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Fungi from the roots of the terrestrial photosynthetic orchid *Himantoglossum adriaticum*

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Background and aims – Mycorrhizae have a pivotal impact on orchid growth and metabolism. We investigated the diversity of root fungal associates in *Himantoglossum adriaticum* H.Baumann, an endangered terrestrial orchid species with a central submediterranean distribution, growing in poor grassland, open woodland and garrigue.
Methods – Fungal diversity in ten *H. adriaticum* adult plants collected from two geographically distinct protected areas of Central Italy was analysed by means of molecular methods. Total DNA from orchid roots was extracted and fungal ITS regions were PCR amplified using the primer pair ITS1F/ITS4; these were cloned and sequenced.
Key results – A variety of fungal species including basidiomycetes and ascomycetes were found to be associated with the orchid under study. Among them, fungal taxa belonging to Tulasnellaceae were identified in most of the analyzed samples whereas ascomycetous fungi such as Helotiales, Sordariomycetes, and *Exophiala* were sporadically recovered. Trophic relationship and function of these fungal associates towards *H. adriaticum* are discussed.
Key words – *Himantoglossum adriaticum*, ascomycetes, basidiomycetes, mycorrhizal fungi, ITS primers, orchids.

INTRODUCTION

The establishment of a relationship with mycorrhizal fungi is what all orchids need at some point in their life, at least in the early stages of their growth and development (Rasmussen 1995). Not one out of the estimated 26000 orchid species (Royal Botanic Gardens, Kew 2009), i.e. about 10% of flowering plants, is likely to be fully autotrophic if the entire life cycle is considered (Rasmussen & Rasmussen 2007). The important role of fungal symbionts in the germination of the extremely small (they measure 0.07–0.40 mm × 0.11–1.97 mm in terrestrial orchids; Arditti et al. 1980) and nearly devoid of nutritional reserves orchid seeds has been known for over 100 years (Bernard 1909). The degree to which orchids depend on fungi for the germination of dust seeds varies in different species. Many orchid taxa, are able to germinate asymbiotically in water and survive for some weeks without infection (Rasmussen 1995), during the so called 'waiting time' (Vermeulen 1947). However, for orchid species in which the first step of germination does not depend on the presence of a fungal partner, a fungus is required in the achlorophyllous protocorm stage, which is the next stage of life (Kull & Arditti 2002, Rasmussen & Rasmussen 2009). All orchid seedlings utilize mycorrhizal fungi for carbon and nutrient uptake, and the growth-promoting effect of symbionts on the seedlings is widely accepted as the fundamental role of endophytes in seedling establishment (Rasmussen 2002, Dearnaley 2007).

About 170 orchid species are completely achlorophyllous, so they maintain a fully heterotrophic lifestyle even as adults (Leake 1994). Most orchids photosynthesize in adulthood but remain reliant on the fungal partner for water, mineral salts, carbohydrates and other organic compounds with varying extents (Rasmussen & Whigham 2002, Girlanda et al. 2006, Waterman & Bidartondo 2008). Some species that develop green leaves at maturity are likely to obtain some carbon heteroterotrophically, using a dual strategy that combines mycoheterotrophy and photoassimilation simultaneously or over time (Gebauer & Meyer 2003, Liebel et al. 2010, Preiss et al. 2010, Girlanda et al. 2011).

This intriguing and complex relationship of orchids with mycorrhizal fungi may play an important role in orchid distribution and rarity, particularly for specialist orchid taxa which form mycorrhizae with a small number of fungal partners (Swarts et al. 2010, Phillips et al. 2011a). Environmental conditions limiting the presence and abundance of appropriate fungi could ultimately restrict the potential range of suitable habitats for orchids that are strictly dependent on them (Phillips et al. 2011b). Orchidaceae have a high proportion of threatened genera and species, more than any other plant family (Kull & Hutchings 2006, Swarts & Dixon 2009), and knowledge of the identities and roles of mycorrhizal fungi is of primary importance for understanding the biology of orchids and contributing to their conservation (Batty et al. 2001, Liu et al. 2010).

Recent advances in studying of orchid mycorrhizae are linked to the development of new techniques that enable identification and enumeration of fungal symbionts (Selosse & Cameron 2010). Molecular methods have been recently used to overcome the experimental problems associated with the *in vitro* isolation and morphological characterization of orchid endophytes as most of them are recalcitrant to isolation and growth in axenic culture (Pereira et al. 2005, Pellegrino & Bellusci 2009, Pecoraro et al. 2012a). Moreover, the identification of the great majority of orchid mycorrhizal fungi, belonging to the form-genus *Rhizoctonia*, is very problematic because of a paucity of

distinctive and stable morphological features, even when fungal isolation is undertaken (Taylor & Bruns 1999, Taylor & McCormick 2008, Pecoraro et al. 2012b).
Himantoglossum adriaticum H.Baumann is a terrestrial green orchid with a central submediterranean distribution (northern and central Italy, Slovenia, Croatia, eastern Austria, Slovakia and Hungary); it has been reported from all Italy, except for the Val d'Aosta, Apulia, Sardinia and Sicily (Delforge 1995, Rossi 2002) and has been included in the Italian lists of endangered species (Conti et al. 1997, Alonzi et al. 2006). This orchid usually grows in full sun, on dry calcareous soil, in poor grassland, banks, verges, open woodland and garrigue, up to 1600 m (Rossi 2002). *H. adriaticum* is characterized by 30–105 cm tall plants, with pale green and quite large leaves. The elongate and lax inflorescence is constituted by 15–40 flowers with brownish red, frequently bifid lip and with a typical unpleasant smell (Delforge 1995, Rossi 2002).
The objective of this study was to characterize, for the first time, fungi associated with this terrestrial orchid species, by employing molecular techniques. Total DNA was extracted from roots of plants and fungal ITS DNA was amplified, cloned and sequenced.

MATERIAL AND METHODS

Collection of root samples Orchid roots were collected from each of ten adult individuals from four dry calcareous grassland sites (at 969–1047 m a.s.l.) located in two geographically distinct protected areas in Central Italy, 'Cornate di Gerfalco' Natural Reserve (UTM coordinates site1: 32T 659256E/4779872N, site2: 32T 659440E/4779729N, site3: 32T 659610E/4779621N) and 'Monte Cetona' Natural Reserve (UTM coordinates site4: 32T 734929E/4756615N). Sampling was carried out during the flowering period, in the early summer of 2007 and 2008. Root samples were collected together with a soil core (maximum diameter 20 cm, and depth 30 cm) and transferred to the laboratory. Root fragments were washed with tap water, scrubbed with a brush and subsequently treated in an ultrasonic bath (three cycles of 30 s each) to remove soil particles and to minimize the detection of soil microorganisms. After checking for fungal colonization on thin cross sections under the microscope, infected root portions were frozen in liquid nitrogen for 3 min and kept at -80°C for molecular analysis.

Molecular identification of mycorrhizal fungi DNA extraction and ITS-DNA PCR –
Total DNA extraction from *H. adriaticum* root fragments was carried out using the cetyltrimethyl ammonium bromide (CTAB) procedure (Henrion et al. 1992). The internal transcribed spacer (ITS) regions of nuclear rDNA were amplified by polymerase chain reaction (PCR) with primers ITS1F and ITS4 (Gardes & Bruns 1993) in 50 µL reaction volume, containing 38 µL steril distilled water, 5 µL 10 × buffer (100 mm Tris–HCl pH 8.3, 500 mm KCl, 11 mm Mg Cl2, 0.1% gelatine), 1 µL 10 mm dNTP, 1 µL of each primer (ITS1F and ITS4), 1.5 U of RED *Taq*TM DNA polymerase (Sigma) and 2.5 µL of extracted genomic DNA at the appropriate dilution. Amplifications were performed in a PerkinElmer/Cetus DNA thermal cycler, under the following thermal conditions: 1 cycle of 95°C for 5 min, 30 cycles of 94°C for 40 s, 55°C for 45 s, 72°C for 40 s, 1 cycle of 72°C for 7 min. PCR products were electrophoresed in 1% agarose gels with ethidium bromide and visualized under UV

light. Purification of ITS--PCR products involved use of QIAquick PCR Purification Kit (Qiagen).

Cloning and sequencing

Cloning of fungal ITS regions utilized the pGEM–T (Promega, Madison, Wisconsin, USA) vector system. XL–2 Blue ultracompetent cells (Stratagene) were transformed following the manufacturer's instructions. After transformation, white colonies were randomly taken and transferred to a fresh LB (Luria Broth) plate and the bacterial cells lysed at 95°C for 10 min. Plasmid inserts were amplified using the ITS1F and ITS4 primers under the following conditions: 94°C for 5 min (1 cycle); 94°C for 30 s, 55°C for 45 s, 72°C for 1 min (25 cycles); 72°C for 7 min (1 cycle).

Cloned ITS inserts of extracted plasmids (Plasmid Purification Kit, QIAGEN), representative of the major PCR products initially present, were sequenced with the same primer pair used for amplification. Dye sequencing was carried out on ABI 310 DNA Sequencer (Applied Biosystems, Carlsbad, California, USA). Sequences were edited to remove vector sequence and to ensure correct orientation and assembled using the program Sequencher 4.1 for MacOS 9. Sequence analysis was conducted with BLAST searches against the National Center for Biotechnology Information (NCBI) sequence database (GenBank, http://www.ncbi.nlm.nih.gov/ BLAST/index.html) to determine closest sequence matches.

RESULTS

Fungal hyphae forming pelotons were microscopically observed in cortical cells of all Himantoglossum adriaticum roots. The majority of samples were heavily colonized by fungi with the exception of sample CG2 that was only lightly colonized.

Sequences from the root fungal associates were obtained for all the analyzed H. adriaticum samples and they were deposited in GenBank under accession numbers JQ685234–JQ685249 (table 1). Six out of ten individuals, from both investigated areas, were colonized by fungi belonging to Tulasnellaceae. The closest match for the sequences amplified from samples CG1 (97% over 994 bp), CG2 (97% over 987 bp), CG5 (97% over 1053 bp), MC2 (96% over 989 bp) and MC3 (97% over 1031 bp) was with uncultured Tulasnellaceae from the roots of Cypripedium montanum Douglas (DQ925572) sampled in northern California and the main root fungal endophyte of sample CG7 was a fungus with identity to Tulasnellaceae found in Ophrys bergonii A.Camus in France (EU583691). The second closest match for the same sequences (from four out of six samples, table 1) was with Epulorhiza sp. (anamorphic Tulasnella) found in roots of Cypripedium macranthos Sw. var. rebunense Miyabe & Kudô (AB369928) and C. macranthos Sw. var. speciosum Rolfe (AB369938) in Rebun Island (Japan).

Roots of sample CG4 were colonised by fungi (clone C) with close identity (96%) to Helotiales obtained from Cephalanthera longifolia (L.) Fritsch (DQ182427) in Saaremaa Island (West Estonia).

Table 1 – Closest matches from BLAST searches of fungal sequences amplified from *H. adriaticum* roots collected in Cornate di Gerfalco (samples CG1-CG7) and Monte Cetona (samples MC1-MC3).
Deposited accession code, accession code for the closest GenBank matches, sequence identity (% match) and overlap of each match are reported.

Sample	Clone	GenBank accession code	Best BLAST match(es)	Accession code	Overlap length	% match
CG1	A	JQ685234	Tulasnellaceae (from *Cypripedium montanum*)	DQ925572	994	97%
	C	JQ685235	*Exophiala pisciphila*	DQ826739	917	93%
CG2	A	JQ685236	Tulasnellaceae (from *Cypripedium montanum*)	DQ925572	987	97%
			Epulorhiza sp. (from *Cypripedium macranthos* var. *speciosum*)	AB369938	805	86%
	B	JQ685237	*Fusarium* sp.	DQ682576	948	99%
CG3	A	JQ685238	*Fusarium* sp. (from cyst of *Heterodera schachtii*)	AY729065	868	96%
	E	JQ685239	*Gibberella* sp. (from *Protea nitida*)	EU552132	978	99%
CG4	A	JQ685240	Sordariomycetes (from exoskeleton of *Spodoptera ornithogali*)	EU680539	838	94%
			Gibberella moniliformis	EU364864	838	94%
	B	JQ685241	*Fusarium lateritium*	AF310980	973	98%
	C	JQ685242	Helotiales (from *Cephalanthera longifolia*)	DQ182427	899	96%
			Leohumicola minima	AY706329	870	95%
CG5	A	JQ685243	Tulasnellaceae (from *Cypripedium montanum*)	DQ925572	1053	97%
			Epulorhiza sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369928	717	87%
CG6	A	JQ685244	Mycorrhizal fungus (from *Epipactis helleborine*)	AB428793	941	97%
			Exophiala salmonis	AF050274	835	94%
CG7	B	JQ685245	Tulasnellaceae (from *Orchis bergonii*)	EU583691	643	92%
MC1	B	JQ685246	*Fusarium* sp.	DQ061112	320	87%
	C	JQ685247	Hypocreales (from soil and roots of oilseed rape)	EU754933	747	94%
			Fusarium redolens	EF495234	747	94%
MC2	A	JQ685248	Tulasnellaceae (from *Cypripedium montanum*)	DQ925572	989	96%
			Epulorhiza sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369928	697	85%
MC3	B	JQ685249	Tulasnellaceae (from *Cypripedium montanum*)	DQ925572	1031	97%
			Epulorhiza sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369928	699	86%

Sequences from sample CG6 matched instead (97% over 941 bp) an uncultured mycorrhizal fungus colonizing Epipactis helleborine (L.) Crantz in Japan (AB428793).
Fungal associates with sample CG1 (clone C) shared 93% similarity with Exophiala pisciphila McGinnis & Ajello (DQ826739) whereas the sequences obtained from sample CG4 corresponded to a fungus with identity (94% over 838 bp) to Sordariomycetes obtained from the exoskeleton of Spodoptera ornithogali Guenée (Lepidoptera) and to Gibberella moniliformis Wineland.
Fungal sequences from the remaining samples mostly belonged to Hypocreales, the closest match being with Fusarium species found in roots of Taurantha concinna (R.Br.) D.L.Jones & M.A.Clem. (sample MC1, clone B), rhizomes of Paris polyphylla Sm. var. yunnanensis (Franch.) Hand.- Mazz. (MC1, clone C), tissues of coffee plants (sample CG2, clone B), cyst of the nematode Heterodera schachtii Schmidt (sample CG3).

DISCUSSION

The finding of a constant and heavy fungal colonization of Himantoglossum adriaticum roots supports the hypothesis of a trophic relationship between this orchid and associated fungi. Fungal coils called pelotons, microscopically observed in the root cortical cells, represent the primary evidence of the establishment of the orchid mycorrhiza (Rasmussen 1995, Shefferson et al. 2008). The observation of mycorrhizal hyphal coils growing intracellularly within the H. adriaticum roots was not surprising as some authors have recently showed the dependency on fungal symbionts in several photosynthetic orchid species, also in the adult stage. With regard to green orchid species growing in forests, Julou et al. (2005) and Abadie et al. (2006) respectively demonstrated that Cephalanthera damasonium (Mill.) Druce and C. longifolia use an additional mycorrhizal carbon source, although they are able to photosynthesize (mixotrophy). Partial mycoheterotrophy in Cephalanthera species has been confirmed by Preiss et al. (2010) by showing that these orchids obtain carbon from fungal symbionts under low light conditions, but become completely autotrophic when they are exposed to high irradiances. A trophic relationship with fungal partners has been also demonstrated in green orchids of open habitats, such as Orchis purpurea Huds., Barlia robertiana (Loisel.) Greuter and Habernaria tridactylites Lindl. (Liebel et al. 2010), Ophrys fuciflora (F.W.Schmidt) Moench, Anacamptis laxiflora (Lam.) R.M.Bateman, Pridgeon & M.W.Chase, Orchis purpurea Huds. and Serapias vomeracea Briq. (Girlanda et al. 2011) by means of C and N stable isotope analyses.

Although Himantoglossum species have for long held a certain fascination for scientists and early researches on these orchids described regulatory mechanisms of orchid mycorrhiza (Gäumann et al. 1961), little is known about the diversity of fungi associated with this orchid genus and our work constitutes the first molecular analysis of mycorrhizal symbionts in H. adriaticum. In this work we identified several fungal species occurring in H. adriaticum roots and we cannot exclude that symbiotic nutrient exchanges exist between these organisms, although physiological analyses are needed to confirm this hypothesis.

Sequences of basidiomycetes belonging to Tulasnellaceae were found in most of the analyzed samples (table 1). We found sequences that matched closely with uncultured Tulasnellaceae collected by Shefferson et al. (2007) from roots of adult Cypripedium montanum showing a narrow mycorrhizal specificity. These authors suggested that fungal carbon may still be needed during adulthood in Cypripedium plants and may be most necessary in taxa in which dormancy is most common. The closest match for the sequences amplified from sample CG7 was with Tulasnellaceae found in Ophrys bergonii in the frame of a study about mycorrhizal interactions in this hybrid Mediterranean orchid (Schatz et al. 2010). In the latter study, Tulasnellaceae taxonomically similar to the symbionts of both parent species were found in hybrids allowing them to germinate and establish mycorrhizae.

The second closest match, for sequences sharing the highest similarity with these uncultured Tulasnellaceae, was with Epulorhiza sp. isolated by Shimura et al. (2009), from adult plant roots of C. macranthos var. rebunense and C. macranthos var. speciosum and successfully used for seed germination showing their symbiotic status.

Tulasnellaceae was confirmed as a family with a range of orchid mycorrhiza forming species (Kristiansen et al. 2001, 2004, McCormick et al. 2004, Bougoure et al. 2005,

Shefferson et al. 2005, Suárez et al. 2006, Bonnardeaux et al. 2007). Several green orchid species growing in the same habitat of H. adriaticum, sometimes with similar distribution, were recently found to be associated with tulasnelloid fungi. Among them, species of the genus Orchis showed a tendency to select Tulasnella from the potential fungal community as the best partner to meet their nutritional demands both in protocorms and adult individuals (Jacquemyn et al. 2010, 2011a, 2011b). Both Shefferson et al. (2008) and Vendramin et al. (2010) showed that Tulasnellaceae were the primary associates in Orchis militaris L. Tulasnelloid fungi have been shown to be the dominant symbionts in Anacamptis laxiflora, Orchis purpurea, Ophrys fuciflora and Serapias vomeracea (Girlanda et al. 2011). Moreover, Tulasnellaceae were also identified in Orchis pauciflora Ten. and O. tridentata Scop. sampled in the same study areas where H. adriaticum roots collection was carried out (Pecoraro et al. 2012a, 2012b). We suggest a symbiotic relationship between H. adriaticum and tulasnelloid fungi. Further analyses are therefore needed to clarify the functional role of these putative mycorrhizal fungi we found in the roots of the investigated orchid species.

Tulasnella calospora (Boud.) Juel was proposed as an universal orchid symbiont being the most common root and seedling endophyte, with the capacity to establish mycorrhiza with plenty of orchid species in the laboratory (Hadley 1970, Rasmussen 1995). However, taxonomic problems still stand in the way of unbiased Tulasnella species delimitation and molecular identification (Suárez et al. 2006, Cruz et al. 2011). A very effective primer pair for the amplification of ITS region from Tulasnellaceae was developed by Taylor & McCormick (2008). These authors showed that new basidiomycete ITS primers (ITS1–OF/ITS4-–OF) increase the success of Tulasnella amplification from orchid roots DNA whereas the universal fungal primers ITS1F and ITS4 fail to amplify some taxa in family Tulasnellaceae. In our work, the lack of Tulasnella sequences from five analyzed samples could be affected by the use of ITS1F-ITS4 primer pair and further molecular studies based on the use of different primer combinations will allow an in-depth analysis of tulasnelloid fungi associated with *H. adriaticum*.

Another potential symbiont with close identity to an uncultured mycorrhizal fungus found in *Epipactis helleborine* (Ogura-Tsujita & Yukawa 2008) was identified in the sample CG6. The results of the latter study suggested that *E. helleborine* thrives in the deepest woodland shade with nutritional supplementation from its fungal partners. The same mycorrhizal fungi could sustain development of H. adriaticum by transferring carbon from environmental sources to orchid plants.

Several ascomycetous fungi, with obscure ecological function, were found in *H. adriaticum* roots. Helotiales with identity to a sequence from roots of *Cephalanthera longifolia* analyzed by Abadie et al. (2006) were found in sample CG4. A trophic relationship with *H. adriaticum* was difficult to assess for these ascomycetes as Helotiales are an ecologically diverse taxon including plant symbionts (both ericoid and ectomycorrhizal), endophytes, saprotrophs and pathogens.

The closest two matches for the sequence amplified from sample CG4, clone A, were with Sordariomycetes found by Feldman et al. (2008) on the exoskeleton of nocturnal moths and *Gibberella moniliformis* collected from cucurbits crops (Zheng et al. 2008). In the former study, Sordariomycetes were considered asymptomatic fungi occurring in aboveground tissues of Paspalum grasses. The authors suggested that these endophytes,

vectored by *Spodoptera ornithogali*, could inhibit colonization of plant tissues by Claviceps paspali F.Stevens & J.G.Hall a common fungal pathogen of Paspalum species. With regard to *G. moniliformis*, Zheng et al. (2008) showed that this ascomycete is a pathogen of cucurbits and we cannot hypothesize any relationship between the same fungal taxon and *H. adriaticum*.

In addition, we found sequences that matched with *Fusarium* species collected from different sources (table 1) including orchid roots (Taurantha concinna, Otero et al. 2011), plant tissues (Posada et al. 2007, Li et al. 2008) and cyst of nematode (Gao et al. 2008). Because of the wide set of ecological functions of Fusarium species, the trophic relationship between these fungi and orchids is unclear and some authors consider them just like root surface contaminants (Girlanda et al. 2006). Fusarium species are primarily plantassociated fungi, even if some taxa are insect pathogens and some cause human mycoses (Bacon & White 2000). Fusarium species establish endophytic colonization in both cultivated and wild plants. There are examples of species with short endophytic phases that develop into pathogenic infection and also endophytes that never become pathogens. Moreover, the response of plants to fungal infection depends on several environmental factors and even Fusarium species that are known to be virulent can be asymptomatic under optimal growth conditions (Bacon & White 2000). The asymptomatic association between Fusarium and plants are sometimes considered mutualistic as the fungus receives nutrients from the plant that is protected against pathogenic infections by the fungal partner (Van Wyck et al. 1988, Yates et al. 1997). As Fusarium endophytes-plant associations range from latent pathogenic infections to mutualistic interactions, we cannot exclude the possibility of symbiotic relationship between Fusarium species and H. adriaticum. The same hypothesis was recently proposed by Chutima et al. (2011) for endophytic Fusarium strains colonizing Pecteilis susannae (L.) Rafin, whereas Ovando et al. (2005) and Johnson et al. (2007) have already reported that Fusarium isolated from orchids can promote the growth of orchid seedlings and form endomycorrhiza.

This study showed that H. adriaticum associates with a range of fungi that we found in the roots of all analyzed orchid individuals by means of molecular analysis. Our finding of pelotons in the root cells has provided the first evidence of mycorrhiza formation between *H. adriaticum* and some of the associated fungi. The results suggest that the study orchid species may have a dominant trophic relationship with basidiomycetes in the family Tulasnellaceae which are known to form mycorrhizas on several orchid taxa. We also identified a variety of ascomycetous fungi that have been already found in the roots of orchid species with unclear ecology. Their detection suggests a potential role as orchid symbionts although further investigations are needed in order to clarify the relationship between these ascomycetes and orchids. Similarly, very little is known about physiology and biochemistry of the obscure interactions between orchids and ascomycetous taxa with the putative ecology of endophytes, such as Hypocreales we found in *H. adriaticum* roots. A deeper understanding of the endophytic lifestyle of these fungi is essential.

However, the molecular assessment of fungal diversity associated with *H. adriaticum*, reported for the first time in this work, constitutes an advance in our understanding of the ecology of this orchid species and may represent the starting point for the analysis of the undoubtedly complex interaction between this delicate plant and fungi.

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