Molecular identification of root fungal associates in Orchis pauciflora Tenore

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L. Pecoraro , M. Girlanda , T. Kull , C. Perini & S. Perotto

Abstract

The terrestrial orchid, *Orchis pauciflora* Ten., growing in poor grassland and garrigue of Central Mediterranean region, is local and rare and has been included in the red lists of several Italian regions. We investigated the diversity of fungal associates in *O. pauciflora* adult plants collected in two protected areas of Tuscany (Central Italy). Genomic DNA was extracted from mycorrhizal roots of 12 orchid plants and the fungal ITS were amplified and sequenced. Several fungal associates, belonging to different *taxa* of basidiomycetes (*Tulasnellaceae*) and ascomycetes such as *Leptodontidium*, *Exophiala* and *Phialophora* species, were recovered. The trophic role of these fungi and their impact on *O. pauciflora* growth and conservation are discussed.

Keywords: Ascomycota , Basidiomycota , conservation, fungal symbionts, Orchidaceae ,

Introduction

Orchidaceae is one of the most threatened plant families. Due to their rarity level and endemic status many orchid species have been included in the lists of endangered species at the world (CITES, Bern Convention) and national level (Conti et al. 1992 Conti, F, Manzi, A and Pedrotti, F. 1992. *Libro rosso delle piante d'Italia*, Roma: Poligrafica Editrice. Ministero dell'Ambiente, Ass. Ital. per il WWF, S.B.I, 1997 Conti, F, Manzi, A and Pedrotti, F. 1997. *Liste rosse regionali delle piante d'Italia. WWF Italia, Società Botanica Italiana*, Camerino: TIPAR Poligrafica Editrice.) and protected under several international statements (IUCN 1994 IUCN. 1994. *IUCN red list categories*, Gland, Switzerland: IUCN.). One of the possible reasons for their rarity could be the obligatory dependence of orchids on fungi (Kull & Kull 2005 Kull, T and Kull, K. How orchids regulate their numbers. Proceedings of the 17th world Orchid Conference. 2002, Kota Kinabalu, Shah Alam, Sabah. pp.173–177. Malaysia: Natural History Publications (Borneo).).

Association with symbiotic fungi has a crucial impact on orchid growth and metabolism from germination through the seedling stage and in many cases throughout the life (Kull & Arditti 2002 Kull, T and Arditti, J. 2002. *Orchid biology: Reviews and perspectives, VIII*, Dordrecht: Kluwer Academic Publishers. ; Dearnaley 2007 Dearnaley, J DW. 2007. Further advances in orchids mycorrhizal research. *Mycorrhiza*, 17: 475–486. ; Valletta et al. 2008 Valletta, A, Attorre, F, Bruno, F and Pasqua, G. 2008. In vitro asymbiotic germination of *Orchis mascula* L. *Plant Biosyst*, 142: 653–655. ; Sgarbi et al. 2009 Sgarbi, E, Grimaudo, M and Del Prete, C. 2009. In vitro asymbiotic germination and seedling development of *Limodorum abortivum* (*Orchidaceae*). *Plant Biosyst*, 143: 114–119. ; Pierce et al. 2010 Pierce, S, Ferrario, A and Cerabolini, B. 2010. Outbreeding and asymbiotic germination in the conservation of the endangered Italian endemic orchid *Ophrys benacensis*. *Plant Biosyst*, 144: 121–127.). The great seed production in orchids suggests that the mortality of seeds and seedlings is very large. To germinate, orchid seeds need not only suitable substrate and physical conditions, but also a compatible mycobiont providing water and mineral nutrients as well as organic carbon, since the minuscule seed has minimal nutritional reserves. At the

adult stage, most orchids retain their mycorrhizal partnerships, and due to their characteristically poorly developed root systems, they are thought still to be heavily reliant on mycorrhizal fungi for mineral nutrition (Waterman & Bidartondo 2008 Waterman, R J and Bidartondo, M I. 2008. Deception above, deception below: Linking pollination and mycorrhizal biology of orchids. *J Exp Bot*, 59: 1085–1096.).

There is still more to be learnt about fungal specificity in the *Orchidaceae* and its impact on the conservation of individual species. The identification of the mycorrhizal symbionts will be of critical importance to understand the ecology of orchids and conservation efforts (Kristiansen et al. 2001 Kristiansen, K A, Taylor, D L, Kjøller, R, Rasmussen, H N and Rosendahl, S. 2001. Identification of mycorrhizal fungi from single pelotons of *Dactylorhiza majalis* (*Orchidaceae*) using SSCP and mitochondrial ribosomal LsDNA sequences. *Mol Ecol*, 10: 2089–2093.). Some photosynthetic orchids, even when sampled over a wide range, have a single dominant mycorrhizal fungus (McCormick et al. 2004 McCormick, M K, Whigham, D F and O'Neill, J. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytol*, 163: 425–438. , 2006 McCormick, M K, Whigham, D F, Sloan, D, O'Malley, K and Hodkinson, B. 2006. Orchid-fungus fidelity: A marriage meant to last?. *Ecology*, 87: 903–911. ; Shefferson et al. 2005 Shefferson, R P, Weiss, M, Kull, T and Taylor, D L. 2005. High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. *Mol Ecol*, 14: 613–626. ; Dearnaley & Le Brocque 2006 Dearnaley, J DW and Le Brocque, A F. 2006. Molecular identification of the primary root fungal endophytes of *Dipodium hamiltonianum* (Yellow hyacinth orchid). *Aust J Bot*, 54: 487–491.). Fungal specificity and orchid rarity may be linked if the fungal partner is rare or patchily distributed in the landscape (Bonnardeaux et al. 2007 Bonnardeaux, Y, Brunndrett, M, Batty, A, Dixon, K, Koch, J and Sivasithamparam, K. 2007. Diversity of mycorrhizal fungi of terrestrial orchids: Compatibility webs, brief encounters, lasting relationships and alien invasions. *Mycol Res*, 111: 51–61.).

Information on the identity of *Orchis pauciflora* mycorrhizal symbionts could be very important for both *in situ* and *ex situ* conservation of this orchid. In this research, we identify fungi associated with *O. pauciflora* by means of molecular techniques. Our goal was to identify the root fungal endophytes of adult individuals, collected in similar habitats at different sites.

Materials and methods

Study species

Orchis pauciflora Ten. is a terrestrial orchid species that grows in poor grasslands and garrigues, on dry, calcareous soils, in central and southern Italy, but not in Sardinia, up to 1800 m a.s.l.

The geographical distribution of this orchid includes the Central Mediterranean region, from Corsica to Crete. The species is local and rare (Delforge 1995 Delforge, P. 1995. *Orchids of Britain & Europe*, Basingstoke: Harper Collins.) and has been included in the red lists of several Italian regions (Alonzi et al. 2006 Alonzi, A, Ercole, S and Piccini, C. 2006. La protezione delle specie della flora e della fauna selvatica: Quadro di riferimento legislativo regionale. *APAT Rapporti*, 75).

The plant is 10–30 cm tall, with pale green leaves that are relatively small, narrowly lanceolate. It bears a compact inflorescence, usually composed of few (3–10, rarely more) yellow flowers (Rossi 2002 Rossi, W. 2002. *Orchidee d'Italia. Quad. Cons. Natura, 15, Min. Ambiente-Ist. Naz. Fauna Selvatica* 333).

Sample collection

We collected 12 root samples of adult *O. pauciflora* plants, during the flowering period in early summer in the years 2007–2008, in two protected areas of Tuscany (Central Italy). Six samples were collected in the "Monte Cetona" and six in the "Cornate di Gerfalco" Natural Reserves, in meadow habitats, on the top of calcareous mountains (about 1000 m a.s.l.). Root fragments were extensively rinsed with tap water, kindly brushed and subjected by sonication in an ultrasonic bath (three cycles of 30 s each) to remove soil particles and microorganisms adhering to the root surface.

Molecular identification of fungal symbionts

Mycorrhizal morphology of fresh orchid roots was observed under a light microscope; root sections exhibiting higher fungal colonization were frozen in liquid nitrogen and stored at −80°C for subsequent molecular analysis.

Total DNA from root samples was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Henrion et al. 1992 Henrion, B, Le Tacon, F and Martin, F. 1992. Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. *New Phytol*, 122: 289–298.).

Fungal ITS regions were PCR amplified using the primer pair ITS1F/ITS4 (Gardes & Bruns 1993 Gardes, M and Bruns, T D. 1993. ITS primers with enhanced specificity for basidiomycetes – applications to the identification of mycorrhizae and rusts. *Mol Ecol*, 2: 113–118.) 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 Cl_2, 0.1% gelatine), 1 µL 10 mm dNTP, 1 µL of each primer (ITS1 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, with 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. The resulting PCR products were electrophoresed in 1% agarose gel with ethidium bromide 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; the vectors were used to transform XL-2 Blue ultracompetent cells (Stratagene). 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 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 Genetic Analyzer (Applied Biosystems).

Sequences were analysed using the BLASTN algorithm available through the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/index.html).

Results

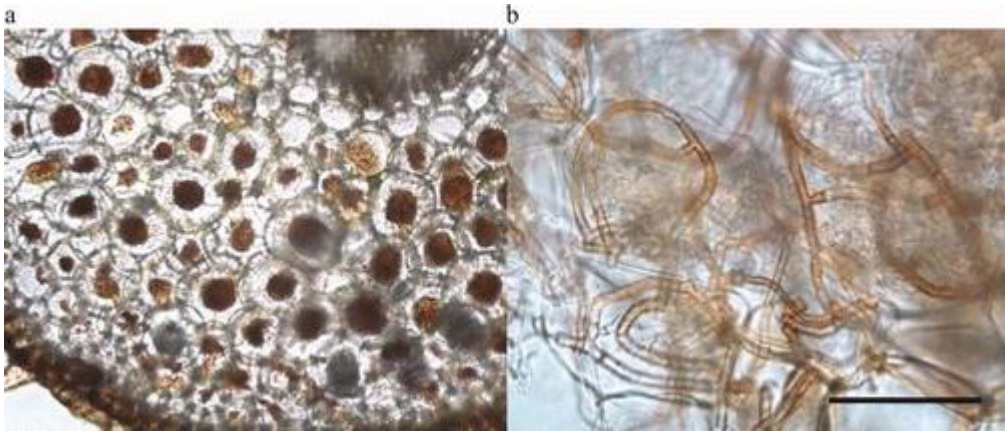
The cortical cells of the sampled *O. pauciflora* roots were heavily colonized by hyphae forming dense intracellular coils (Figure 1a). Root sections showed occurrence of both hyaline (3–5 μm in diameter) and brown larger (6–8 μm in diameter) hyphae exhibiting typical *Rhizoctonia* features (branches produced at right and acute angles to the main hypha, the branch hypha being slightly constricted at the branch origin, and septum often occurring near the branch origin; Figure 1b). Sequencing of the cloned ITS fungal inserts corresponding to the major PCR products obtained from amplification with the ITS1F and ITS4 primers revealed a variety of fungal species occurring in *O. pauciflora* roots from both "Cornate di Gerfalco" and "Monte Cetona" (Table I). Fungal sequences from sample CG4 mostly belonged to *Tulasnellaceae*, the closest match being with *Tulasnellaceae* obtained from *Orchis mascula* (Linnaeus) Linnaeus (96–97% over 863–878 bp). The sequences obtained from five plants collected either at "Cornate di Gerfalco" or "Monte Cetona" (CG1, CG2, CG3, MC3 and MC6) matched (96–99% over 545–1052 bp) a sequence from a fungal endophyte from the rhizomes of *Paris polyphylla* Smith var. *yunnanensis* (Franch.) Hand-Mazz. Sequences from samples CG3 (clone d) and CG5 matched instead (95–98% over 704–924 bp) an uncultured ascomycete colonizing *Cephalanthera damasonium* (Miller) Druce. Sequences from sample MC1 matched *Leptodontidium elatius* (F. Mangenot) de Hoog (clone a) and *Exophiala salmonis* J.W. Carmich. (clone b) (90–95% over 295–906 bp). *Phialophora europaea* de Hoog, Mayser & Haase was found in the roots of sample CG5 (clone e). For the remaining samples (CG6, MC2, MC4 and MC5), the closest match was with *Fusarium* species.

Table I. Blast search results for the fungal sequences amplified from *O. pauciflora* roots collected in Cornate di Gerfalco (samples CG1-CG6) and Monte Cetona (samples MC1-MC6)

Sample	Clone	Best BLAST match(es)	Accession code	Overlap length	% match
CG1	a	Fungal endophyte (from *Paris polyphylla*)	EF495231	897	99
CG2	a	*Fusarium* sp. from *Terminalia arjuna* (root)	AY924269	681	90
	b	Fungal endophyte (from *Paris polyphylla*)	EF495231	834	96
	d	*Fusarium redolens*	EF495234	890	98
CG3	b	Fungal endophyte (from *Paris polyphylla*)	EF495231	545	92
	c	*Scolecobasidium tshawytschae*	AB161066	191	96
	d	Ascomycete (from *Cephalanthera damasonium*)	AY833032	704	95
		Calycina herbarum	AY348594	540	87
CG4	a	*Tulasnellaceae* (from *Orchis mascula*)	DQ925635	878	97
	c	*Tulasnellaceae* (from *Orchis mascula*)	DQ925615	863	96
CG5	a	Ascomycete (from apple seedling)	EU003079	132	78
		Leptodontidium orchidicola (from *Salix* sp.)	AY237626	127	80
	c	*Fusarium oxysporum*	DQ906172	123	91
	e	Ascomycete (from *Cephalanthera damasonium*)	AY833038	924	98
		Phialophora europaea	EF540756	636	87
CG6	a	*Fusarium oxysporum*	AF176656	733	96
	b	*Scolecobasidium tshawytschae*	AB161066	339	96
	c	*Phoma* sp. (from oilseed rape)	EU754897	719	95
MC1	a	*Epacris microphylla* root associated fungus	AY268186	297	91
		Leptodontidium elatius	AY805569	295	90
	b	Fungus (from *Phragmites australis*)	AJ875365	1047	98
		Exophiala salmonis	AF050274	906	95
	c	*Leohumicola minima*	AY706329	930	97
MC2	d	*Fusarium oxysporum*	X78259	729	95
MC3	a	*Helotiales* (from ectomycorrhizal root tip)	EU326174	836	97
		Ericoid mycorrhizal	AF269067	720	92
	d	*Fusarium* sp.	EU750682	908	99
	e	Fungal endophyte (from *Paris polyphylla*)	EF495231	1052	99
		Exophiala salmonis	AF050274	951	95
MC4	a	Uncultured soil fungus	DQ421009	596	98
		Hypocreales (from oilseed rape)	EU754934	590	98
	b	*Fusarium* sp. (from *Liriope spicata*)	DQ098907	744	93
	e	Fungal endophyte (from *Streptogyna americana*)	EU686805	634	87
		Stilbella byssiseda	AF335453	545	83
MC5	a	*Neonectria radicicola*	AJ875336	585	99
	e	*Fusarium oxysporum*	AF176656	619	92
MC6	a	Ascomycete (from apple seedling)	EU003079	830	94
		Cadophora sp.	DQ317329	758	92
	b	*Fusarium oxysporum*	EU839400	904	99
	e	Fungal endophyte (from *Paris polyphylla*)	EF495231	975	98

Note: 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.

Figure 1. Morphological features of *O. pauciflora* mycorrhizal roots. (a) Cross-section showing large numbers of intracellular fungal pelotons (hyphal coils). (b) Details of Rhizoctonia-like hyphae emanating from pelotons. Scale bar represents 100 μm.



Discussion

All analysed *Orchis pauciflora* roots were heavily colonized by fungi. This result was not surprising as several photosynthetic terrestrial orchids are highly dependent on soil fungi for their nutrition, also in the adulthood (Benzing 1981 Benzing, D H. 1981. Why is *Orchidaceae* so large, its seeds so small, and its seedlings mycotrophic?. *Selbyana*, 5: 241–242. ; Hadley 1985 Hadley, G. Mycorrhiza in tropical orchids. Proceedings of the Fifth Asian Orchid Conference Seminar. pp.154–159. Singapore: Singapore Parks and Recreation Department, Ministry of National Development. ; Richardson et al. 1993 Richardson, K A, Currah, R S and Hambleton, S. 1993. Basidiomycetous endophytes from the roots of neotropical epiphytic *Orchidaceae*. *Lindleyana*, 8: 127–137. ; Otero et al. 2002 Otero, J T, Ackerman, J D and Bayman, P. 2002. Diversity and host specificity of endophytic *Rhizoctonia*-like fungi from tropical orchids. *Am J Bot*, 89: 1852–1858. ; Girlanda et al. 2011 Girlanda, M, Segreto, R, Cafasso, D, Liebel, H T, Rodda, MErcole, E. 2011. *Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations*, Am J Bot 98: 1148–1163.). Stable isotope (^{13}C) natural abundance analysis has indeed shown that these orchids may acquire fungus-derived organic C (Liebel et al. 2010 Liebel, H T, Bidartondo, M I, Preiss, K, Segreto, R, Stöckel, MRodda, M. 2010. C and N stable isotope signatures reveal constraints to nutritional modes in orchids from the Mediterranean and Macaronesia. *Am J Bot*, 97: 903–912. , Girlanda et al. 2011 Girlanda, M, Segreto, R, Cafasso, D, Liebel, H T, Rodda, MErcole, E. 2011. *Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations*, Am J Bot 98: 1148–1163.).

Culture-independent polymerase chain reaction (PCR) amplification used in this study to directly identify fungi within roots using fungal specific primers provided evidence that *O. pauciflora* was associated with a variety of fungi, that may establish different trophic relationships with the host plant. PCR-based approaches are helping to overcome the problems associated culture biases in orchid mycorrhizal research. However, while potentially providing access to unculturable fungi, culture-independent molecular methods, as fungal isolation, do not distinguish between mycorrhizal and non-mycorrhizal (e.g. rhizoplane) fungi, what may lead to suspect conclusions concerning the biology of the symbiosis.

The primer pair used mostly yielded sequences of ascomycetous fungi (Table I). Among them, endophytes with close identity to a sequence from rhizomes of *Paris polyphylla* (*Trilliaceae*) collected by Zhou et al. (2007 – Unpublished) were found, from five *O. pauciflora* plants (Table I). Even if we do not know the exact identity of these endophytes and their ecology, we could suppose they have an important trophic relationship with orchids as they probably have with *P. polyphylla*. Indeed, in a later study, Li et al. (2008 Li, J, Zhao, J, Xu, L, Zhou, L, Li, X and Wang, J. 2008. Endophytic fungi from rhizomes of *Paris polyphylla* var. *yunnanensis*. *World J Microbiol Biotechnol*, 24: 733–737.) deeply analysed the diversity of endophytic fungi from *P. polyphylla* and these included the same uncultured ascomycete. The authors supposed this fungus was mycorrhizal. After all, the role of symbionts for endophytes of *P. polyphylla* was suggested by several authors. Tan et al. (2006 Tan, X M, Guo, S X, Zhou, Y Q, Yu, L Y and Miu, J H. 2006. Microstructure and endophytic fungus distribution of the root of *Paris polyphylla*. *Mycosystema*, 25: 227–233.), for instance, analysed microstructure and distribution of endophytic fungi of the roots of this perennial herb and they found the hyphae were colonized in cortex of rhizomes and adventitious roots. They infected the cortex parenchyma through epidermis and exodermis, and then, formed pelotons and expanded their occupying area of cortex. The pelotons were digested and absorbed in some cells. As there is some evidence to suggest that the asymptomatic association between endophytes and several plant species may be mutualistic (Bacon & White 2000 Bacon, C W and White, J F. 2000. *Microbial endophytes*, New York, NY: Marcel Dekker.) we cannot exclude that endophytic fungi form orchid mycorrhizae.

Sequences of ascomycetous fungi corresponding to several genera were also found in most of the studied orchid samples (Table I). We found sequences that matched closely with ascomycetes collected by Julou et al. (2005 Julou, T, Burghardt, B, Gebauer, G, Berveiller, D, Damesin, C and Selosse, M A. 2005. Mixotrophy in Orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalantera damasonium*. *New Phytol*, 166: 639–653.) from roots of *C. damasonium* that was suggested to achieve mixotrophy by using C from their mycorrhizal fungi. In the same study, were amplified sequences that had their closest GenBank relatives in *Exophiala salmonis* strain reported in a work pertained to molecular systematics of the *Herpotrichiellaceae* (Untereiner & Naveau 1999 Untereiner, W A and Naveau, F A. 1999. Molecular systematics of the *Herpotrichiellaceae* with an assessment of the phylogenetic positions of *Exophiala dermatitidis* and *Phialophora Americana*. *Mycologia*, 91: 67–83.). We also amplified sequences with identity to the same *E. salmonis* (with the same accession code) from roots of *O. pauciflora*. Moreover, we found sequences with identity to fungi belonging to the genera *Leptodontidium* and *Phialophora*. Julou et al. (2005 Julou, T, Burghardt, B, Gebauer, G, Berveiller, D, Damesin, C and Selosse, M A. 2005. Mixotrophy in Orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalantera damasonium*. *New Phytol*, 166: 639–653.) amplified sequences relating to the same genera from *C. damasonium* roots and they suggested the putative ectomycorrhizal ecology for *Phialophora*.

Some fungal sequences found during the molecular analysis may reflect fungal contaminants from the roots surface or non-mycorrhizal root endophytes in the *O. pauciflora* samples. Among them, we want to focus on *Fusarium* sequences as they are not considered mycorrhizal, but their continuous and heavy presence in the roots of a lot of orchid species (Girlanda et al. 2006 Girlanda, M, Selosse, M A, Cafasso, D, Brilli, F, Delfine, SFabbian, R. 2006. Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal *Russulaceae*. *Mol Ecol*, 15: 491–504.) leaves us in a state of uncertainty in the matter of a trophic role that they could play.

Ascomycetes are known to occur either as non-mycorrhizal endophytes or mycorrhizal partners in orchid roots (see e.g. Selosse et al. 2004 Selosse, M A, Faccio, A, Scappaticci, P and Bonfante, P. 2004. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (*Neottieae*,

Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. *Microbiol Ecol*, 47: 416–426. ; Julou et al. 2005 Julou, T, Burghardt, B, Gebauer, G, Berveiller, D, Damesin, C and Selosse, M A. 2005. Mixotrophy in Orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalantera damasonium*. *New Phytol*, 166: 639–653. ; Girlanda et al. 2006 Girlanda, M, Selosse, M A, Cafasso, D, Brilli, F, Delfine, SFabbian, R. 2006. Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal *Russulaceae*. *Mol Ecol*, 15: 491–504. , 2011 Girlanda, M, Segreto, R, Cafasso, D, Liebel, H T, Rodda, MErcole, E. 2011. *Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations*, Am J Bot 98: 1148–1163.). Further studies are therefore needed to clarify the function of the ascomycete associates towards *O. pauciflora*.

Sequences of *Tulasnellaceae* (basidiomycetes) were instead obtained from a single (sample CG4) *O. pauciflora* plant (Table I). Detection of *Tulasnellaceae* is consistent with the observation of *Rhizoctonia*-like hyphae emanating from mycorrhizal pelotons in *O. pauciflora* roots (Figure 1b), suggesting a symbiotic status. So far, fungi belonging to this family are the most commonly encountered fungal symbionts of orchids. Tulasnelloid fungi have been indeed found as mycorrhizal partners of orchids with different ecology, such as epiphytic orchids (Pereira et al. 2005 Pereira, O L, Kasuya, M CM, Borges, A C and de Araujo, E F. 2005. Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. *Can J Bot*, 83: 54–65. ; Suárez et al. 2006 Suárez, J P, Weiss, M, Abele, A, Garnica, S, Oberwinkler, F and Kottke, I. 2006. Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an andean cloud forest. *Mycol Res*, 110: 1257–1270.), green terrestrial forest orchids (McCormick et al. 2004 McCormick, M K, Whigham, D F and O'Neill, J. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytol*, 163: 425–438. ; Bougoure et al. 2005 Bougoure, J J, Bougoure, D S, Cairney, J WG and Dearnley, J DW. 2005. ITS-RFLP and sequence analysis of endophytes from *Acianthus*, *Caladenia* and *Pterostylis* (*Orchidaceae*) in southeastern Queensland. *Mycol Res*, 109: 452–460. ; Shefferson et al. 2005 Shefferson, R P, Weiss, M, Kull, T and Taylor, D L. 2005. High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. *Mol Ecol*, 14: 613–626. ; Bonnardeaux et al. 2007 Bonnardeaux, Y, Brunndrett, M, Batty, A, Dixon, K, Koch, J and Sivasithamparam, K. 2007. Diversity of mycorrhizal fungi of terrestrial orchids: Compatibility webs, brief encounters, lasting relationships and alien invasions. *Mycol Res*, 111: 51–61.), and green terrestrial orchids of open habitat (*Dactylorhiza majalis* (Reichenbach) P.F. Hunt et Summerhayes, in Kristiansen et al. (2001 Kristiansen, K A, Taylor, D L, Kjøller, R, Rasmussen, H N and Rosendahl, S. 2001. Identification of mycorrhizal fungi from single pelotons of *Dactylorhiza majalis* (*Orchidaceae*) using SSCP and mitochondrial ribosomal LsDNA sequences. *Mol Ecol*, 10: 2089–2093.); *Epipactis atrorubens* (Hoffmann ex Bernhardi) Besser and *Platanthera chlorantha* (Custer) Reichenbach, in Bidartondo et al. (2004 Bidartondo, M I, Burghardt, B, Gebauer, G, Bruns, T D and 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*, 271: 1799–1806.); *Orchis antropophora* (Linnaeus) Allioni, *O. mascula*, *O. militaris* Linnaeus, *O. purpurea* Hudson and *O. simia* Lamarck, in Jacquemyn et al. (2010 Jacquemyn, H, Honnay, O, Cammue, B PA, Brys, R and Lievens, B. 2010. Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis*. *Mol Ecol*, 19: 4086–4095.), Lievens et al. (2010 Lievens, B, van Kerckhove, S, Juste, A, Cammue, B PA, Honnay, O and Jacquemyn, H. 2010. From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi. *J Microbiol Methods*, 80: 76–85.), Schatz et al. (2010 Schatz, B, Geoffroy, A, Dainat, B, Bessière, J M, Buatois, B, Hossaert-McKey, M and et, al. 2010. A case study of modified interactions with symbionts in a hybrid mediterranean orchid. *Am J Bot*, 97: 1278–1288.), Vendramin et al. (2010 Vendramin, E, Gastaldo, A, Tondello, A, Baldan, B, Villani, M and Squartini, A. 2010. Identification of two fungal endophytes associated with the endangered

orchid *Orchis militaris* L. *J Microbiol Biotechnol*, 20: 630–636.); *O. purpurea*, *Ophrys fuciflora* (F.W. Schmidt) Moench, *Anacamptis laxiflora* (Lamarck) R.M. Bateman, Pridgeon & M.W. Chase and *Serapias vomeracea* (N.L. Burman) Briquet, in Girlanda et al. 2011 Girlanda, M, Segreto, R, Cafasso, D, Liebel, H T, Rodda, MErcole, E. 2011. *Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations*, Am J Bot 98: 1148–1163.). We could suppose that *O. pauciflora* has a symbiotic relationship with *Tulasnellaceae* even if this is the first study on the identification of fungal symbionts in this green orchid species and much is still to be learned about its ecology and physiology. Moreover, tulasnelloid fungi have proven difficult to characterize using standard PCR primers sets, apparently because of accelerated evolution of the nuclear ribosomal operon and consequent mutation of bases in conserved regions to which primers hybridize (Taylor et al. 2002 Taylor, D L, Bruns, T D, Leake, J R and Read, D J. 2002. "Mycorrhizal specificity and function in myco-heterotrophic plants". In *Mycorrhizal ecology. Ecol Studies* Edited by: MGA, Van der Heijden and I, Sanders. Vol. 157, 375–413.). Sequences from *O. pauciflora* closely matched fungal sequences from *O. mascula* plants collected in Belgium (Jacquemyn et al. 2010 Jacquemyn, H, Honnay, O, Cammue, B PA, Brys, R and Lievens, B. 2010. Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis*. *Mol Ecol*, 19: 4086–4095.). In the latter study, while the other investigated *Orchis* species (*O. antropophora*, *O. militaris*, *O. purpurea* and *O. simia*) exhibited low mycorrhizal specificity (individual plants associating with more than one fungus simultaneously), all the analysed *O. mascula* plants were found to be associated with a single tulasnelloid fungus. Our finding indicates a wide distribution area for this fungal symbiont.

In conclusion, our data show *O. pauciflora* is associated with several fungi colonizing its roots and some of them, such as *Tulasnellaceae,* should be mycorrhizal symbionts. The results suggest that *O. pauciflora* may not have highly specific relationships with fungal symbionts as we did not find a dominant associated *taxon*. This hypothesis fit well with data derived from recent studies that divide orchids into groups based on their photosynthetic ability: Myco-heterotrophy in many non-photosynthetic orchid species is associated with high specificity towards narrow clades of fungi; in contrast, photosynthetic mycorrhizal plants are typically generalists towards mycorrhizal fungi (Bidartondo & Read 2008 Bidartondo, M I and Read, D J. 2008. Fungal specificity bottlenecks during orchid germination and development. *Mol Ecol*, 17: 3707–3716. ; Smith & Read 2008 Smith, S E and Read, D J. 2008. *Mycorrhizal symbiosis.* , 3th ed, San Diego, CA: Academic Press.).

However, there are still doubts on molecular identification of orchid mycorrhizal symbionts (Taylor & McCormick 2008 Taylor, D L and McCormick, M K. 2008. Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. *New Phytol*, 177: 1020–1033.) and much is still to be learned about the identity of these fungi and especially about their trophic relationship with *O. pauciflora*. The use of different primer pairs (such as those developed by Taylor & McCormick (2008 Taylor, D L and McCormick, M K. 2008. Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. *New Phytol*, 177: 1020–1033.) to specifically target tulasnelloid orchid-associated fungi) will allow further characterization of the fungal symbionts of this threatened orchid species. Fungal isolation followed by symbiotic seed germination assays may help clarifying the functional roles of the root-associated fungi. Improved understanding of the identity and ecology of *O. pauciflora* mycobionts may represent a crucial aspect for the conservation of this orchid species and its habitats, considering that pasture grasslands are indicated as a priority for protection and conservation at European level, in part due to the numerous fungal species closely linked to these ecosystems (Arnolds 2001 Arnolds, E. 2001. "The future of fungi in Europe: threats, conservation and management". In *Fungal conservation. Issues and solutions*, Edited by: D, Moore, MM, Nauta, SE, Evans and M, Rotheroe. 64–80. Cambridge: Cambridge University Press.).

Acknowledgements

We thank the Estonian Science Foundation and the European Social Fund (Mobilitas Postdoctoral Research Grant MJD135) for financial support.

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