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THE MOST RECENT RESULTS ON ORCHID MYCORRHIZAL FUNGI IN HUNGARY

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Symbionts and endophytes of Hungarian orchids were studied at diverse habitats. Mycobionts of roots and *in situ* germinated protocorms of 15 orchid species were identified by molecular methods. Four fungal groups could be distinguished from orchids living at diversely wet treeless habitats: Ceratobasidiaceae, *Epulorhiza* 1, *Epulorhiza* 2 and Sebacinaceae. While the groups Ceratobasidiaceae and Sebacinaceae were detected only at habitats with medium water supply, members of clade *Epulorhiza* occurred at all of the treeless study sites. These observations suggest that fungi belonging to the genus *Epulorhiza* are more tolerant of water-stress than the other investigated genera. An ascomycetous fungus from the family Pezizaceae could be identified from the roots of *Orchis coriophora*. Further Ascomycetes were identified at forest habitats. *Tuber maculatum* was detected from the roots of *Epipactis helleborine* and *Cephalanthera damasonium*, and *Tuber excavatum* from *Epipactis microphylla*.

Keywords: *Epulorhiza* – orchid mycorrhiza – species specificity – *Tuber* – wetland

INTRODUCTION

Orchidaceae is one of the most diverse plant families around the world. Members of this family develop endomycorrhizal relationships with symbiotic fungi, called orchid mycorrhiza. The nutrient supply for germinating orchid seeds containing hardly any nutrient reserve is provided obligatorily by an appropriate fungus. The association becomes facultative in the case of adult photosynthetic orchids, while it remains obligatory between adult mycoheterotrophic orchids and their mycorrhizal fungal partners. This functional heterogeneity as well as the widespread occurrence and various habitats of orchids could denote the similar diversity of orchid mycorrhizal fungi [13].

Conversely, it was previously supposed that orchid mycorrhizal fungi belong to a narrow basidiomycetous group. Fungal strains developing scarcely ever fruit bodies and basidia were classified in the form genus *Rhizoctonia* based on morphological characteristics [19, 21]. Later, according to the improved morphological analyses (electron microscopy) and the results of induced basidium generation the *Rhizoctonia*

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group proved to be more heterogeneous. The development of molecular methods enabled researchers to come to know more and more orchid symbionts. Due to the application of molecular methods, uncultured fungi could also be detected, while earlier trials focused on the morphological identification of fungi cultured from roots. The use of sequence analysis of the nrITS region (internal transcribed spacer between ribosomal genes) of the root-inhabiting fungi allowed to identify much more mycorrhizal species but also generated numerous sequences of endophytic and other fungi [17]. Consequently, it is better to examine directly the hyphal coils of root cortex (i.e. pelotons). Sequences resulting from the culturing (if possible) or direct sequencing of pelotons provide reliable data on orchid mycorrhizal fungi.

We know better the fungal partners of orchids living in treeless habitats of Hungary than those of forest orchids because the former group is easier to be cultured [6]. With studying the symbionts of orchids living in diverse habitats not only the symbiont-pool of an orchid could be assessed but also the habitat preferences of fungi. This information is very useful and essential for conservation of orchids.

Mycoheterotrophic orchids live in forests. It has been previously established that they live in a tripartite mycorrhiza with the surrounding trees and ectomycorrhizal fungi [8]. Fungal partners of this orchid group are different from the abovementioned basidiomycetous *Rhizoctonia* fungi (e.g. *Cortinarius* spp., *Inocybe* spp., *Russula* spp., *Hymenogaster* spp. etc.) [2]. Furthermore, ascomycetous fungi also could be partners of mycoheterotrophic orchids (e.g. *Tuber* spp.) [12].

It is more and more possible that only a few orchid species is mycorrhized exclusively by a single fungal species in all of its habitats. Subsequently an orchid species can be mycorrhized with different fungal partners in different countries with diverse climatic and soil properties [6, 17]. It also means that same orchids could use different fungal partners in different countries, so studies of fungal partnerships of orchids are the same important for species represented in the international literature or not. Another important research is the identification of the potential orchid mycorrhizal fungi of the different Hungarian habitats.

In this study we aimed at summarising the most recent results of the Hungarian orchid mycorrhizal research. Data are presented on the symbionts of orchids of treeless Hungarian habitats with various water regimes. We further studied if the identified mycorrhizal fungi have habitat preferences or not. Another objective of this work was to detect the fungal partners of some orchid species living in forests.

MATERIALS AND METHODS

Orchid species and study sites

Different types of Hungarian orchid habitats were investigated in the present study. Four habitats were graded according to their water regimes. Floating reeds and rushes are extremely wet habitats where individuals of *Dactylorhiza incarnata* (Dunaharaszti, Szigetcsép), *Epipactis palustris* (Dunaharaszti), *Hammarbya paludo-*

sa (Gelénes), *Liparis loeselii* (Dunaharaszti, Pákozd) and *Orchis palustris* (Dunaharaszti) were collected from the sites indicated in brackets. From terrestrial reedbeds, fens, marshes and meadows samples of *Dactylorhiza incarnata* (Aszód, Domonyvölgy, Dunaharaszti, Kunpeszér, Ócsa, Szabadszállás), *Epipactis palustris* (Kistómalom, Kunpeszér), *Liparis loeselii* (Ceska Lípa – Czech Republic), *Orchis palustris* (Balatonszentgyörgy, Dinnyés, Kunpeszér, Ócsa, Pákozd, Szabadszállás) and *Gymnadenia conopsea* (Kunpeszér, Ócsa) were collected. *Ophrys sphegodes* (Ócsa), *Ophrys oestrifera* (Kunpeszér), *Orchis coriophora* (Székesfehérvár) and *Orchis militaris* (Ócsa, Tokod) were sampled in dry steppe meadows and hillsides in the vicinity of wetter habitats. Individuals of *Epipactis palustris* (Mogyorósbánya, Sárisáp), *Orchis militaris* (Érd, Mogyorósbánya, Pusztavám, Tokod), *Orchis morio* (Kács) and *Orchis purpurea* (Kács) were collected at really dry habitats not attached to any wetlands.

Orchids living in forest habitats were also studied. Individuals of *Epipactis helleborine* agg. (Algyő), *Epipactis microphylla* (Szigetcsép) and *Cephalanthera damasonium* (Szigetcsép) were collected from forests. In these study sites fruit bodies of hypogeous fungi were also found: small white truffles (*Tuber* spp.) and *Arcangeliella stephensii* were collected in Algyő, and fruit bodies of *Tuber aestivum*, *Tuber rufum* and *Genea* sp. were gathered in Szigetcsép.

Isolation and identification of mycorrhizal fungi

Fungi were isolated from *in situ* germinated protocorms (p) [4] and from roots (r) of the following orchid species: *Dactylorhiza incarnata* (r), *Epipactis palustris* (r), *Liparis loeselii* (p, r), *Gymnadenia conopsea* (r), *Hammarbya paludosa* (*p*), *Orchis militaris* (p, r), *Orchis morio* (r), *Orchis palustris* (r), *Orchis purpurea* (r), *Ophrys sphegodes* (r) and *Ophrys oestrifera* (p, r). The roots and protocorms were surface sterilised by immersion in 0.1% $AgNO_3$-solution for 1–3 min. After washing them three times in sterile tap water, 1 cm long root sections or protocorms were placed on PDA medium, and the hyphae of growing fungi were isolated [18]. Pure cultures were maintained on PDA medium, and multiplied in liquid culture for the DNA extraction.

DNA was extracted from isolated fungal strains following the method of Kårén et al. [7] and directly from orchid roots and protocorms [10]. The nrITS region of fungal DNA was amplified by polymerase chain reaction (PCR), using the following program: 4.5 min/94 °C preliminary denaturation, followed by 33 three-step cycles of 30 s/94 °C, 30 s/51 °C and 45 s/72 °C, then 7 min/72 °C final synthesis. For pure cultures of mycorrhizal fungi the universal primers ITS1 and ITS4 were used for the amplification [20], while for DNA extracts obtained from orchid roots and protocorms the fungus-specific primers ITS1F and ITS4B [5] were used as these DNA extracts contain fungal and plant-DNA simultaneously.

A dideoxy-cycle DNA sequencing with fluorescent terminators was performed using a BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems). Capillary

electrophoresis was carried out using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems), according to the manufacturer's instructions. Blast search was conducted on all the sequences to determine the most similar sequences [1]. The ClustalW algorithm [16] of the MEGA 4 program package [15] was applied for the exact alignment of sequences. MEGA 4 program package was also used to construct phylogenetic trees. The ITS sequences of the identified fungi were deposited in the NCBI database. Accession numbers are as follows: AJ549120-AJ549123; AJ549126-AJ549127; AJ549132; AJ549182; AM697888-AM697889; AM697891; AM697893; AM697895; AM697901-AM697902; AM697905-AM697907; AM697909; AM697911; AM697913-AM697914; AM697917-AM697918; AM697921; AM697923; AM697933-AM697934; AM697937; AM697939-AM697940; AM697943; AM697945; AM697947; AM697949; AM697951; AM697954; AM711606-AM711607; AM711614; AM711616-AM711623; AM999882; AM999884-AM999885; FM177769; FR676937-FR676940.

RESULTS

A total of 56 sequences were used to construct the two phylogenetic trees. 52 Basidiomycetes were identified from the studied orchids of the different habitats (Fig. 1). Four main groups of fungal partners could be distinguished according to the neighbor-joining bootstrap consensus tree of nrITS sequences. The Ceratobasidiaceae clade forms a homogeneous group containing 16 fungal strains and 5 reference sequences. The fungi of this group were detected on all the orchid genera examined except for the *Hammarbya* genus, and they originated from all habitat types apart from dry habitats. The other three main groups proved to be more heterogeneous. Sequences belonging to genus *Epulorhiza* appear on the tree as two distant groups. *Epulorhiza* 1 group consists of 17 fungal strains and 6 reference sequences belonging to *Tulasnella* spp. Sequences were obtained from all types of habitats. *Epulorhiza* 2 group was represented by 13 sequences and 5 reference sequences. Members of this group occurred mainly in dry habitats, steppe meadows and hillsides, but they were also found in smaller numbers at terrestrial marshes. Mycorrhizal fungi belonging to Sebacinaceae formed the smallest group of the tree consisting of 6 sequences and 3 reference sequences. Members of Sebacinaceae were detected on terrestrial fens, marshes and steppe meadows.

Besides Basidiomycetes 4 ascomycetous fungi could be identified also from the roots of Hungarian orchids (Fig. 2). An ascomycetous fungus was shown out from the roots of *Orchis coriophora* in a dry steppe meadow at Székesfehérvár. Initial Blast searches indicated that this sequence belonged to genus *Terfezia*. 11 reference sequences (*Terfezia* spp. and further members of Pezizales) are presented in this group of the phylogenetic tree in order to define the taxonomic status of this sequence. The results did not support its status in genus *Terfezia* as the sequence mapped in a branch distinct from the confirmed *Terfezia* and *Tirmania* species.

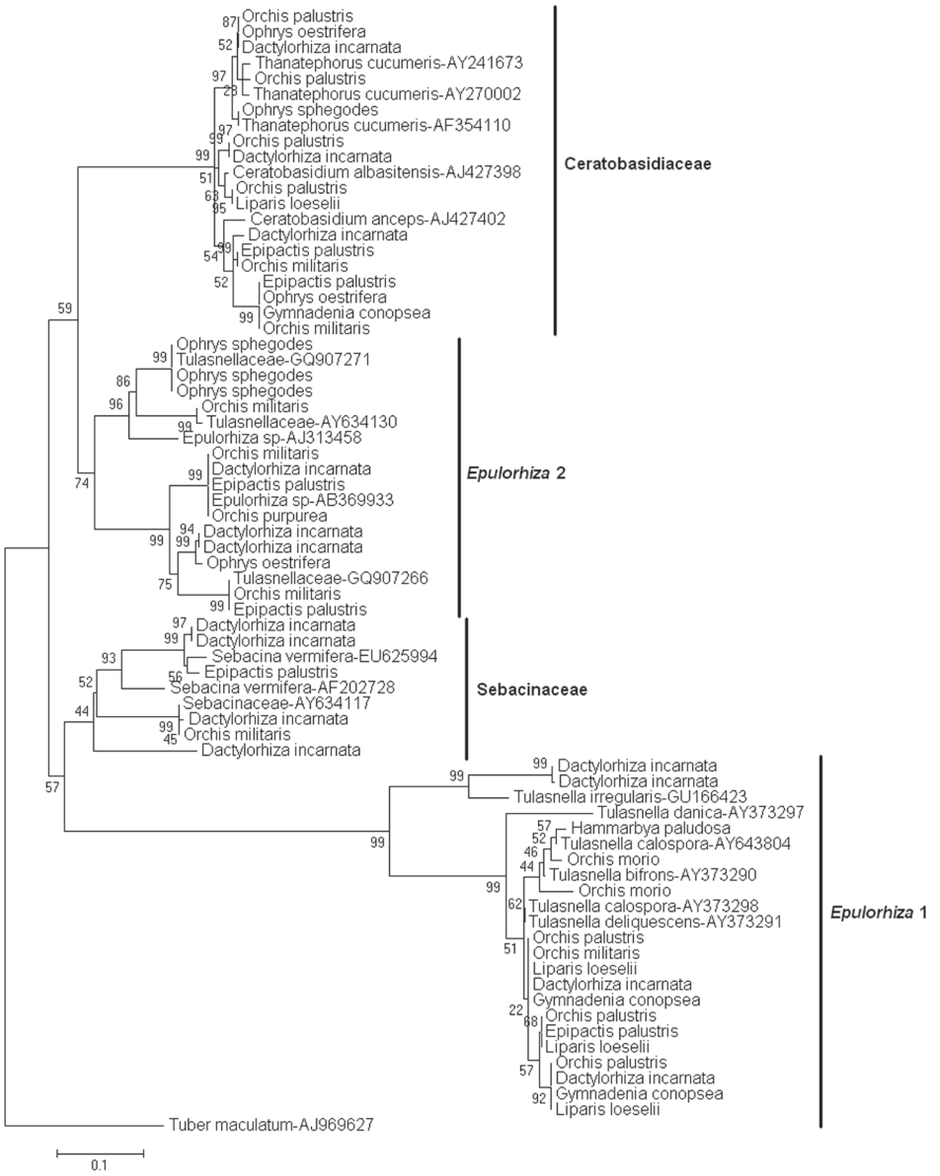

Fig. 1. Neighbor-Joining phylogenetic bootstrap consensus tree based on ITS sequences (ITS-1 and ITS-2) of basidiomycetous symbiotic fungi and other reference sequences. Symbiotic fungi are denoted with their GenBank accession number followed by the name of host orchid species, while reference sequences are represented with their name before their GenBank accession number. Scale bar indicates number of nucleotide changes per site

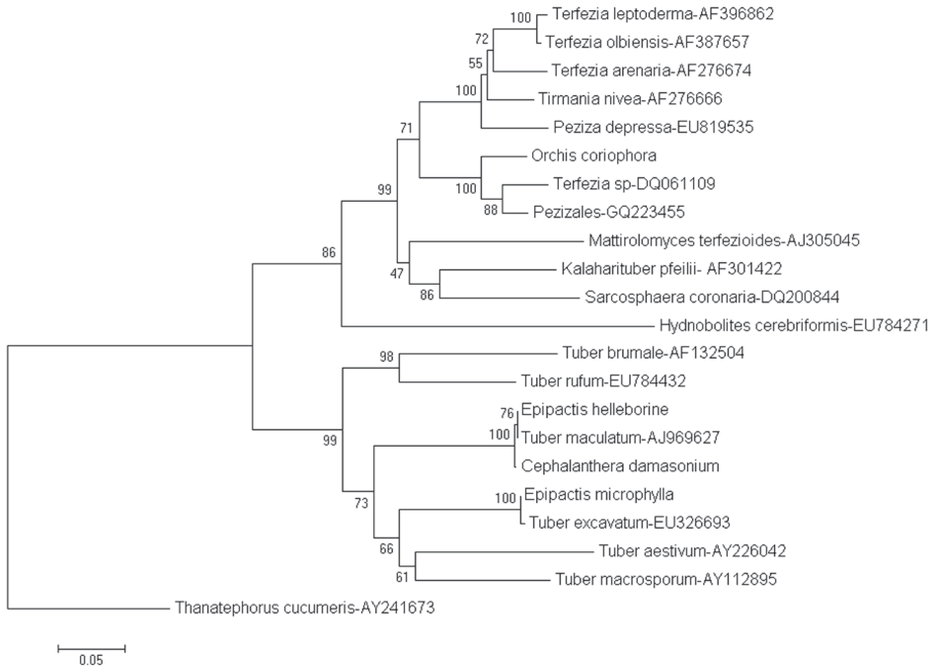

Fig. 2. Neighbor-Joining phylogenetic bootstrap consensus tree based on ITS sequences (ITS-1 and ITS-2) of ascomycetous orchid mycorrhizal fungi and other reference sequences. Orchid mycorrhizal fungi are denoted with their GenBank accession number followed by the name of host orchid species, while reference sequences are represented with their name before their GenBank accession number. Scale bar indicates number of nucleotide changes per site

Three sequences were identified from forest habitats. All three sequences belonged to the ascomycetous genus *Tuber*. This group is represented by 3 sequences and 6 reference sequences in the phylogenetic tree. Two specimens obtained from the roots of *Cephalanthera damasonium* and *Epipactis helleborine* agg. proved to be almost identical with *Tuber maculatum* (GenBank accession number AJ969627). The sequence identified from the roots of *Epipactis microphylla* was very close to *Tuber excavatum* (GenBank accession number EU326693).

DISCUSSION

The sequences of symbionts originated from the Hungarian treeless study sites proved to belong to the most common orchid mycorrhizal fungal families, i.e. Ceratobasidiaceae, Tulasnellaceae and Sebacinaceae. The sequences clustered in four groups in the phylogenetic tree: Ceratobasidiaceae, *Epulorhiza* 1 (teleomorphic equivalent *Tulasnella*), *Epulorhiza* 2 (teleomorphic equivalent not identified so far)

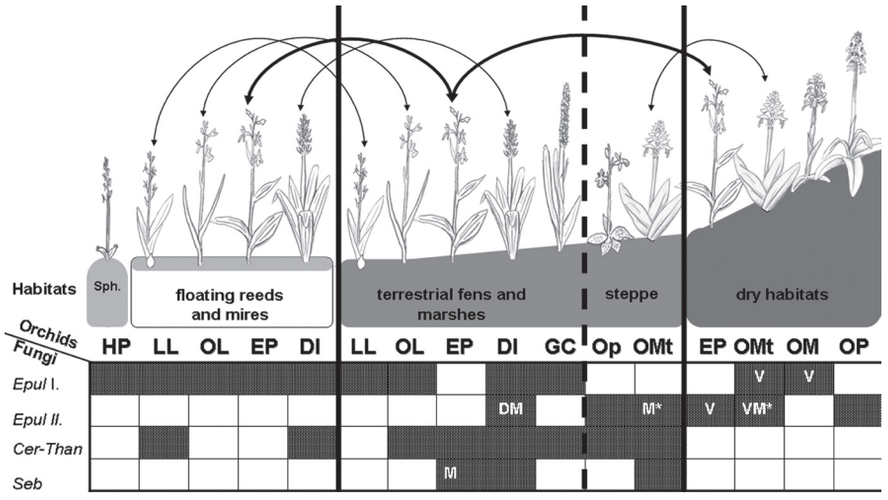

Fig. 3. Distribution of orchid mycorrhizal fungi according to orchid species and habitats. Dark grey cells indicate the occurrence of a fungus. The presented orchid species are HP – *Hammarbya paludosa*, LL – *Liparis loeselii*, OL – *Orchis palustris*, EP – *Epipactis palustris*, DI – *Dactylorhiza incarnata*, GC – *Gymnadenia conopsea*, Op – *Ophrys sphegodes* and *Ophrys oestrifera*, OMt – *Orchis militaris*, OM – *Orchis morio* and OP – *Orchis purpurea*. All the fungi were obtained from natural habitats, except for cells indicated with at least one of the following characters: D – disturbed; V – abandoned vineyard; M – abandoned mine; * – fungus obtained also from natural habitats. Sph – sphagnum mires

and Sebacinaceae groups. The most numerous symbionts (representatives of all the abovementioned families) were identified from the roots of *Dactylorhiza incarnata*, *Epipactis palustris* and *Orchis militaris* (Fig. 3). The other orchid species associated with a narrower diversity of fungi. However, the orchid species accepting the widest range of symbionts also occur in the widest ranges of habitats from marshes to steppe meadows. Therefore it is possible that these orchids are widespread due to their ability to associate with diverse fungi. This assumption is supported by the fact, that *Epipactis palustris* is colonized by species of different fungal genera in floating reeds, terrestrial fens and dry habitats.

The distribution of fungal genera among habitats deserves attention. The taxonomically diverse *Epulorhiza* genus occurred at all types of habitats, while the other taxa, Ceratobasidiaceae and Sebacinaceae appeared mainly at habitats with moderate water supplies. Members of *Epulorhiza* 1 group could be identified at all types of the examined habitats, although it was dominant only in the wet ones. These observations indicate that fungi of this group can tolerate disturbance. This assumption is confirmed by Bonnardeaux et al. [3] who stated that *Epulorhiza* species are common in disturbed and undisturbed habitats. The other *Epulorhiza* group (group 2) was dominant at the dry and disturbed habitats. Members of this group may sustain the effects of drought stress. Further study will be necessary to cast some further light on the stress-tolerance of species belonging to this fungal genus.

The sequence obtained from the roots of *Orchis coriophora*, is similar in 89% to an uncultured species of Pezizales detected in the roots of *Gymnadenia conopsea* (GenBank accession number GQ223455) [14]. Stark et al. identified their Pezizales-like sequence as *Terfezia* sp. based on an unpublished sequence (GenBank accession number DQ061109) which is similar in 84% to our sequence from *Orchis coriophora*. Comparing these with other *Terfezia* species and other genera of Pezizaceae (Fig. 2) the abovementioned sequences proved to be distinct from genus *Terfezia*. These results indicate that the fungus from the roots of *Orchis coriophora* belongs to Pezizaceae, but it needs further research to define its taxonomical state.

Our results add support to the notion that orchids associate with members of the genus *Tuber*. We presented for the first time that *Tuber maculatum* could be present in the roots of orchids [11]. It is remarkable that in Szigetcsép where fruit bodies of *Tuber aestivum*, *Tuber rufum* and *Genea* sp. were previously detected, two other hypogeous fungal species were shown out from orchid roots: *Tuber maculatum* from the roots of *Cephalanthera damasonium* and *Tuber excavatum* from the roots of *Epipactis microphylla*. These observations suggest that, besides the fruit body developing species, further macrofungal symbionts could be found by molecular methods, from the same site. According to the data of a hypogeous fungal database [9] the fungal genera *Tuber* and *Hymenogaster* are the most expected to associate with orchids as these are the most frequently found along with *Cephalanthera* and *Epipactis* spp.

In this study, we presented our results on identification of mycorrhizal fungi of Hungarian orchids. Besides the classical Basidiomycete orchid symbiont fungi we could identify ascomycetous fungi from orchids mainly living in forests. It is becoming increasingly apparent that Hungarian orchids associate with a wide diversity of fungi at most of the treeless study sites. It seems that the role of environmental circumstances in the specificity of orchid mycorrhiza is much more significant than previously assumed. Future work will be devoted to investigations on the stress tolerance of the isolated fungal strains. In addition attempts to study the symbionts and endophytes of further Hungarian orchid species and habitats will be carried out.

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