Molecular analysis of fungi associated with the Mediterranean orchid Ophrys bertolonii Mor

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Abstract

Mycorrhizal fungi are fundamental in orchid growth and metabolism and influence the distribution and rarity of these delicate plants. Fungal molecular systematics has been instrumental for the identification of orchid mycorrhizal symbionts because it overcomes the limits associated with in vitro isolation and morphological characterization of orchid endophytes. In this paper, fungal diversity in the Mediterranean orchid *Ophrys bertolonii* was analyzed using molecular methods. Total DNA from the roots of 12 adult orchid individuals, collected in Central Italy, was extracted and fungal ITS regions were PCR amplified using the primer pair ITS1F/ITS4, cloned and sequenced. Several fungal taxa belonging to Basidiomycota and Ascomycota were found in the analyzed orchid root samples. Results suggest that the studied orchid species may establish symbiotic relationships with a dominant fungal partner belonging to Tulasnellaceae.

Keywords

Ascomycetes .Basidiomycetes .ITSsequencing . Orchidaceae . Orchid mycorrhiza

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Introduction

In all orchids, the establishment of a relationship with mycorrhizal fungi is a requirement for germination and early development, as they grow from extremely small seeds, which are nearly lacking in nutritional reserves (Rasmussen 1995). The intimate association between orchids and their mycorrhizal fungi is unique from the physiological point of view because the plant depends on carbon nutrition supplied from fungal partners. Starting from the experiments of Smith (1967), several studies have shown that carbohydrates move from the mycobionts to the developing orchid plant. By contrast, the transport of carbon, in any form, from orchid to fungus has not been consistently demonstrated (Alexander and Hadley 1985). Although mutualism in orchid mycorrhiza has been recently documented, by showing a bidirectional transfer of carbon between the orchid *Goodyera repens* and its fungal associate (Cameron et al. 2006), the results were obtained under extreme experimental circumstances which can hardly occur in nature. However, the support for considering orchid mycorrhiza as a predominantly unilateral relationship is given by the obligate nutritional dependence of orchids on fungi at least for a part of their life cycle. All orchid seedlings utilize fungal symbionts for carbon and nutrient uptake, and the dependence on the mycorrhizal fungi is complete at the achlorophyllous protocorm stage. In contrast, all fungal partners known to be involved in orchid mycorrhiza are capable of an independent existence (Rasmussen and Rasmussen 2009). This trophic strategy, in which orchid seedlings are entirely nutritionally dependent upon fungi, is called mycoheterotrophy (Leake 1994).

After the myco-heterotrophic juvenile stage, the plant–fungus relationship follows three divergent directions in the adult orchids. The majority of orchids are photosynthetic at maturity, but remain dependent on their mycobionts at least for water uptake and mineral nutrition (Jacquemyn et al. 2011). Over 200 species remain achlorophyllous at the adult stage, lacking photosynthetic capability during their entire life span. These species depend on symbionts for their nutrition throughout their life, and their sole source of carbon is constituted by the fungal partners (Bidartondo et al. 2004). In many obligate myco-heterotrophic orchid species like *Neottia nidus-avis*, the ultimate carbon source is the photosynthesised carbon of autotrophic plants associated with the same fungal symbionts. These orchids are cheating parasites that obtain their carbohydrates from the surrounding photoautotrophs via a shared mycorrhizal fungus (Selosse et al. 2002). Some other achlorophyllous orchids such as *Gastrodia sesamoides* exploit the fungal partners as direct parasites since they associate with free-living saprotrophic fungi (Dearnaley and Bougoure 2010).

A significant number of forest orchids utilize a third nutritional mode, known as partial mycoheterotrophy or “mixotrophy” (Selosse et al. 2004). This strategy allows putatively photosynthetic orchid species to be independent of irradiance and to grow in shaded forests, because the symbiotic fungi provide these plants with both organic and inorganic nutrients. Mixotrophic orchids such as *Cephalanthera damasonium* and *C. rubra* obtain part of their carbon via mycorrhizal fungi under low light availability, while the proportion of heterotrophic nutrition decreases with increasing irradiance (Preiss et al. 2010).

Modern techniques have been used to throw light on physiological aspects of orchid mycorrhiza. Measurements of carbon transfer using isotopic labelling provided the experimental evidence of nutrients flow from ectomycorrhizal trees to the orchid *Corallorhiza trifida* via a shared mycelium (McKendrick et al. 2000). Moreover, the recent application of carbon and nitrogen stable isotope analyses (Gebauer and Meyer 2003) represented a powerful tool for understanding the orchid–fungus resource exchange. Nonetheless, the physiology of mycoheterotrophy and the fundamental mechanisms such as recognition and selection of fungi, nutrient transfer across the plant–fungus interface and metabolic pathways regulating this intriguing relationship between orchids and mycorrhizal symbionts are still almost entirely unexplained (Selosse and Cameron 2010). For this reason, it is not surprising that the exact trophic relationship between several orchids and their associated fungi is unknown. In many fungal taxa identified from orchid roots, the limit between their role as real symbionts or simple endophytes is often indistinct, while their physiological importance is also uncertain (Rasmussen and Rasmussen 2014).

Concerning the identity of the fungi involved in orchid mycorrhiza, both morphological and molecular studies have shown that the great majority of the Orchidaceae associates with basidiomycetes (Dearnaley et al. 2012; Pecoraro et al. 2012a, b; Jacquemyn et al. 2014) and only a few orchid species form symbioses with ascomycetes (Selosse et al. 2004). The use of molecular PCR-based techniques has largely facilitated the identification of fungal partners, especially those which are recalcitrant to isolation and growth in axenic culture (Taylor and McCormick 2008). DNA sequencing has also removed a major obstacle to the identification of culturable orchid symbionts characterized by a paucity of distinctive and stable morphological features. Indeed, most fungi isolated from orchids belong to the anamorphic form-genus *Rhizoctonia* (Otero et al. 2002; Perotto et al. 2013), which has rarely been induced to produce sexual stages in culture.

The aim of the present study was to determine whether the terrestrial green orchid *Ophrys bertolonii* Mor. associates with mycorrhizal fungi, and to investigate the diversity of its rootcolonizing endophytes by means of molecular methods. Information on the interactions between the studied orchid species and fungi may represent a valuable contribution to the knowledge of the

factors governing *O. bertolonii* distribution and population dynamics. Given that *O. bertolonii* is a threatened taxon, included in the Italian lists of endangered species (Conti et al. 1997; Alonzi et al. 2006), a better understanding of its ecology may be of critical importance for the long-term conservation of this delicate orchid.

Materials and methods

Study species and sample collection

Ophrys bertolonii is a terrestrial photosynthetic orchid growing in dry grassland, roadside banks, and forest margin, with a Mediterranean distribution (Balearics, Corsica, Corfu, southern France and Spain, central–southern Italy). Plants are 15– 35 cm tall, with 5–7 lanceolate leaves. Spikes carry 3–8 flowers characterized by a velvety blackish-purple lip, with a blue-violet patch (Davies et al. 1983). *Ophrys bertolonii* roots were collected during the early summer in 2007 and 2008 from 12 adult flowering individuals, in five dry calcareous grassland sites located in 'Monte Cetona' Natural Reserve, at an altitude ranging from 1006 to 1047 m a.s.l., in central Italy. Roots fragments were carefully cleaned under running water, scrubbed with a brush and treated in an ultrasonic bath (three cycles of 30 s each) in order to remove soil particles and microorganisms from root surfaces. Hand cross-sections were observed under a light microscope to check for fungal colonization. Mycorrhizal root portions were frozen in liquid nitrogen and kept at −80 °C for molecular analysis.

Molecular identification of root fungal associates

Total root DNA was extracted from *O. bertolonii* analyzed samples, following the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990).

Fungal ITS regions were amplified by polymerase chain reaction (PCR), using the primer pair ITS1F/ITS4 (Gardes and Bruns 1993) in 50 μL reaction volume, containing 38 μL sterile distilled water, 5 μL 10× buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 11 mM MgCl2, 0.1 % gelatine), 1 μL 10mMdNTP, 1 μL of each primer (ITS1F and ITS4), 1.5 U of RED TaqTMDNA 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, and 1 cycle of 72 °C for 7 min.

PCR products were electrophoresed in 1 % agarose gels with ethidium bromide, visualised under UV light, and purified with the QIAEX II Gel Extraction Kit (QIAGEN) following the manufacturer's instructions.

The purified ITS fragments were cloned into pGEM-T (Promega) vectors. XL-2 Blue ultracompetent cells (Stratagene) were transformed following the manufacturer's advice. 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); and 72 °C for 7 min (1 cycle).

Cloned ITS inserts were purified with Plasmid Purification Kit (QIAGEN) and sequenced with the same primer pair used for amplification. Dye sequencing was carried out on ABI 310 DNA Sequencer (Applied Biosystems, Carlsbad, CA, USA). Sequences were edited, assembled using the program Sequencher 4.1 for MacOS 9, and analysed with BLAST searches against the National Center for Biotechnology Information (NCBI) sequence database (GenBank, http://www.ncbi.nlm.nih.gov/BLAST/index.html).

Results

Microscopic analyses showed that the cortical cells of the orchid root samples were heavily colonized by hyphae forming dense intracellular coils (Fig. 1a, b). The majority of these hyphae appeared to be hyaline and thin-walled (6–8 μm diameter), sometimes exhibiting typical *Rhizoctonia* features.

Fungal sequences were amplified from all investigated *O. bertolonii* root samples. The top-scoring matches from BLAST searches are reported in Table 1. Most of the orchid samples (9 out of 12) were associated with tulasnelloid fungi (basidiomycetes). The closest match for the sequences obtained from samples 2, clone b (96 %over 1081 pb), 3 (96 %over 1107 bp), 5, clone c (97 % over 1009 bp), 9 (96 % over 1061 bp), 10, clone c (96 % over 1075 bp), and clone e (96 % over 1059), 11 (93 % over 555 bp), and 12, clone b (96 % over 1066 bp) was with *Epulorhiza* sp. (anamorphic stage of *Tulasnella*) from *Cypripedium macranthos* var. *rebunense* (AB369931 and AB369932) sampled in Rebun Island, Japan. The sequences obtained from samples 2, clone a, 10, clone d, and 12, clone a matched (98-99 % over 1027- 1103) *Epulorhiza* sp. from *Ophrys sphegodes* whereas the primary fungal endophyte for roots 6 (clone a) and 7 was a fungus with close identity (99 % over

1173-1188) to Tulasnellaceae found in *C. arietinum* (DQ925600). Sequences from sample 12 (clone e) matched instead *Rhizoctonia* sp.

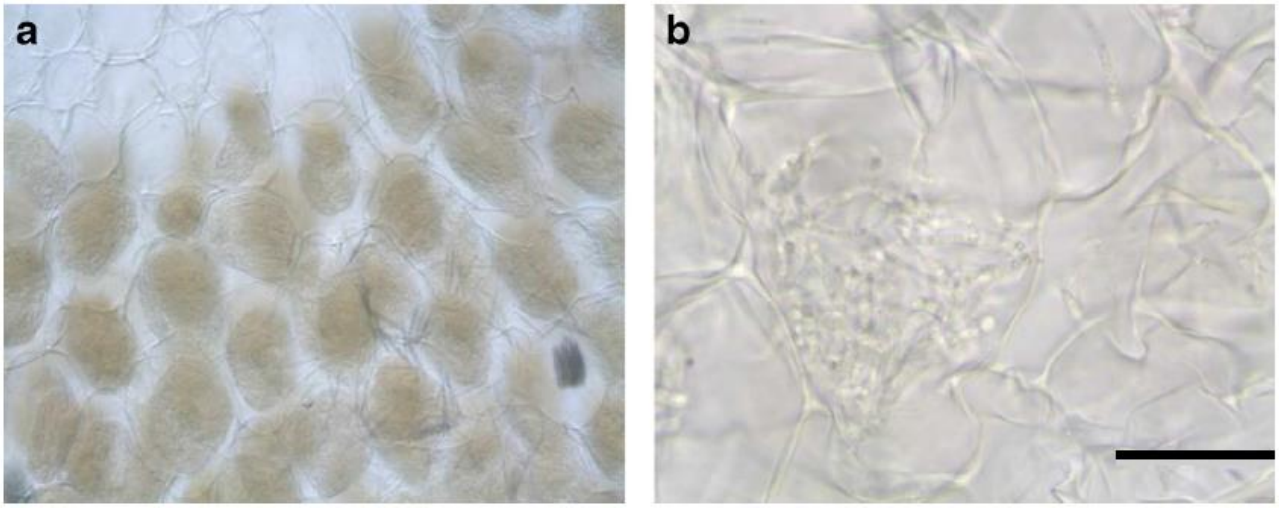

Fig. 1 Light microscopy of Ophrys bertolonii roots. a Crosssection showing intracellular hyphal coils. b Details of hyaline hyphae forming pelotons. Scale bar 100 μm

Fungi associated with sample 5 (clone b) and 6 (clone e) shared 97-98 % similarity with a fungal endophyte from *Parys polyphylla*. The remaining orchid samples (three out of twelve) 1, 4, and 8 were colonized by ascomycetes belonging to *Fusarium*, *Gibberella* (anamorph *Fusarium*), and *Alternaria* (Table 1).

Discussion

Pelotons (intracellular hyphal coils) represent the typical features of orchid mycorrhiza (Rasmussen 1995). Microscopic observations, showing large numbers of fungal pelotons in the root cells of all the sampled *O. bertolonii* individuals, therefore provided morphological evidence of the establishment of a trophic relationship between the orchid plants and their fungal associates. Fungi associated with *O. bertolonii* adult plants have been identified using molecular methods, and several fungal taxa have been found in the root samples. Among them, Basidiomycota occurred in the majority of orchid samples, whereas Ascomycota were amplified from only 4 out of 12 orchid individuals. Results suggest that the studied orchid species may establish symbiotic relationships with a dominant fungal partner belonging to Tulasnellaceae. Indeed, sequences of tulasnelloid fungi were amplified from the majority of analyzed samples. Fungal endophytes from seven *O. bertolonii*

Table 1 BLAST search closest matches of fungal sequences amplified from the twelve analyzed *Ophrys bertolonii* plant samples

Sample	Clone	Best BLAST match(es)	Accession code	Overlap length	% match
1	A	*Fusarium oxysporum* (from onychomycosis)	EU839400	974	99 %
	b	*Fusarium* sp.	EU750682	979	99 %
2	a	*Epulorhiza* sp. (from *Ophrys sphegodes*)	AM697902	1103	99 %
		Epulorhiza sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369931	1086	96 %
	b	*Epulorhiza* sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369932	1081	96 %
3	a	*Epulorhiza* sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369932	1107	96 %
4	a	*Gibberella moniliformis*	EU717682	1003	99 %
	d	*Fusarium oxysporum*	X78259	970	99 %
	e	*Fusarium* sp. (from *Discorea zingiberensis*)	DQ446211	1014	99 %
5	a	*Fusarium redolens* (from *Dianthus* sp.)	X94169	1014	99 %
	b	Fungal endophyte (from *Paris polyphylla*)	EF495231	1114	98 %
	c	*Epulorhiza* sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369932	1109	97 %
6	a	*Tulasnellaceae* (from *Cypripedium arietinum*)	DQ925600	1173	99 %
	e	Fungal endophyte (from *Paris polyphylla*)	EF495231	1037	97 %
7	a	*Tulasnellaceae* (from *Cypripedium arietinum*)	DQ925600	1188	99 %
8	a	*Alternaria* sp.	DQ491090	968	99 %
	b	*Alternaria arborescens* (from *Lycopersicum esculentum*)	AY154706	972	98 %
9	a	*Epulorhiza* sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369932	1061	96 %
10	c	*Epulorhiza* sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369932	1075	96 %
	d	*Epulorhiza* sp. (from *Ophrys sphegodes*)	AM697902	1038	98 %
	e	*Epulorhiza* sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369931	1059	96 %
11	a	*Epulorhiza* sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369931	555	93 %
12	a	*Epulorhiza* sp. (from *Ophrys sphegodes*)	AM697902	1027	98 %
		Epulorhiza sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369931	1016	94 %
	b	*Epulorhiza* sp. (from *Cypripedium macranthos* var. *rebunense*)	AB369932	1066	96 %
	e	*Rhizoctonia* sp.	AJ318440	710	90 %

The best BLAST hit is reported as described in the GenBank accession. Accession codes for the closest GenBank matches, sequence identity (% match) and overlap of each match are reported

plants found the best similarity in BLAST searches with *Epulorhiza* sp. isolated by Shimura et al. (2009) from populations of the threatened orchid *C. macranthos* var. *rebunense*. These authors demonstrated the functional role of the *Cypripedium* fungal isolates as orchid mycorrhizal partners by testing their ability to induce symbiotic orchid seeds germination. Sequences obtained from samples 2, 10, and 12 matched closely with *Epulorhiza* sp. collected by Illyés et al. (2009) from roots of *O. sphegodes* growing in treeless wetlands in higher-lying areas of the Carpathian Basin which are flooded for short periods. These authors suggested a fundamental role of the fungal symbionts in *O. sphegodes* seedling establishment and habitat adaptation, as this association with *Epulorhiza* fungi was peculiar of drier sites among different habitats, with diverse water regime. Fungal sequence from two *O. bertolonii* plants matched Tulasnellaceae found in *C. arietinum* sampled in three populations in the Upper Peninsula of Michigan (Shefferson et al. 2007). High specificity was found in the *Cypripedium*–fungus association and a mycorrhizal relationship in which carbon is provided by the fungus as well as phosphorus and nitrogen was described. The sequence obtained from *O. bertolonii* sample 12, clone e, corresponded to a fungus with identity to *Rhizoctonia* sp. isolated in Singapore during a study on the identification and molecular phylogeny

of *Epulorhiza* associated with roots and protocorms from tropical orchids (Ma et al. 2003). All the fungal isolates obtained by these authors could induce in vitro germination of orchid seeds, thus showing their potential as symbionts. In the present paper, we propose the role of symbionts for the Tulasnellaceae associated with *O. bertolonii* roots. This hypothesis, supported by the microscopic observations of *Rhizoctonia*-like hyphae forming pelotons, is in agreement with several studies that have recently documented the association of tulasnelloid fungi with many orchids, especially with photosynthetic terrestrial species, from both forest (Bonnardeaux et al. 2007) and open habitats (Girlanda et al. 2011; Pecoraro et al. 2013). However, further analyses are needed to confirm the symbiotic relationship between *O. bertolonii* and Tulasnellaceae and to investigate physiological aspect of this intriguing association. Symbiotic seed germination experiments could be performed to clarify the functional role of fungal strains collected from *O. bertolonii* roots, using single peloton isolation methods (Zhu et al. 2008). Moreover, additional PCR amplification using Tulasnellaceae-specific primers could lead to a more precise identification of *O. bertolonii* mycobionts (Taylor and McCormick 2008). Indeed, Tulasnellaceae are problematic to amplify using the universal fungal ITS primers, due to bases mutation occurring in regions to which primers hybridise, as a consequence of accelerated evolution of the nuclear ribosomal operon (Dearnaley et al. 2012). Ascomycetes were sporadically found in *O. bertolonii* analyzed roots. Sequences that matched a fungal endophyte, isolated from rhizomes of the traditional Chinese medicinal plant *P. polyphylla* var. *yunnanensis*, were amplified from two orchid samples. Li et al. (2008) cultured this non-sporulating fungus isolated from *P. polyphylla* plant samples collected in Yunnan Province (southwest China) and identified the isolate as a mycorrhizal ascomycete, based on its morphological and molecular features. We cannot exclude that the same fungus may play a trophic role associating with orchids. *Fusarium* sequences were instead found in samples 1, 4, and 5, respectively sharing similarity with *F. oxysporum* isolated from patients with onychomycosis in Colombia (López et al. 2008), *Fusarium* sp. colonizing the rhizomes of the perennial medicinal plant *Dioscorea zingiberensis* growing in China (Xu et al. 2008), and *F. redolens* isolated by Waalwijk et al. (1996) from *Dianthus* sp. collected in Germany. *Fusarium* fungi are ecologically diverse and their function in many ecosystems is still unclear (Bacon and White 2000). They are cosmopolitan soil fungi including a large number of plant endophytes, as well as plant, animal and human pathogens. The same *Fusarium* strain can be simultaneously an etiological agent of onychomycosis and a plant pathogen (see Lopez et al. 2008). Endophytic *Fusarium* strains range from asymptomatic pathogens to mutualistic partners. For instance, the endophytes found by Xu et al. (2008) in *D. zingiberensis* did not cause significant damage to the plant, and their secondary metabolites, displaying antibacterial activity, were supposed to protect *D. zingiberensis* against

pathogens. The real meaning of the relationship between *Fusarium* and orchids is still almost unknown and should be deeply analysed in order to understand whether this group of fungi contributes to orchid growth and nutrition. However, some evidence of a possible symbiotic association between *Fusarium* species and orchids has been reported by several authors showing that *Fusarium* strains can stimulate orchid seed germination and seedling growth (Vujanovic et al. 2000; Ovado et al. 2005; Johnson et al. 2007).

This study showed that *O. bertolonii* associates with a range of fungi, belonging to Basidiomycota and Ascomycota identified in all the analysed orchid root samples, by means of molecular methods. Results suggest that basidiomycetes in the family Tulasnellaceae may play a dominant trophic role in the investigated orchid species. Although the relationships between *O. bertolonii* and its fungal associates need to be confirmed by further analyses, information provided in the present work could be fundamental for conservation efforts. The vast majority of orchid taxa are well known for their rarity so that the entire family Orchidaceae is included in the CITES (Convention on International Trade in Endangered Species of wild flora and fauna) appendix listing species of varying degrees of protection. Many orchid species are currently threatened or endangered in several countries throughout the world and are included in the lists of protected species at international level (IUCN 1994). In particular, *O. bertolonii*, together with the whole genus *Ophrys*, is protected under several national statements in Italy (Conti et al. 1997; Alonzi et al. 2006). Because orchids are critically dependent on fungal partners for seed germination and plant growth, knowledge of the identities and roles of mycorrhizal fungi is of primary importance for understanding the biology of orchids and contributing to their conservation (Liu et al. 2010; McCormick and Jacquemyn 2014).

The molecular analysis of fungi associated with *O. bertolonii*, performed for the first time in this work, provides valuable information on the biology of this threatened orchid species that could be of great value for its conservation.

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