Volume 52(1):229-232, 2008
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Could orchids indicate truffle habitats? Mycorrhizal association between orchids and truffles

Noémi Ouanphanivanh*, Zsolt Merényi, Ákos Kund Orczán, Zoltán Bratek, Zoltán Szigeti, Zoltán Illyés

Department of Plant Phisiology and Molecular Plant Biology, Eötvös Loránd University, Budapest, Hungary

ABSTRACT Each member of the Orchidaceae family is in symbiosis with fungi. It has been explicitly stated that some forest orchid species are mycorrhizated by ectomycorrhizal fungi connected to trees, instead of classic orchid symbionts. These orchids supply their required nutrients via fungi. Our investigation aimed at analysing the data of a computerized database on hypogeous fungi. This database contains the data on hypogeous fungi gathered in the Carpathian basin, and also the coenological relevés made in their quadrat. We have found 16 hypogeous fungal genera with close presence of orchids (genus *Cephalanthera* and genus *Epipactis*), that is l3 % of all relevés. In case of two fungal genera (*Tuber* and *Hymenogaster*) the orchid co-occurence proved to be more frequent than average, so these fungal taxons were supposed to be in mycorrhizal connection with the orchids. *Tuber maculatum*, and *Tuber excavatum* species were identified based on nrITS sequence analysis of three orchid individuals. *Tuber maculatum* has been firstly identified from orchid root in this case. **Acta Biol Szeged 52(1):229-232 (2008)**

KEY WORDS

mycorrhiza
Epipactis
Cephalanthera
Tuber
Hymenogaster

Each member of the Orchidaceae family takes up (at least in initial, non-photosynthetic phase of its development) required nutrients with the help of symbiotic fungi, because orchid seeds do not contain, or scarcely contain stocks of nutrients (Arditti and Ghani 2000). In case of temperate zone orchid species mycorrhiza connection remains in the adult phase too, it is optional in photosynthetic species, and obligatory in mycoheterotrophic species.

Considered in classic way, the orchid symbiont fungi belong to *Rhizoctonia*-like fungi (phylum Basidiomycota), they live in either saprotrophic or parasitic manner. The anamorph-teleomorph genera couples so far identified are as follows: *Ceratorhiza*-*Ceratobasidium*, *Moniliopsis*-*Thanatephorus*, *Epulorhiza*-*Tulasnella* and *Epulorhiza*-*Sebacina* (Bratek et.al. 2001). It has been established that various groups of anamorph genus *Epulorhiza* can only grow in extremely humid floating mat habitats, and in dry habitats (for example in lawns), whereas in moorlands, which are moderately humid and very rich in orchids, all classic orchid symbiont genera can be found. From *Epipactis palustris*, which is the only species of the genus living in treeless habitats, all symbiotic fungal groups of the co-occurred orchids (*Orchis* spp., *Dactylorhiza* spp., *Liparis loeselii*, *Gymnadenia conopsea*) were detected (Illyés et al. 2005, 2006).

It has been recently shown that orchid species living in forest habitats often live in symbiosis with fungi related to trees ectomycorrhiza, beyond symbionts mentioned earlier, or instead of them, taking up such a way their nutrients from the trees, via fungi (Taylor and Bruns 1997). It is particularly interesting that forest orchids are capable of creating symbiosis not only with basidiomycete fungi: individuals of ascomycete genus *Tuber* actually occur as symbionts of forest orchids (Selosse et al. 2004). It has been defined explicitly on the hypogeous basidiomycete genus *Hymenogaster* that they are orchid symbionts, and lots of other fungal species have been demonstrated from orchid roots, which species traditionally were considered as non orchid symbionts: *Wilcoxina*, *Cortinarius*, *Inocybe*, *Thelephora*, *Tomentella*, *Leptodontidium*, *Phialophora*, *Russula* and *Peziza* (Bidartondo et al. 2004).

Main goals of the present work were to reveal that which fungal species could be in symbiotic connection with some Hungarian individuals of genera *Epipactis* and *Cephalanthera* and how exactly the orchids can indicate the presence of hypogeous fungi.

Materials and methods

Analysis of hypogeous fungal database

The base of our investigation has been formed by a database, which contained the hypogeous fungi found in Carpathian basin since 1990, and related coenological relevés (Merényi et al. 2008). In this database the following row data are presented: species name, herbarial number, the time of gathering (year, month and day), the gatherer, geographical unit, the settlement, type of the habitat, exposure of the habitat, name of the truffle dog, type of the soil, association, partner plant

*Corresponding author. E-mail: ouanoe@freemail.hu

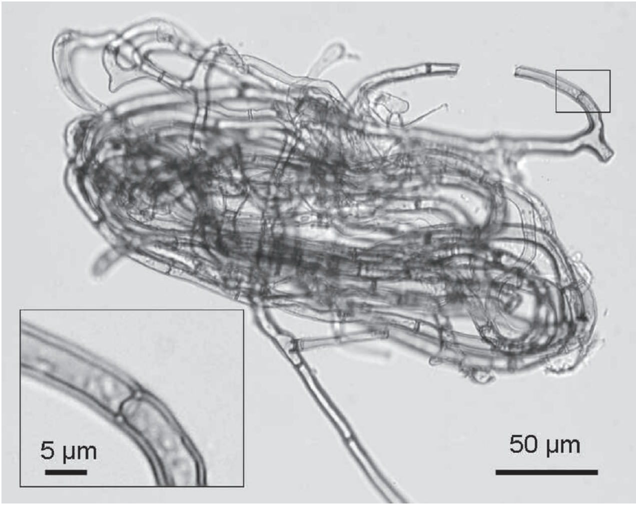

Figure 1. Hyphae taken from the root of *Epipactis microphylla* under light microscope. Not having clamp connexions (see insert), it is feasible, that they are originated from an ascomycete fungus. Hyphae are 5-9 µm wide.

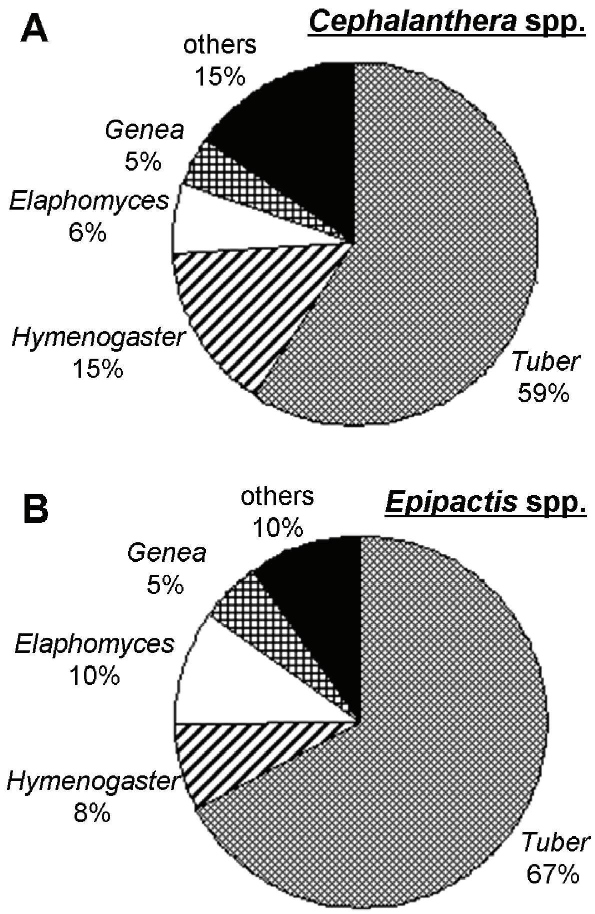

Figure 2. Distribution of hypogeous fungal genera belonging to *Cephalanthera* (A) or *Epipactis* (B) data of the database.

and observation. Morphological characterisation of certain fungal taxa is being accomplished in due course. The micro- and macromorphological features generally used for the characterisation of taxa are as follows: microscopic characters of the spores and of the peridium, the size and the mass of the carpophores, characteristics and colours of the peridium and the gleba; the latter made after the Colour Identification Chart (Royal Botanical Garden of Edinburgh, 1969). This database contains data for soil measurement as well as coenological data. The coenological relevés have been carried out concerning fungi in 10x10 meters quadrats; they have presented plant species occurred at the canopy and subcanopy level, shrub level and ground level together with the percentage of covering values. This coenological database mostly contains data prepared at the ripening time of hypogeous fungi (in summer and in autumn), that is not an optimal period yet for identifying certain orchid taxa. Although autogamous microspecies of the genus *Epipactis* can occur in the relevés they have been introduced into the analyses as *Epipactis helleborine* agg. We have accomplished the analysis of the relationship between orchid occurrences (stated upon coenological data) and the hypogeous fungal taxa.

Field studies

In order to find out the actual mycorrhizal relation we took samples from orchid roots in two habitats.

Habitat 1: Algyő (time of gathering: 25th September, 9th November 2007); carpophores so far gathered are: small white *Tuber*, *Archangeliella stephensii*. Association: Fraxino pannonicae-Ulmetum. Tree species: *Populus* x *canescens*. Orchid root sample: *Epipactis helleborine* agg. (time of gathering: 9th November 2007).

Habitat 2: Szigetcsép (time of gathering: 19th May 2007, 11th May 2008); carpophores so far gathered: *Tuber aestivum*, *Tuber rufum*, *Genea* sp. Association: Convallario – Quercetum roboris. Tree species: *Quercus robur*. Orchid root sample: *Epipactis microphylla*, *Cephalanthera damasonium* (time of gathering: 2nd May 2008).

From the roots of three orchid individuals (one *Epipactis helleborine* agg., one *Epipactis microphylla*, and one *Cephalanthera damasonium*) we have identified mycorrhizal fungi by molecular biological methods. After light-microscopic control (Fig. 1) we have extracted DNA from the mycorrhizated root sections revealing cells by the method of „vibrating pearls" (Ouanphanivanh et al. 2007), further on we followed the protocol of Kårén et al. (1997); afterwards we have multiplied the nuclear ITS region (Gardes et al. 1991) by polymerase chain reaction (PCR). We used the primers ITS1F (Gardes and Bruns 1993), Tw13, ITS1, ITS4 (White et al. 1990), ITS5, and ITS6 (Bertini et al. 1999) for multiplication. After a purifying step we accomplished sequencing of the successful PCR products; we used the ITS1 and ITS4

Table 1. Hypogeous fungal species that have orchid occurrences in more than 13% of their coenological relevés.

hypogeous fungal species	number of coenological relevés	coenological relevés containing orchid	rate
Tuber aestivum	169	54	32 %
Tuber excavatum	98	18	18 %
Hymenogaster luteus	15	5	33 %
Hymenogaster bulliardii	7	2	29 %
Hymenogaster citrinus	9	2	22 %

primers for the sequencing reaction. We have found the sequences standing closest to the electropherograms from the NCBI database (http://www.ncbi.nlm.nih.gov). The accession numbers of our sequences are as follows: AM999882, AM999883, AM999884, AM999885, and AM999886.

We have made the determination of small white *Tuber* ascocarps gathered from the investigated habitats according to Halász et al. (2005) and by molecular biological methods described earlier; for the extraction of DNA we followed the protocol of Kårén et al. (1997).

Results and Discussion

Analysis of hypogeous fungal database

Among 904 coenological relevés accomplished in hypogeous fungal habitats 119 relevés contain orchids, that is 13 % of all relevés. Individuals from two orchid genera occurred in the coenological relevés; besides all Hungarian *Cephalanthera* species (*Cephalanthera damasonium*, *Cephalanthera longifolia*, *Cephalanthera rubra*) species occurred from genus *Epipactis* as follows: *Epipactis helleborine* agg., *Epipactis atrorubens*, *Epipactis purpurata*, *Epipactis microphylla*, *Epipactis muelleri* and *Epipactis palustris*. Because of the latency period of orchids (sprouting does not develop every year from the tuber or the rhizome of the orchid), it is possible that orchids can occur in much more relevés than noted but they just have been undetected.

It happened very seldom that one relevé contain numerous orchid species, but it was very frequent that lots of hypogeous fungal species turned up from one quadrat; this way the number of possible relations between orchids and hypogeous fungi is 171. We defined sixteen hypogeous fungal genera totally, where we could state close presence of orchid species. So that the possible orchid symbiont partners can be ranged in the following fungal genera: Zygomycota: *Glomus*; Ascomycota: *Tuber*, *Elaphomyces*, *Hydnobolites*, *Genea*, *Hydnotria*, *Choiromyces*, *Stephensia*, *Balsamia*; Basidiomycota: *Hymenogaster*, *Melanogaster*, *Rhizopogon*, *Zelleromyces*, *Octaviania*, *Sclerogaster*, and *Archangeliella*. It is quite probable, that all of the possible fungal partners do not actually go in symbiosis with orchids, but in the cases where the coenological relevés of hypogeous fungi presented orchid occurrence with a higher frequency than the average, we can suppose the presence of a symbiotic relation. In the case of five hypogeous fungal species presented in the database occurred orchid in the coenological relevés with more than 13 % probability (Table 1).

We have also made an analysis in order to detect that hypogeous fungal genera found in the same quadrats as *Epipactis* and *Cephalanthera* species in what proportion occur with investigated orchids (Fig. 2). *Tuber* species occur in the highest extent with individuals of these two orchid genera. The participation of *Tuber* species is higher at genus *Epipactis*, whereas *Hymenogaster*, an other group of hypogeous fungi pointed out from orchid roots (Bidartondo et al. 2004) occurred more frequently together with *Cephalanthera* species. These two fungal groups (*Tuber* and *Hymenogaster*) together but in different rates make 75 % of hypogeous fungi relatively to both orchid genera. We should also emphasise the presence of the species of genus *Elaphomyces*, which is

Table 2. Our ITS sequence data obtained from orchid roots and a taxonomically ambiguous fungus. The samples represent: EH-51 and EH-53 – fungal ITS sequences obtained from root sections of *Epipactis helleborine* agg.; CD-3 – fungal ITS sequence obtained from the root of *Cephalanthera damasonium*; EM-1 – fungal ITS sequence obtained from the root of *Epipactis microphylla*; ZB3641 – fungal ITS sequence obtained from the ascocarp of a small white *Tuber* gathered at Algyő. Rows contain items of the NCBI database most similar to our sequences.

sample	accession number	hypogeous fungal species	maximum identity (%)	publication
EH-51	AJ969627	Tuber maculatum	99	Tedersoo et.al. (2006) 20082006
EH-53	AJ969627	Tuber maculatum	99	Tedersoo et.al. (2006)
CD-3	AJ969627	Tuber maculatum	99	Tedersoo et.al. (2006)
EM-1	EU326693	Tuber excavatum	99	Hilszczanska et.al. (2008) 2008
ZB3641	AJ969627	Tuber maculatum	98	Tedersoo et.al. (2006)

higher than the presence of genus *Hymenogaster* in case of *Epipactis* species. Taking all this in consideration we suppose that besides the ascomycete genus *Tuber* the genus *Elaphomyces* also participates in the mycorrhizal association formed together with forest orchid species.

Field studies

We succeeded in identifying hypogeous fungal sequences from every individual of examined three orchids; these belong to the genus *Tuber* if we consider their similarity with the sequences found in the NCBI database. We managed to elicit *Tuber maculatum* from *Epipactis helleborine* agg. and from *Cephalanthera damasonium*, and *Tuber excavatum* from examined *Epipactis microphylla* individual (Table 2).

In the hypogeous fungal habitat of Algyő, where we could not previously find carpophores of hypogeous fungi mentioned in Table 1, we have manifested small white *Tuber* (*Tuber maculatum*) from the *Epipactis* species growing in this habitat. The presence of *Tuber maculatum* in orchid roots was unknown so far, but we supposed its presence, as we found ascocarps of small white *Tuber*s at Algyő.

In the habitat of Szigetcsép since there *Tuber aestivum* was present, we assumed that this species would be shown out from the investigated orchids, but from the examined *Cephalanthera* individual we also elicited *Tuber maculatum*. From the roots of *Epipactis microphylla* we identified the sequence of *Tuber excavatum*, which is the second most probable *Tuber* species mycorrhizating orchids according to Table 1. Carpophores of this species have not turned up yet from the habitat, but it is quite probable upon the abovementioned results that carpophores should come to light.

Acknowledgements

The authors wish to thank Judit Vikor, Mihály Hajdú and Imre Zagyva for collecting hypogeous fungal fruiting bodies.

References

Arditti J, Ghani AKA (2000) Tansley review no. 110. Numerical and physical properties of orchid seeds and their biological implications. New Phytol 145:367-421.

Bertini L, Amicucci A, Agostini D, Polidori E, Potenza L, Guidi C, Stocchi V (1999) A new pair of primers designed for amplification of the ITS region in *Tuber* species. FEMS Microbiol Lett 173:239-245.

Bidartondo MI, Burghadt B, Gebauer G, Bruns TD, Read DJ (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proc Royal Soc 271:1799-1806.

Bratek Z, Illyés Z, Szegő D, Vértényi G (2001) Az orchidea-típusú mikorrhiza képződésének és működésének egyes kérdései. Bot Közlem 88:185-193.

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol Ecol 2:113-118.

Gardes M, White TJ, Fortin JA, Bruns TD, Taylor JW (1991) Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Can J Bot 69:180-190.

Halász K, Bratek Z, Szegő D, Rudnóy S, Rácz I, Lásztity D, M. Trappe J (2005) Tests of species concepts of the small, white, European group of *Tuber* spp. based on morphology and rDNA ITS sequences with special reference to *Tuber rapaeodorum*. Mycological Progress 4:281-295.

Hilszczanska D, Sierota Z, Palenzona M (2008) New *Tuber* species found in Poland. Mycorrhiza (in press)

Illyés Z, Eszéki E, Ouanphanivanh N, Garay T, Halász K, Geösel A, Lukács N, Bratek Z (2006) Conservation methods of Hungarian native orchids and identification of symbiotic mycorrhizal fungi. 1st European Congress of Conservation Biology, Eger, 2006. augusztus 22-26. Book of Abstracts:119p.

Illyés Z, Rudnóy S, Bratek Z (2005) Aspects of *in situ*, *in vitro* germination and mycorrhizal partners of *Liparis loeselii*. VIII. Magyar Növényélettani Kongresszus, Szeged 2002. június 24-27. Acta Biol Szeged 49:137-139.

Kårén O, Högberg N, Dahlberg A, Jonsson L, Nylund J-E (1997) Inter- and intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. New Phytol 136: 313-325.

Merényi Zs, Pintér Zs, Orczán ÁK, Illyés Z (2008) A Kárpát-medence földalatti gombafajainak biogeográfiai és ökológiai kutatása számítógépes adatbázisok létrehozásával és integrálásával. Mikol Közl (in press)

Ouanphanivanh N, Illyés Z, Rudnóy Sz, Bratek Z (2007) Hazai *Orchis militaris* élőhelyek orchidea-mikorrhiza gombáinak vizsgálata. Tájökológiai Lapok 5:325-332.

Selosse M-A, Faccio A, Scappaticci G, Bonfante P (2004) Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottiae, Orchidaceae) are associated with ectomycorrhizal Septomycetes, including truffles. Microb Ecol 47:416-426.

Taylor DL, Bruns TD (1997) Independent, specialized invasions of ectomycorrhizal mutualism by two non photosynthetic orchids. Proc Natl Acad Sci U S A, 94:4510-4515.

Tedersoo L, Hansen K, Perry BA, Kjoller R (2006) Molecular and morphological diversity of pezizalean ectomycorrhiza. New Phytol 170:581-596.

White TJ, Bruns T, Lee S, Taylor JW (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*, eds., Innis MA, Gelfand DH, Sninsky JJ, White TJ), pp. 315-322. Academic Press, New York.