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# Analysis of fungal diversity in *Orchis tridentata* Scopoli

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## Abstract

We have assessed the identities of fungi associated with *Orchis tridentata*, an endangered orchid species growing in open woodland and poor grassland of Central and Southern Europe. Fungal diversity in ten *O. tridentata* adult individuals collected in two protected areas of Central Italy was analysed by means of morphological and molecular methods. Sequencing of the cloned ITS fungal inserts corresponding to the dominant PCR products obtained from amplification of total root DNA with ITS1F and ITS4 primers revealed a variety of fungal species occurring in *O. tridentata* roots. Among them, members of the basidiomycete families Ceratobasidiaceae, Tulasnellaceae and Hymenogastraceae were recovered, together with ascomycetes belonging to *Leptodontidium* and *Terfezia*. The implications of these results in the understanding of *O. tridentata* biology and for the conservation of this threatened orchid species are discussed.

Keywords: Ascomycetes; Basidiomycetes; Endangered species; Fungal associates; Photosynthetic orchids

## 1. Introduction

The family Orchidaceae, with about 24000 species, is the largest in the plant kingdom [ 1 ] and the vast majority of orchid taxa is well known for its rarity and is threatened in several countries throughout the world [ 2 , 3 ]. Mycorrhizae have a pivotal impact on orchid growth and metabolism in several plant life stages as well as they influence the distribution and rarity of these delicate plants, more than other important environmental factors such as edaphic conditions or pollinators limitation [ 4 ]. Indeed, orchid mycorrhizal symbioses are known to affect pollinators visitation as they modify number and size of flowers or nectar production [ 5 ]. Mycorrhizae also influence orchid tolerance to different soil conditions by conferring resistance to toxic metals [ 6 ].

All orchids need to establish a relationship with mycorrhizal fungi for seed germination and subsequent growth and development [ 7 , 8 ]. After initial development, leading to the protocorm, a non-photosynthetic stage that depends on fungi for carbohydrates, the destiny of the relationship between orchids and fungal symbionts diverges in different species. Achlorophyllous orchids are nutritionally dependent on their fungal partners throughout their life, a strategy named mycoheterotrophy [ 9 ], mixotrophic orchids are photosynthetic at the adult stage, but augment their carbon requirements via mycorrhizal fungi [ 10 ], photosynthetic orchids, that include the majority of species, become fully autotrophic at maturity, but they still

depend on their fungal associates for carbon nutrition in particular living conditions, as is usual in such cases under low light availability [ 11 ].

Although mycorrhizae are considered essential for orchid biology, little is known about the diversity of mycorrhizal fungi associated with orchids in nature, especially as far as photosynthetic terrestrial species are concerned [ 12 , 13 ]. Molecular PCR-based methods have been applied over the last few years to directly identify symbionts in orchid mycorrhizas and have improved our knowledge of the intriguing relationships between fungi and orchids [ 14 ]. Culture-dependent and morphological methods previously used for characterization of orchid mycorrhizas were often not effective in discriminating the fungal symbionts. Indeed, the most of the orchid symbionts are Rhizoctonia-like fungi [ 15 , 16 ] belonging to a variety of teleomorphic taxa ( *Ceratobasidium* , *Sebacina* and *Tulasnella* ), but sexual stages are rarely observed in nature and isolates have very rarely been induced to fruit in culture. As a consequence, morphological discrimination of teleomorphic species for the orchid-associated *Rhizoctonia* is very difficult [ 17 ]. Molecular techniques were recently used for the identification of mycorrhizal fungi in some orchids species belonging to the genus *Orchis* [ 5 , 18 - 22 ]. The identification of symbionts would be very important for the in situ and ex situ conservation of this orchid taxon that shows declining geographic ranges and population extinctions in several species [ 3 ]. In this work, we assessed the diversity of fungi associated with *Orchis tridentata* Scop. by means of morphological and molecular methods. Knowledge of the identities and roles of mycorrhizal fungi associated with *Orchis* species, such as *O. tridentata* , is of primary importance for understanding the biology of this threatened orchid genus and contributing to the conservation of these delicate plants. The primary objective of this research was to verify whether the fully photosynthetic orchid *O. tridentata* , growing in open habitats under high light conditions, was associated with mycorrhizal fungi in adulthood and, in case the study species was colonized by mycobionts, to identify its fungal associates.

## 2. Experimental Procedures

### 2.1 Study species and sampling

*Orchis tridentata* is a photosynthetic terrestrial orchid with green bluish leaves, the basal ones (3-8) lanceolate, the cauline leaves (1-3) narrower, acute, bract-like. The plant is 15-40 cm tall with a short, compact, ovoid inflorescence constituted by small, acuminate flowers. Sepals and petals are entirely lilac or pinkish purple veined; labellum is trilobed, white to pale violet, marked with purple spots [ 23 ]. *O. tridentata* grows in full sun to semi-shade poor grassland, mountain pastures, open woodlands and garrigue on calcareous soil up to 1600 m. This orchid species is widespread in Central and Southern Europe, from Pyrenees to the Caucasus and is quite rare in the west of its area [ 24 ]; it is present in all the Italian regions where the genus *Orchis* or the whole family *Orchidaceae* are generally protected [ 25 ]. *O. tridentata* was collected during the early summer of 2007 and 2008, in two protected areas of Tuscany (Central Italy). We sampled a total of ten flowering adult individuals, five in the "Monte Cetona" and five in the "Monte Penna" (specifically on Monte Rotondo) Natural Reserves, in mountain poor grasslands, on calcareous soil. Root portions were rinsed in water and scrubbed with a brush to remove the most of soil debris. They were subsequently cleaned by sonication with three cycles of 30 s each in an ultrasonic bath, in order to remove remaining soil particles and microorganisms from root surface. Some samples were immediately processed for microscopic analysis and fungal isolation. Root fragments to be used for molecular identification were frozen and stored at -80°C.

### 2.2 Morphological observations

Fresh root samples were cut in 1 cm long portions and cross sectioned to identify regions colonised by pelotons. One section per root portion was checked for the presence of fungal pelotons, that are the primary evidence of the establishment of the orchid mycorrhiza [ 6 ],

under a light microscope. Root portions with high mycorrhizal colonisation were processed immediately for fungal isolation.

## 2.3 Fungal isolation

Two or three roots per plant were surface-sterilized with consecutive washes of 1:5 sodium hypochlorite (30 s) and three rinses of sterile water. Eight 3-5 mm pieces from each root were cultured in malt extract agar (MEA) and potato dextrose agar (PDA) amended with gentamycin (40 mg/l) and/or chloramphenicol (50 µg/ml). Petri dishes were incubated at room temperature in the dark for up to two months to allow the development of slow-growing mycelia.

## 2.4 DNA extraction and ITS amplification

Total DNA from root samples was extracted following the cetyltrimethyl ammonium bromide (CTAB) procedure modified from Doyle and Doyle [ 26 ]. The polymerase chain reaction (PCR) was performed using the primers ITS1F and ITS4 [ 27 ] 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 Mg Cl<sub>2</sub>, 0.1% gelatine), 1 µl 10 mM dNTP, 1 µl of each primer (ITS1 and ITS4), 1.5 U of RED Taq DNA polymerase (Sigma) and 2.5 µl of extracted genomic DNA at the appropriate dilution. PCR amplifications were run 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. PCR products were electrophoresed in 1% agarose gel with ethidium bromide and purified with the QIAEX II Gel Extraction Kit (QIAGEN) according to the manufacturer's advice.

## 2.5 Cloning

The purified ITS fragments were cloned into pGEM-T (Promega) vectors that were used to transform XL-2 Blue ultracompetent cells (Stratagene). 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).

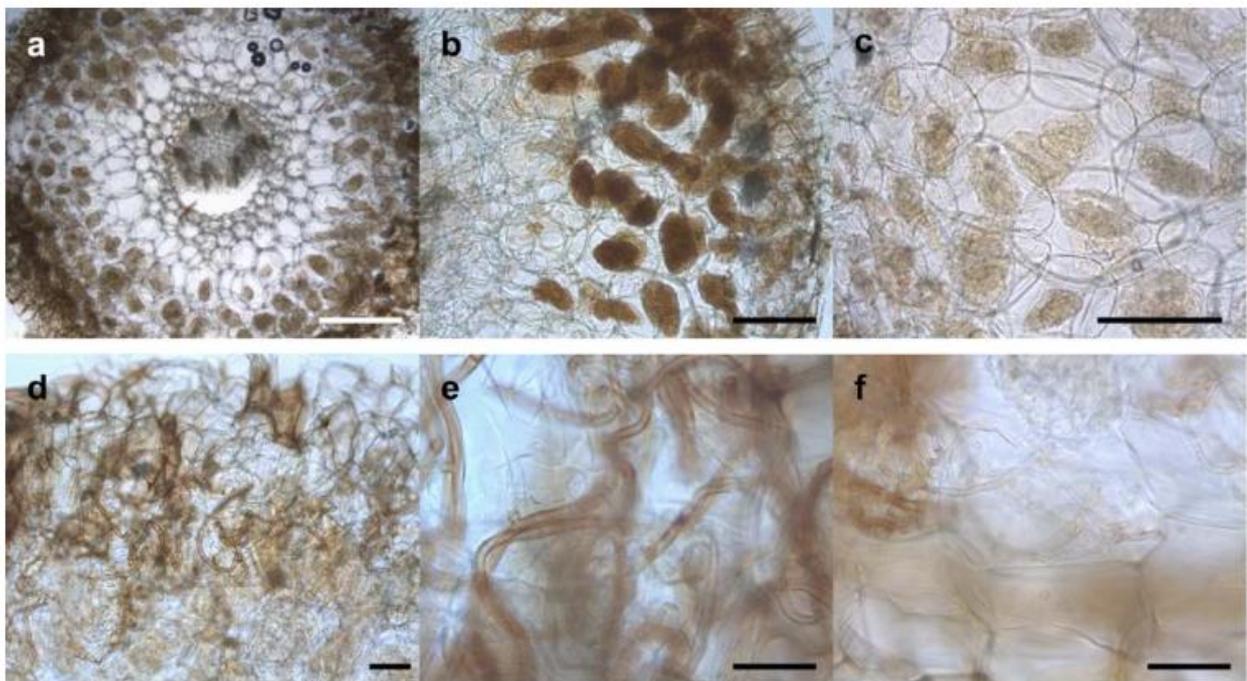
## 2.6 Sequencing of ITS region and sequence analysis

Cloned ITS inserts, representative of the PCR products initially present were purified with Plasmid Purification Kit (QIAGEN) and sequenced with the same primer pair used for amplification. Dye sequencing was performed on a ABI 310 Genetic Analyzer (Applied Biosystems). Searches for similar sequences allowing taxonomic identification were conducted using the BLASTN algorithm available through the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/index.html>).

## 3. Results

Morphological observations clearly showed fungal colonization in all *Orchis tridentata* root samples, from both study sites. Microscopy showed densely colonized root cortical cells cluttered with conspicuous fungal pelotons (Figure 1). These abundant intracellular coils were predominantly confined to the cortex, whereas the central stele appeared free from mycelium (Figure 1 a,b). Fungal hyphae had a dominant morphology and details of mycelial structures are visible in root sections (Figure 1 d,e,f). Hyphae occupied all the cortical cells and were brown, septate, unclamped and thick-walled (8-10 µm diameter). Even though most attempts of in vitro isolation were unsuccessful, one fungal endophyte, putatively symbiont, was isolated from the roots of the *O. tridentata* sample MC1. Mycelium growing in pure culture could be assigned to the form genus *Rhizoctonia* on the ground of morphological features. Many non-mycorrhizal endophytes, such as *Fusarium* species, were isolated together with this

Rhizoctonia -like taxon. The identification of *O. tridentata* associated fungi by molecular techniques was successful for all the collected samples, that could be amplified by PCR. Sequences obtained from *O. tridentata* root tissue with the fungal specific primer pair ITS1F/ITS4 revealed that a dominant fungal symbiont belonging to the Ceratobasidiaceae could be associated with this orchid species, irrespective of the site of origin (Table 1). Of the 10 analysed *O. tridentata* individuals, 6 were associated with fungi showing close similarity to symbionts ascribed to the family Ceratobasidiaceae: The closest match for the sequences amplified from sample MC1 (98% over 885 bp) and MC2 (97% over 854 bp) was with Ceratobasidiaceae found in roots of *Cephalanthera longifolia* (L.) Fritsch (DQ182418); the sequences obtained from samples MR1, MR2 and MR3 corresponded to a fungus with a similarity of 91% (over 880-891 bp) to *Ceratobasidium* sp. from *Fragaria ananassa* Duch in Israel (DQ102402); sequence from sample MR3 (clone e) shared 91% similarity with sequences from *C. cornigerum* (Bourdot) D.P. Rogers; the main root fungal endophytes of sample MR5 were fungi with identity to *Ceratobasidium* sp. (clone a) and Ceratobasidiaceae from *Epipactis gigantea* Douglas ex Hooker (clone b). Fungal associates with orchid sample MR4 shared 96-97% similarity with Hymenogastraceae from *C. damasonium* (Mill.) Druce (clone a) and *E. microphylla* (Ehrh.) Swartz (clone c). As regards sequences found in sample MC4, an identity of 96% was recorded with *Epulorhiza* sp. (anamorphic *Tulasnella*) found within *Cypripedium macranthos* Sw. var. *rubense* (Kudô) Miyabe et Kudô roots and 98% identity with an uncultured fungus found in *Ophrys sphegodes* Mill. in Hungary (clone b); clone c from the same sample, shared 97% of similarity with *Epulorhiza* sp. (EU218890). Sequences from sample MC3 matched to *Leptodontidium* sp. (DQ148411) isolated from the roots of an alpine plant *Saussurea involucrata* Kar. et Kir. ex Maxim and to an unidentified fungus from *Pterostylis nutans* R. Br. whereas the closest match for sample MC5 was with mycorrhizal hypogeous ascomycetes *Terfezia* sp. (DQ061109).



**Figure 1.** *O. tridentata* mycorrhizal roots: (a-c) Cross sections showing fungal pelotons in orchid root cells. (d-f) Details of fungal hyphae forming coils. Scale bars: 500  $\mu$ m (a), 200  $\mu$ m (b, c), 100  $\mu$ m (d, e, f).

Sample	Clone	GenBank	Best BLAST match(es)	Accession	Overlap	% match
			accession code	code	length	
MC1	isolated	JN683860	<i>Ceratobasidiaceae</i> (from <i>Cephalanthera longifolia</i> )	DQ182418	885	98%
			<i>Rhizoctonia</i> sp. (from <i>Teucrium dunense</i> )	DQ865086	795	96%
MC2	a	JN683842	<i>Fusarium</i> sp.	DQ446211	989	99%
	e	JN683843	Basidiomycete (from mixed hardwood soil)	AY970109	872	93%
MC3	c	JN683844	<i>Ceratobasidiaceae</i> (from <i>Cephalanthera longifolia</i> )	DQ182418	854	97%
			Fungus (from <i>Pterostylis nutans</i> )	EF090490	660	94%
			<i>Leptodontidium</i> sp. (from <i>Saussurea involucreta</i> )	DQ148411	658	94%
MC4	b	JN683847	<i>Fusarium oxysporum</i>	DQ635184	992	99%
			<i>Fusarium</i> sp.	AJ279478	1014	100%
			<i>Epulorhiza</i> sp. (from <i>Cypripedium macranthos</i> var. <i>rubense</i> )	AB369932	1077	96%
MC5	a	JN683849	Fungus (host= <i>Ophrys sphegodes</i> )	AM697902	1048	99%
			<i>Epulorhiza</i> sp.	EU218890	267	97%
			<i>Candida</i> sp.	DQ911447	174	84%
MR1	d	JN683850	<i>Terfezia</i> sp.	DQ061109	324	83%
			Basidiomycete (from sandy loam soil)	FM866373	856	93%
MR2	a	JN683853	<i>Ceratobasidium</i> sp. (from <i>Fragaria</i> sp.)	DQ102402	880	91%
			Basidiomycete (from sandy loam soil)	FM866373	959	95%
MR3	a	JN683854	<i>Ceratobasidium</i> sp. (from <i>Fragaria</i> sp.)	DQ102402	883	91%
			Basidiomycete (from sandy loam soil)	FM866373	959	95%
			<i>Ceratobasidium</i> sp. (from <i>Fragaria</i> sp.)	DQ102402	891	91%
MR4	e	JN683855	<i>Ceratobasidium cornigerum</i>	EU273525	872	91%
			<i>Hymenogasteraceae</i> (from <i>Cephalanthera damasonium</i> )	AY634136	1195	97%
			<i>Hymenogaster citrinus</i>	EU784360	1110	95%
MR5	c	JN683857	<i>Hymenogasteraceae</i> (from <i>Epipactis microphylla</i> )	AY351628	1055	96%
			<i>Ceratobasidium</i> sp.	EU218894	874	92%
MR5	b	JN683859	<i>Ceratobasidiaceae</i> (from <i>Epipactis gigantea</i> )	AY634119	769	99%

**Table 1.** Closest matches from BLAST searches of fungal sequences amplified from *O. tridentata* roots collected in Monte Cetona (samples MC1-MC5) and Monte Rotondo (samples MR1-MR5). Deposited accession code, accession code for the closest GenBank matches, sequence identity (% match) and overlap of each match are reported.

## 4. Discussion

This study has analyzed, for the first time, the fungal diversity associated with roots of *Orchis tridentata*, using both morphological culture-dependent and PCR-based methods. A high level of fungal colonization was clearly visible in *O. tridentata* root by morphological observations. Roots of all orchid samples contained fungal pelotons in their cortical cells, showing the typical features of orchid mycorrhiza, as described by several authors [6, 7, 28, 29]. This observation is in agreement with recent works that show the presence of mycorrhizal symbiosis in several green orchids. Cameron et al. demonstrated for the first time mutualism in *Goodyera repens* (L.) R. Brown mycorrhiza, with carbon passing from the orchid to the fungus, in return for mineral nutrient passing from the fungus to the orchid [30]. More recently, Liebel et al. and Girlanda et al. demonstrated that some photosynthetic orchid species belonging to the genus *Orchis* depend on fungi as a source of carbon, using natural abundance analysis of  $^{13}\text{C}$  [19, 31]. Only one *Rhizoctonia*-like fungus, characterized by right-angle branching, a constriction at the branch point, and a septum in the branch hypha near its point of origin, could be isolated from *O. tridentata* analysed roots (sample MC1). The ITS region of this strain showed an identity of 98% with *Ceratobasidiaceae* found in *Cephalanthera longifolia* [28], similarly to sequences obtained from total DNA extracted from roots of other orchid sample (MC2). In the

previous work on *C. longifolia* [ 28 ] two *Ceratobasidium* were found, but they were not reported to form pelotons. For this reason, the authors were not sure about the symbiotic status of these fungi as some *Ceratobasidiaceae* are plant parasites or simply grow endophytically, without being mycorrhizal [ 16 , 32 , 33 ]. Anyway, the majority of fungi that have been recorded as orchid mycorrhizal symbionts belong to the anamorphic form-genus *Rhizoctonia* and most of the orchid-associated *Rhizoctonia* species belong to the *Ceratobasidiaceae* , *Sebacinaceae* and *Tulasnellaceae* [ 7 , 15 , 17 , 34 ]. Even if morphology is the first choice for species discrimination in eukaryotes, in most fungi where complex fruit bodies are absent, such as the three families of orchid symbionts above mentioned, morphological species delimitation is difficult. Moreover, symbionts of a number of orchids are difficult or impossible to cultivate axenically [ 9 , 35 , 36 ], as confirmed from the results obtained through the in vitro isolation attempts performed in our work. As a consequence, molecular methods, based on PCR, used to directly identify fungi within *O. tridentata* roots, have been essential to characterize fungal diversity in this orchid. A large number of *Ceratobasidiaceae* were found after cloning of the PCR amplification products, while in vitro isolation failed to reveal the presence of these fungi in the majority of samples. Sequences from samples MR1, MR2 and MR3 shared similarity with *Ceratobasidium* sequences from *Fragaria ananassa* plants collected in Israel [ 37 ]. The closest match for the sequences amplified from sample MR5 was with *Ceratobasidiaceae* found in roots of *Epipactis gigantea* collected in California and Oregon [ 38 ]. These typical rhizoctonia-forming orchid mycorrhizal fungi dominated the roots of the stream-dwelling *E. gigantea* . As ceratobasidioid fungi have been found as symbionts or putatively symbionts of several orchid species, such as *C. damasonium* [ 10 ], *C. longifolia* [ 28 ], *Cypripedium* spp. [ 39 ], *Dactylorhiza majalis* (Reichenbach) P.F. Hunt et Summerhayes, *E. gigantea* , *E. helleborine* (L.) Crantz, *E. palustris* (L.) Crantz and *Platanthera chlorantha* (Cust.) Rchb. [ 38 ], *Ionopsis utricularioides* (Sw.) Lindl. [ 40 ], *Pterostylis nutans* [ 41 ], *Tolumnia variegata* (Sw.) Braem [ 42 ], the ceratobasidioid fungi reported in this study could have the same trophic relationship with *O. tridentata* . Roots of sample MR4 were colonised by fungi with similarity to *Hymenogastraceae* found in *C. damasonium* [ 38 ] and *E. microphylla* [ 43 ]. These basidiomycetes, that usually produce hypogeous basidiomata during the sexual phase, belong to a family that mostly contains obligate ecto-mycorrhizal fungi. Sequences amplified from sample MC4 matched to *Epulorhiza* sp. from juvenile plant of *C. macranthos* var. *rubense* [ 44 ]. All the fungi isolated from *C. macranthos* grew well on nutrition-poor media, suggesting that the nutritional demands of these isolates differ from those of typical saprophytic *Rhizoctonia* growing well on common nutrition-rich media. After all, *Epulorhiza* belongs to *Tulasnellaceae* that are well-known to associate symbiotically with several orchid taxa , including some *Orchis* species [ 5 , 18 , 19 , 45 ]. Actually, some papers have shown that tulasnelloid fungi are dominant in mycorrhizal associations with *Orchis* spp., for instance in *O. militaris* L. [ 6 , 20 - 22 ] that is closely related to *O. tridentata* (probably a monophyletic group, with five species derived from a common ancestor close to *O. militaris* and isolated by chromosome reorganisation from *O. tridentata* ) [ 24 ]. The small number of sequences related to *Tulasnella* amplified from *O. tridentata* could be influenced by primers used in fungal ITS regions amplification. Indeed, the primer pair ITS1F/ITS4 is very effective for the amplification of ITS region from essentially all *Eumycota* , but it does not effectively amplify some core species within the *Tulasnellaceae* [ 17 ]. Sequences of ascomycetes were obtained from samples MC3 and MC5. Ascomycetous fungi from sample MC3 shared 94% similarity with *Leptodontidium* sp. found in *Saussurea involucreta* [ 46 ]. Resynthesis study was conducted to clarify the relationship between the dark-septate root endophyte and *S. involucreta* . Seedlings of the host plant, inoculated with endophytes appeared healthy with no external symptoms of disease and their dry weights increased significantly compared to controls. Fungi belonging to the genus *Leptodontidium* are common fungal endophytes that have been found in many orchid species worldwide [ 10 , 28 , 38 , 41 , 47 ]. Physiological studies are needed in order to clarify the role of these fungi in orchids. Sequences from sample MC3 also shared 94% similarity with an uncultured fungus from *P. nutans* sampled in Australia [ 41 ], but there was not evidence for a symbiotic association between the unidentified fungus and the terrestrial orchid. The closest match for

sequences amplified from sample MC5 was with *Terfezia* sp. Ascomycetes belonging to this genus, known as “desert truffles”, are mycorrhizal fungi forming hypogeous fruit bodies, associated with shrubs such as *Helianthemum* sp. [ 48 ] in semiarid condition. We cannot fully exclude that they form orchid mycorrhizae. Indeed, although basidiomycetes are the most important group of orchid fungi, several authors have shown through ITS sequencing that ascomycetes can form mycorrhizal association with orchid species [ 10 , 43 ]. In this study, we have demonstrated that *O. tridentata* associates with fungi heavily colonizing the roots. Among them, Ceratobasidiaceae could be dominant, as suggested by PCR amplification and cloning of fungal rDNA, but further studies, based on the use of different primers recently developed to specifically target tulasnelloid fungi [ 17 ], are needed to deeply characterize the diversity of fungal symbionts in *O. tridentata*. Other fungi found in the roots of the study species, such as Hymenogastraceae, *Epulorhiza* sp. and some ascomycetous fungi, could be also mycorrhizal partners. Further ecological and physiological studies will be necessary to flesh out whether these endophytes are truly mycorrhizal. The biology of *O. tridentata* cannot be fully understood without an in-depth analysis of its fungal symbionts. The results of this work could have direct implications for the conservation of this threatened orchid. Indeed, management measures for the conservation and restoration of orchid populations require knowledge of the host specificity, distribution and abundance of fungal symbionts in natural habitats [ 49 ]. For instance, the presence of appropriate fungal species is needed for long-term success of traslocation efforts [ 20 ]. Transplantation into the nature of orchid seedlings associated with their mycorrhizal partners and in situ land inoculation with fungal symbionts could be of primary importance for conservation efforts [ 22 , 50 ].

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