya* genus, and were isolated from all four types of habitat.
The sequences related to the Tulasnellaceae appear on the tree as two distant groups, designated as *Epulorhiza* 1 and *Epulorhiza* 2, based on MA et al. (2003). The similarity between the sequences of the two groups was very low, being 20-28% for ITS and 61-75% for 5.8S. The *Epulorhiza* 1 group could be divided into two clades (a and b), between which the similarity was 38-47% for the ITS region and 83-84% for the 5.8S gene. The *Epulorhiza* 1a clade contained 38 fungal strains. Sequence similarity within the clade was high: ITS 87-100%, 5.8S: 96-100%. Fungi isolated from the roots of *Hammarbya paludosa* differed slightly from the other sequences in the clade, forming a separate subgroup with a similarity of 87-89% to the other sequences in the clade, between which a similarity of over 95% was noted. Apart from *Ophrys* and the *Hammarbya* genus mentioned above, these fungi could be detected on all the orchid species examined. They were dominant mainly on floating habitats, but were found in smaller numbers in all the habitats.
The *Epulorhiza* 1b clade was represented by four fungus strains. Sequence similarity within the clade was extremely high: ITS 97-100%, 5.8S 99-100%. These fungi were only isolated from *Dactylorhiza incarnata* roots in wet and variable habitats.
The ITS sequences of the *Epulorhiza* 2 group were extremely variable, with a similarity of only 49-55% for the entire ITS, though this value was 93-98% for the 5.8S gene. Three clades could be distinguished, the first of which, *Epulorhiza* 2a, consisted only of fungi originating from the roots of *Ophrys sphegodes* from dry and variable habitats. This clade was extremely uniform, with 99-100% similarity for ITS and 100% for 5.8S. The group was separated to some extent from the *Epulorhiza* 2b and 2c clades, which showed a much closer relationship to each other on the phylogenetic tree. The *Epulorhiza* 2b clade included the sequences of fungi isolated from the roots of two orchid species, *Dactylorhiza incarnata* and *Ophrys scolopax*, both from variable habitats. Within the clade, sequence similarity was 94-100% for ITS and 100% for 5.8S. The *Epulorhiza* 2c clade consisted of the completely identical sequences of three fungi, one isolated from the roots of *Dactylorhiza incarnata*, one from *Epipactis palustris* and one from *Orchis militaris*, which were found in dry and variable habitats, as in the case of the *Epulorhiza* 2a clade.

Discussion

The sequences of the 94 strains examined proved to belong to the groups of Heterobasidiomycetes generally widespread on photosynthesising orchids, i.e. the Tulasnellaceae, Ceratobasidiaceae and Sebacinaceae families (RASMUSSEN, 2002; DEARNALEY, 2007). However, these were not present in equal proportions in the various habitats.

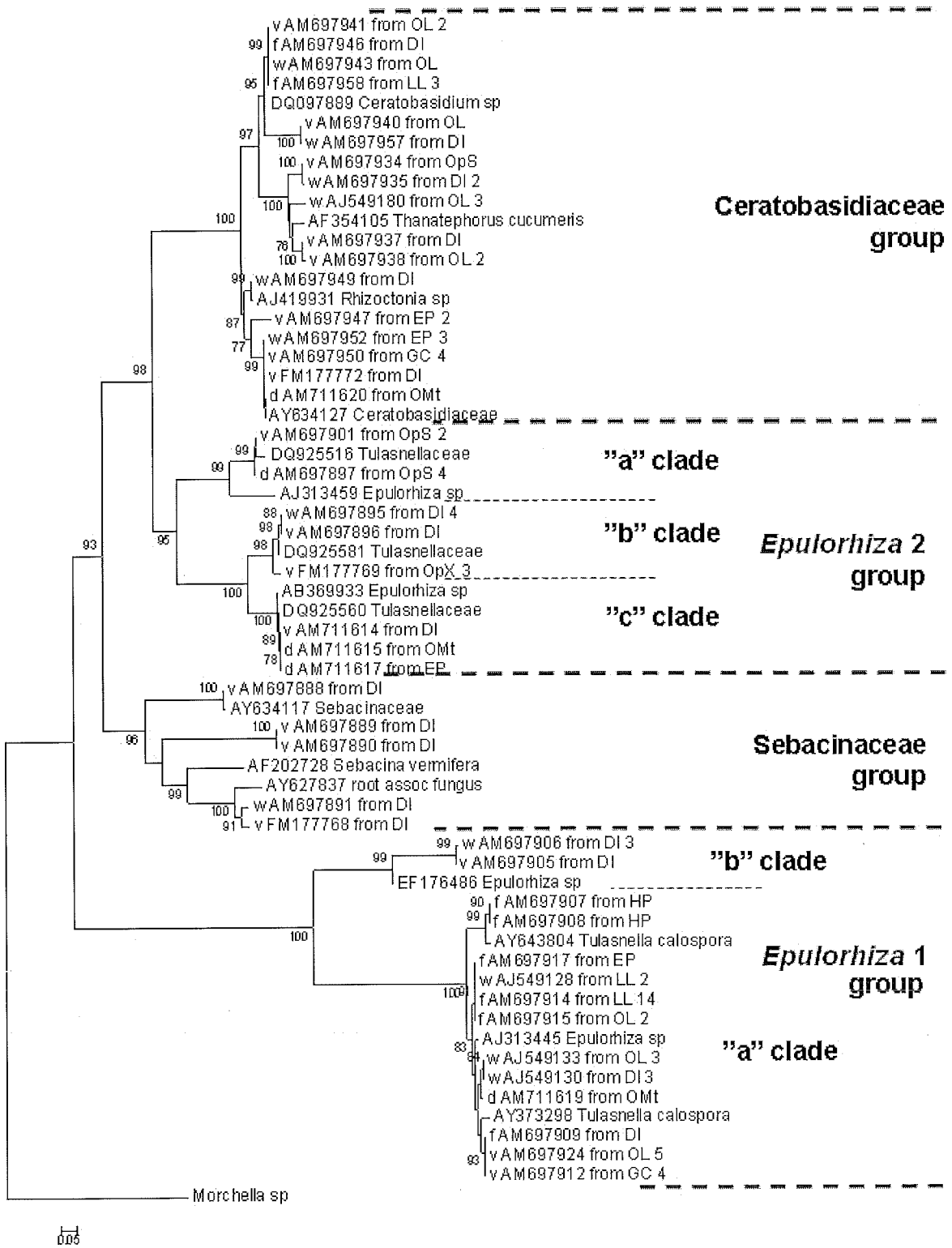

Fig. 1: "Neighbor-Joining" consensus tree based on diverse fungal sequences and references. The tree was prepared with the MEGA 4 program package. Bootstrap values (% of 1000 replications) are given for selected nodes. The outgroup was a *Morchella* sp. sequence isolated from *Ophrys scolopax*. Scale bar indicates nucleotide changes per position. Codes of samples are: f: floating, w: wet, v: variable, d: dry. Accession numbers and two letter abbreviations of orchid hosts are also designated. Numbers at the end of taxon names denote the numbers of identical sequences within the branch.

Epulorhiza-like groups

Treating floating mats as a separate type of habitat was justified by the fact that, although any type of fen may be formed on floating mats (BALOGH, 2000), the almost constant saturation of the peat with water means that this pseudo-land floating on water differs considerably from terrestrial fen types. The present studies confirmed this difference, as the diversity of orchid symbiont fungi was considerably smaller on floating mats than in the other habitats. Orchid species ger-

Tab. 2: Description of the orchid mycobiont groups distinguished on the phylogenetic tree, including all the fungal sequences. Fungal sequences isolated from orchid species and included on the tree are printed in bold, while the accession numbers of sequences identical to these are given in brackets. Among the sequences published in the international database, the accession numbers and similarity values for the most similar data are included in the table. Orchid mycobionts are underlined.

Clade	data of most similar published sequences		
	similarity	acc. nr.	description
Sebacina like			
DI: AM697888	99%	AY634117	Sebacinaceae from *Epipactis gigantea*
DI: AM697889, AM697890	99%	AJ744852	Uncultured fungus from *Robinia pseudoacacia*
DI: AM697891, FM177768	87-88%	AY627837	*Epacris pulchella* root associated fungus
Ceratobasidiaceae			
DI: FM177772; **EP: AM697952** (AM697953, AM697954); **GC: AM697950** (AM697951, AM697955, AM697956); **OMt: AM711620**	99-100%	AY634127	Ceratobasidiaceae from *Epipactis palustris*
DI: AM697935 (AM697936), **AM697937**; **OL: AM697938** (AM697939); **OpS: AM697934**	91-92%	AF354104	*Thanatephorus cucumeris* from potato
OL: AJ549180 (AJ549181, AJ549182)	96%	AF354104	*Thanatephorus cucumeris* from soil
DI: AM697957; **OL: AM697940**	90%	AF472285	*Ceratobasidium* sp. from *Psychilis monensis*
DI: AM697946; **LL: AM697958** (AM697944, AM697945); **OL: AM697941** (AM697942); **AM697943**	97%	DQ097889	*Ceratobasidium* sp.
EP: AM697947 (AM697948)	94%	AY634128	Ceratobasidiaceae from *Epipactis helleborine*
DI: AM697949	99%	AJ419931	*Rhizoctonia* sp. from *Pinus sylvestris*
Epulorhiza 1a			
DI: AM697909, AJ549130 (AJ549131, AJ549132); **EP: AM697917**; **GC: AM697912** (AM697913, AM697922, AM697923); **HP: AM697907, AM697908**; **LL: AM697914** (AM697918, AM697919, AJ549124, AM697925-AM697933, AM040890), **AJ549128** (AJ549129); **OL: AJ549133** (AJ549125, AJ549126), **AM697924** (AM697910, AM697911, AM697920, AM697921), **AM697915** (AM697916); **OMt: AM711619**	95-97%	AY373298	*Tulasnella calospora* (*Rhizoctonia repens*)
	95-97%	AJ313445	*Epulorhiza* sp. from *Spathaglotis plicata*
Epulorhiza 1b			
DI: AM697906 (AM697903, AM697904), **AM697905**	94%	EF176486	*Epulorhiza* sp. from *Disa bracteata*
Epulorhiza 2a			
OpS: AM697897 (AM697898-AM697900), **AM697901** (AM697902)	96%	DQ925516	Tulasnellaceae from *Cypripedium macranthon*
Epulorhiza 2 c			
DI: AM697895 (AM697892-AM697894), **AM697896**; **OpX: FM177769** (FM177770, FM177771)	97-98%	DQ925581	Tulasnellaceae from *Cypripedium parviflorum*
Epulorhiza 2b			
DI: AM711614; **EP: AM711617**; **OMt: AM711615**	99%	DQ925560	Tulasnellaceae from *Cypripedium calceolus*

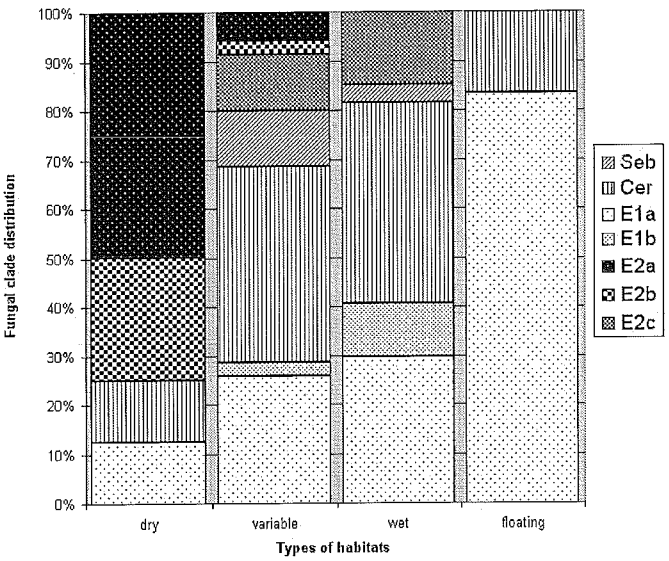

Fig. 2: Mutual ratios of the fungal groups distinguished on the molecular tree in the four habitats investigated. Seb=Sebacinaceae, Cer=Ceratobasidiaceae, E1=*Epulorhiza* 1, E2=*Epulorhiza* 2 (a, b and c represent clades within the *Epulorhiza* groups)

minating in this habitat have to make do with this restricted choice. This habitat specificity may substantially change our understanding of orchid fungal specificity. If an orchid taxon exhibits a preference for a certain type of habitat and this habitat has only a narrow range of potential orchid symbionts, field surveys will demonstrate the presence of few fungi on the orchids, falsely suggesting strict orchid-fungus specificity. This is true of the orchid species *Liparis loeselii*, which is found exclusively on floating mats in Hungary. In the majority of cases fungi of the *Epulorhiza* type (*Epulorhiza* 1a clade) were isolated from both roots and seedlings, and these exhibited very close relationship with the ITS sequences of *Tulasnella calospora*. In the case of a closely related orchid species, *Liparis liliifolia*, a photosynthesising orchid found in America, a single symbiotic fungal species was detected (McCormick et al., 2004), which was attributed to specificity between the two organisms. The sequence of the fungus isolated by McCormick et al. and identified as a *Tulasnella* species (acc.nr.AY310901) was extremely similar to that isolated from *L. loeselii* in the present work, with similarity values of 91% for ITS and 95% for 5.8S. This would seem to suggest that the relationship is also specific for *Liparis loeselii*.

However, the comparative analysis of the fungal diversity of the different habitats urges caution in stating the existence of species specificity, as *L. loeselii* does not appear to have any choice but to establish a relationship with the only fungus available in its habitat, which does not necessarily indicate the existence of specificity. The dominance of this fungus on floating mats is confirmed by the fact that orchid species found both on floating mats and in terrestrial habitats (*Dactylorhiza incarnata, Epipactis palustris, Orchis laxiflora* ssp. *palustris*) also formed symbioses with the fungus found on *Liparis loeselii* when they grew on floating mats, but had other fungal symbionts in other habitats.

In addition to studies on species-specific symbiotic relationships, the occurrence of potential symbiont fungal partners is another important aspect that must be considered when trying to explain the rare occurrence of *L. loeselii*. The absence of suitable fungal partners could be an important limiting factor for plants in certain habitats. Fungi belonging to the *Epulorhiza* 1 group, suitable as symbionts for *L. loeselii*, were found in many habitats where this rare orchid did not occur, as they were isolated not only from floating mats (Fig. 2). In fact, *L. loeselii* – *Epulorhiza* 1a symbiosis may also develop in a terrestrial environment, as observed in a wet habitat in Ceska Lipa. In addition to *Epulorhiza* 1a fungi, symbionts belonging to the Ceratobasidiaceae family were also isolated from *L. loeselii* plants in small numbers. These fungi also occur in terrestrial habitats as the symbiotic partners of *O. laxiflora* ssp. *palustris*. These results suggest that the rarity of *Liparis loeselii* is unlikely to be caused by the rarity of its fungal partner. A similar conclusion was reached by Feuerherdt et al. (2005), who demonstrated that the fungal partner of the extremely rare *Arachnorchis behrii* was more widespread than the orchid. It is therefore likely that the exclusive preference of *L. loeselii* for floating mats in Hungary is due not to the influence of the fungal symbiont, but to other factors, e.g. its preference for a cold microclimate. Hungary is at the southern limit of the spread of this species, which could be why it chooses to live in the colder microclimate of the floating mats.

Within the *Epulorhiza* 1a group it was interesting to note the difference between the symbiont fungi of *H. paludosa* and the other sequences in the group (Fig. 1). *H. paludosa* is found on peat moss fens with a low pH, while most of the other *Epulorhiza* 1a samples originated from basic floating mats and terrestrial habitats. There is no difference in water status between the floating mats and the peat moss fen, so this could not be responsible for the occurrence of fungi with different ITS sequences in the two habitats. The difference in pH could explain this, however, as low pH can lead to a great reduction in fungal diversity (Hadley, 1970). Acidity probably produces special conditions for fungi in the extremely wet habitats examined in the present study. The taxonomical differences between the orchid symbiont fungi can thus be compared with the pronounced differences in vegetation observed between basic and acidic habitats (Tab. 1).

In addition to the dominant *Epulorhiza* 1 group, the *Epulorhiza* 2 group, consisting of fungi with extremely diverse sequences that could nevertheless be classified in the Tulasnellaceae family on the basis of sequence similarity, were also identified on the floating mats. Ma et al. (2003) also demonstrated two *Epulorhiza* groups with very distinct sequences on tropical orchids, with ITS sequence similarity of 18-44%. The *Epulorhiza* 1 group described by these authors was very similar to that detected in the present work (88-97% for the entire ITS region), but greater differences were found between the *Epulorhiza* 2 groups, the greatest similarity observed in pairwise alignment being 83%. Ma et al. (2003) suggested that the diverse sequences of the two anamorphic *Epulorhiza* groups represent two diverse teleomorphic genera, and consider that the second of the groups is more closely related to *Sebacina* species than to *Tulasnella* species, though they also raise the possibility that these groups are related to the Agaricales. The relationship of the *Epulorhiza* 2 group in the present work with the Sebacinaceae group was also suggested by the data (Fig. 1). The sequence relationship between the two *Epulorhiza*-like groups identified in Hungary and the fungal groups isolated by Ma et al. (2003) from the roots of tropical orchids in Singapore reflects the wide incidence of these two fungal groups. Similar results were reported by Bonnardeaux et al. (2007), who studied the mycorrhiza of terrestrial orchids. *Epulorhiza* sequences were found to be especially frequent among the fungi detected on expanding species of orchid (e.g. *Disa bracteata*). One of the *Epulorhiza* groups (acc.nr.EF176477) described by these authors, which exhibited great sequence variability, had 94-98% sequence similarity with the *Epulorhiza* 1a clade in the present work, while another (acc.nr.EF176486) exhibited 94% similarity with the present *Epulorhiza* 1b clade. The *Epulorhiza* group described by Bonnardeaux et al. thus appears to be related to the *Epulorhiza* 1 group in the present work. These authors characterised the fungal group as disturbance-tolerant, as many of them were isolated in disturbed habitats. This agrees with the present finding that the *Epulorhiza* 1 group was found in all the habitats examined, indicating that it is not choosy and is tolerant of habitats where the

water supplies are not constant (e.g. dryness, oxygen deficiency), as its numbers increased on floating mats compared with those of other fungal groups. It is interesting to note, however, that the *Epulorhiza* 2 group tended to be found on dry habitats or on habitats that periodically dried out, suggesting that it did not favour being flooded with water, but had good tolerance of dryness (Fig. 2). Only fungal strains from the 'a' and 'b' clades of the *Epulorhiza* 2 group were isolated on *Ophrys* species growing on drier habitats. It is possible that, as in the case of *L. loeselii*, this is due to habitat requirements rather than fungal specificity.

Sebacinaceae group

The species most closely related to the small number of Sebacinaceae fungus strains isolated proved to be *Sebacina vermifera*. Its similarity to the members of this group was only 41-79% for the entire ITS region, but was very high (97-100%) for the 5.8S gene. One group of *Sebacina* taxa was detected primarily from non-photosynthesising orchids (*Neottia nidus-avis* – McKENDRICK et al., 2002; *Hexalectris plicata* – TAYLOR et al., 2003; *Erythrorchis cassythoides* – DEARNALEY, 2006), while SELOSSE et al. (2004) demonstrated an ectomycorrhizal symbiosis between *Sebacina* species and woody plants. Sebacinoid fungi were isolated in Australia from the photosynthesising species *Microtis media*, which is disturbance-tolerant and has a wide range of symbionts (BONNARDEAUX et al., 2007). One interesting parallel with the present experiments is that sebacinoid fungi were only detected on the roots of *D. incarnata*, an orchid species which is widespread in aqueous habitats and was found to have one of the largest ranges of symbionts (Fig. 1). This may be one reason why it is so widespread in many types of habitats.

BIDARTONDO et al. (2004) reported finding sebacinoid fungi on the roots of the species *Epipactis palustris*, also studied in the present work. Another sebacinoid fungus, isolated by these authors from the roots of *Epipactis gigantea*, exhibited a high degree of similarity (99%) with one of the strains found in the present work on the roots of *D. incarnata* (Tab. 2), while SELOSSE et al. (2004) identified sebacinoid fungi on the roots of *Epipactis microphylla*, suggesting that if more habitats were examined it might prove possible to isolate sebacinoid fungi from *Epipactis palustris* in wet habitats in Hungary. In the present work only the dominant fungal group at the given habitat was isolated from *Epipactis palustris* samples, i.e. representatives of *Epulorhiza* 2 in dry habitats, Ceratobasidiaceae in wet habitats and *Epulorhiza* 1 on floating mats. Sebacinoid fungi were detected in variable and wet habitats, but due to the small number of isolates, they could not be said to be dominant in either habitat. This is probably why they were not found on *Epipactis palustris* samples, especially if we consider the fact that rhizomatous species like *Epipactis* have far fewer mycorrhizal root parts than tuberous species such as *Dactylorhiza* (LÁTR et al., 2008). As few fungi were isolated from the roots of *E. palustris*, this species was not considered representative of the orchid mycorrhizal fungus diversity of the given habitats. *Dactylorhiza incarnata* could be a better candidate for this role, as almost all the fungal groups were present on its roots, including some symbiotic fungi that were not isolated from other orchid species, e.g. sebacinoid fungi.

Ceratobasidiaceae group

There is little ITS variability within the Ceratobasidiaceae group (Fig. 1). Nevertheless, the sequences that were most similar to members of this group (Tab. 2) originated from very diverse isolates (soil, potato, pine, orchid roots), suggesting that a wide range of species from this family were isolated. In fact the most similar sequences included two teleomorphic genera, the *Ceratobasidium* and the *Thanatephorus* (Tab. 2). It seems that ITS variability is smaller among this group of related fungus species than in the other groups identified.

The group as a whole did not exhibit strong habitat preference (Fig. 2) and no specific relationship with any one orchid species was found, possibly due to the taxonomic heterogeneity of the group. It is interesting to note that the Ceratobasidiaceae sequences isolated from *Dactylorhiza incarnata* and *Liparis loeselii* plants on floating mats had 100% similarity, suggesting that they belong to the same species. However, just as the *Epulorhiza* 1 group dominant on floating mats could also be detected in terrestrial habitats, this *Ceratobasidium*-type species was also isolated from the roots of *Orchis laxiflora* ssp. *palustris* growing in wet terrestrial habitats, though not in dry habitats. The other subgroup of the Ceratobasidiaceae group was also detected in drier habitats, but in general these species were not dominant in extremely wet habitats or in habitats subject to drying out.

All in all it can be said that the orchid symbiont diversity may vary greatly in different habitats, which could be due to the habitat requirements of certain potential symbiotic fungi. The present work focussed on the water supplies of the habitats, thus drawing attention to changes associated with the drying out of habitats. Naturally, many other environmental factors may also influence the fungal diversity in soils, such as the pH of the soil, as demonstrated in the present work. In addition, salinisation and the accumulation of nutrients may also cause major changes. A combination of studies on the fungal specificity of individual orchid species and on the habitat requirements and spread of fungal groups could provide useful information for environmental protection projects designed to reconstruct habitats and carry out active species protection.

Acknowledgements

Thanks are due to Attila Molnár (Hortobágy National Park) for contributing to research on *Hammarbya paludosa*, to the Duna-Ipoly National Park for regular financial and professional assistance in research on *Liparis loeselii*, and to András Máté (Kiskunság National Park) for his work on *Ophrys scolopax*. Special thanks are also due to Dr. Márton Balogh, Dr. András Attila Takács and Dr. Gábor Sramkó for their field and professional assistance.

References

ALBERT, L., ZÖLD-BALOGH, Á., BABOS, M., BRATEK, Z., 2004: Characteristic cap fungi on floating mats of the Carpathian Basin. (in Hungarian with English abstract) Mikol. Közlem., Clusiana, 43, 61-74.

ALTSCHUL, S.F., MADDEN, T.L., SCHÄFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W., LIPMAN, D.J., 1997: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

BALOGH, M., 2000: Questions of floating mire succession I. (in Hungarian with English abstract) Kitaibelia 5, 9-16.

BIDARTONDO, M.I., BURGHARDT, B., GEBAUER, G., BRUNS, T.D., READ, D.J., 2004: Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proc. R. Soc. Lond., B, Biol. Sci. 271, 1799-1806.

BONNARDEAUX, Y., BRUNDRETT, M., BATTY, A., DIXON, K., KOCH, J., SIVASITHAMPARAM, K., 2007: Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. Mycol. Res. 111, 51-61.

BRATEK, Z., ZÖLD-BALOGH, Á., 2002: Reporting Discomycetes from Hungarian floating mats. (in Hungarian with English abstract) Mikol. Közlem., Clusiana, 41, 53-62.

CURRAH, R.S., ZETTLER, L.W., MCINNIS, T.M., 1997: *Epulorhiza inquilina* sp. nov. from *Platanthera* (Orchidaceae) and a key to *Epulorhiza* species. Mycotaxon 61, 335-342.

DEARNALEY, J.D.W., 2006: The fungal endophytes of *Erythorchis cassythoides* – is this orchid saprophytic or parasitic? Australasian Mycol. 25, 51-57.

DEARNALEY, J.D.W., 2007: Further advances in orchid mycorrhizal research. Mycorrhiza 17, 475-486.

FEUERHERDT, L., PETIT, S., JUSAITIS, M., 2005: Distribution of mycorrhizal fungus associated with the endangered pink-lipped spider orchid (*Arachnorchis* (syn. *Caladenia*) *behrii*) at Warren Conservation Park in South Australia. *New Zealand* J. Bot. 43, 367-371.

FROSLEV, T.G., JEPPESEN, T.S., LAESSOE, T., KJOLLER, R., 2007: Molecular phylogenetics and delimitation of species in *Cortinarius* section Calochroi (Basidiomycota, Agaricales) in Europe. Mol. Phylogenet. Evol. 44, 217-227.

GARDES, M., WHITE, T.J., FORTIN, J.A., BRUNS, T.D., TAYLOR, J.W., 1991: Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Can. J. Bot. 69, 180-190.

GIRLANDA, M., SELOSSE M.A., CAFASSO, D., BRILLIS F., DELFINE, S., FABBIAN, R., GHIGNONE, S., PINELLI, P., SEGRETO, R., LORETO, F., COZZOLINO, S., PEROTTO, S., 2006: Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. Mol. Ecol. 15, 491-504.

HADLEY, G., 1970: Non-specificity of symbiotic infection in orchid mycorrhiza. New Phytol. 69, 1015-1023.

ILLYÉS, Z., 2006: Hungarian localities of *Liparis loeselii* and vegetation mapping of its locality on Lake Velencei. (in Hungarian with English abstract) Journal of Landscape Ecology 4, 149-168.

ILLYÉS, Z., RUDNÓY, S., BRATEK, Z., 2005: Aspects of *in situ*, *in vitro* germination and mycorrhizal partners of *Liparis loeselii*. Acta Biol. Szeged. 49, 137-139.

IERWIN, M.J., BOUGOURE, J.J., DEARNALEY, J.D.W., 2007: *Pterosylis nutans* (Orchidaceae) has specific association with two *Ceratobasidium* root-associated fungi across its range in eastern Australia. Mycoscience 48, 231-239.

KRÖEL-DULAY, GY., BARABÁS, S., RÉDEI, T., SZURDOKI E., 1995: New orchid in the Hungarian flora: bog orchid (*Hammarbya paludosa* (L.) O. Kuntze). (in Hungarian with English abstract) Bot. Közlem. 82, 35-38.

LÁTR, A., ČUŘIKOVÁ, M., BALÁŽ, M., JURČÁK, J., 2008: Mycorrhizas of *Cephalanthera longifolia* and *Dactylorhiza majalis*, two terrestrial orchids. Ann. Bot. Fennici 45, 281-289.

MA, M., TAN, T.K., WONG, S.M., 2003: Identification and molecular phylogeny of *Epulorhiza* isolates from tropical orchids. Mycol. Res. 107, 1041-1049.

MARKUS, N., THORMANN, M.N., RICE, A.V., 2007: Fungi from peatlands. Fungal Diversity 24, 241-299.

MASUHARA, G., KATSUYA, K., 1994: In situ and in vitro specificity beetween *Rhizoctonia* spp. And *Spiranthes sinensis* (Persoon) Ames. Var. amoena (M. Bieberstein) Hara (Orchidaceae). New Phytol. 127, 711-718.

MCCORMICK, M.K., WHIGHAM, D.F., O'NEILL, J., 2004: Mycorrhizal diversity in photosynthetic terrestrial orchids. New Phytol. 163, 425-438.

MCKENDRICK, S.L., LEAKE, J.R., TAYLOR, D.L., READ, D.J., 2002: Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. New Phytol. 154, 233-247.

MOLNÁR, V.A., 1999: *Hammarbya paludosa* (L.) O. Kuntze. In: Farkas, S. (ed.), Protected plants of Hungary, (in Hungarian) 323. Mezőgazda Kiadó, Budapest.

NEUBERT, K., MENDGEN, K., BRINKMANN, H., WIRSEL, S.G.R., 2006: Only a few fungal species dominate highly diverse mycofloras associated with the common reed. Appl. Environ. Microb. 72, 1118-1128.

OUANPHANIVANH, N., MERÉNYI, ZS., ORCZÁN, Á.K., BRATEK, Z., SZIGETI, Z., ILLYÉS, Z., 2008: Could orchids indicate truffle habitats? Mycorrhizal association between orchids and truffles. Acta Biol. Szeged. 52, 229-232.

RASMUSSEN, H.N., 2002: Recent developments int he study of orchid mycorrhiza. Plant Soil 244, 149-163.

RYDIN, H., JEGLUM, J. (eds.), 2006: The Biology of Peatlands (Biology of Habitats). 1-350. Oxford University Press, Oxford.

SELOSSE, M.A., FACCIO, A., SCAPPATICCI, G., BONFANTE, P., 2004: Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microb. Ecol. 47, 416-426.

SHEFFERSON, R.P., WEI, M., KULL, T., TAYLOR, D.L., 2005: High specificity generally characterises mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. Mol. Ecol. 14, 613-626.

TAMURA, K., DUDLEY, J., NEI, M., KUMAR, S., 2007: MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.

TAYLOR, D.L., BRUNS, T.D., 1997: Independent, specialized invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. Proc. Natl. Acad. Sci. USA 94, 4510-4515.

TAYLOR, D.L., BRUNS, T.D., SZARO, T.M., HODGES, S.A., 2003: Divergence in mycorrhizal specialization wihin *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. Am. J. Bot. 90, 1168-1179.

THOMPSON, J.D., HIGGINS, D.G., GIBSON, T.J., 1994: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

WARCUP, J.H., 1981: The mycorrhizal relationships of Australian orchids. New. Phytol. 87, 371-381.

WHITE, T.J., BRUNS, T.D., LEE, S., TAYLOR, J.W., 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: INNIS, M.A., GELFAND, D.H., SNINSKY, J.J., WHITE T.J. (eds.), PCR protocols – A guide to methods and applications, 315-322. Academic Press, London.

ZÖLD-BALOGH, Á., PARÁDI, I., BRATEK, Z., 2002: Mycorrhizal relations of the floating mat plants on the Lake Fekete – Őrség. (in Hungarian with English abstract) Kanitzia 10, 217-224.

Address of the author:

Eötvös Loránd University, Department of Plant Physiology and Plant Molecular Biology, H-1117 Pázmány Péter sétány 1/C, Budapest, Hungary (Zoltán Illyés, Szabolcs Rudnóy, Noémi Ouanphanivanh, Tamás Garay, Zoltán Bratek)

Corvinus University of Budapest, Department of Plant Biology and Plant Biochemistry, H-1118 Villányi út 35-43. Budapest, Hungary (Krisztián Halász)