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Fungal diversity and specificity in *Cephalanthera damasonium* and *C. longifolia* (Orchidaceae) mycorrhizas

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Running title: **Mycorrhizal associations in *Cephalanthera* species**

Abstract

Orchids depend on mycorrhizal fungi for their nutrition, at least in the early stages of their growth and development and in many cases throughout the life. In spite of the increasing number of studies describing fungal diversity in orchids, there is still more to be learnt about the identity of fungal partners and specificity in orchid mycorrhizal associations. We investigated the fungal communities associated with the roots of *Cephalanthera damasonium* and *C. longifolia* adult plants, using morphological methods and fungal ITS-DNA PCR amplification, cloning and sequencing. A range of fungi belonging to Basidiomycota and Ascomycota was uncovered in the roots of the two investigated orchid species, showing a low degree of mycorrhizal specificity. At least 11 fungal taxa, including *Cenococcum geophilum*, *Ceratobasidium* sp., *Exophiala salmonis*, Hymenogastraceae, and Sebacinaceae colonized *C. damasonium* roots, while about 9 fungal types, such as *Bjerkandera adusta*, *Phlebia acerina*, Sebacinaceae, *Tetracladium* sp., and *Tomentella* sp. associated with *C. longifolia*. Phylogenetic and statistical analyses indicated significant differences in the fungal communities associated with the two studied *Cephalanthera* species, as well as distinct mycorrhizal partners associated with each orchid plant. Our results strongly suggest that both *C. damasonium* and *C. longifolia* are generalist in their mycorrhizal associations.

Keywords Ascomycetes; basidiomycetes; fungal symbionts; mycorrhizal specificity; Orchidaceae; orchid mycorrhiza.

1 Introduction

Orchid mycorrhiza is recognized as a distinct type of endomycorrhiza where individual fungal hyphae cross the orchid cell wall by simple penetration and form pelotons (intracellular coils) in the cortical tissues of protocorms, roots, tubers and rhizomes, in close connection with the invaginated plant plasmalemma (Rasmussen, 1995; Smith & Read, 2008).

As well as from the anatomical point of view, orchid-fungus associations are unique in their physiological and nutritional aspects. Indeed, the great majority of plants associate with soil fungi that usually exchange mineral nutrients for plant carbon (Smith & Read, 1997), whereas orchids are dependent upon mycorrhizal fungi for the provision of carbohydrates, at least in the early development. Thus, the polarity of carbon movement in orchid mycorrhiza, occurring from the fungal partner to the plant, is inverted as compared with the general condition existing in the other mycorrhizal types (Smith, 1967). Moreover, the relationship between orchids and symbiotic fungi is considered a one-way system in favour of the plant (Merckx et al., 2009; Rasmussen & Rasmussen, 2009), instead of a mutualistic association as in the arbuscular mycorrhizas and ectomycorrhizas, with a very few exceptions (Cameron et al., 2006).

This fungus-dependent life style, called mycoheterotrophy (Leake, 1994; Bidartondo & Read, 2008), is a necessity for all orchid species, during the achlorophyllous protocorm stage. At the adult stage, most orchids develop a photoassimilating apparatus and become autotrophic, although they continue to rely on their fungal associates for water and mineral salts (Liebel et al., 2010; Girlanda et al., 2011; Jacquemyn et al., 2011a). On the contrary, some orchid species, growing in forest habitats, remain dependent on fungal sugars even at maturity. Based on their carbon nutrition, these forest species can be divided into two physiological types (Dearnaley & Bougoure, 2010). Fully mycoheterotrophic orchids are achlorophyllous and completely depend on mycorrhizal fungi for their carbon, throughout their life cycle (Bidartondo et al., 2004; Roy et al., 2009). Partially mycoheterotrophic orchids, are green and perform photosynthetic carbon fixation, but also obtain additional carbon from their mycobionts with varying extents, depending on light availability (Preiss et al., 2010). All studied achlorophyllous orchids associate specifically with narrow phylogenetic fungal groups (Taylor et al., 2002; Selosse & Roy, 2009), while partially mycoheterotrophic orchids, using this newly discovered, dual nutritional strategy, also called mixotrophy (Selosse et al., 2004), show a variable level of mycorrhizal specificity (Julou et al., 2005; Girlanda et al., 2006).

The genus *Cephalanthera* Rich. mainly belongs to the latter trophic group. This orchid taxon comprises a total of 15 species with a mostly Eurasian distribution (Delforge, 2006). Only one species *C. austiniae* (A. Gray) A. Heller, characterised by permanent loss of chlorophyll and strong leaf reduction, has been found in North America (Colemann, 1995). Seven species are the European representatives of the genus (Delforge, 2006). Among them, *C. damasonium* (Mill.) Druce and *C. longifolia* (L.) Fritsch have been found to obtain carbon both through photoassimilation and from their fungal root associates (Gebauer & Mayer, 2003; Julou et al., 2005; Abadie et al., 2006), thus indicating that these species constitute typical examples of mixotrophic orchids. These two phylogenetically close orchid species (Bateman et al., 2004) are rhizomatous plants with perennial roots, summer-green and overwinter underground, growing in broadleaved, coniferous, and mixed forests, often colonizing shadowy forest edge, between shrubs and trees (Rossi, 2002; Delforge, 2006). Previous studies have focused on the identification of their mycorrhizal fungi. Fungal diversity in *C. damasonium* has been assessed in three different works carried out in France, Germany, and Hungary showing that this orchid associates with a variety of both basidiomycetes and ascomycetes (Bidartondo et al., 2004; Julou et al., 2005; Illyes et al., 2010), while Abadie et al. (2006) and Liebel et al. (2010) have respectively demonstrated that *C. longifolia* associates with several fungal taxa, such as Thelephoraceae and Sebacinaceae, in Estonia, whereas *Hebeloma*, *Russula*, and *Tomentella* are the main mycorrhizal fungi in the same orchid species collected in Italy. All these studies have reported on mycorrhizal diversity in only one of the two *Cephalanthera* species, analysing a small number of samples (four *C. damasonium* individuals in Julou et al. (2005) and Bidartondo et al. (2004), only two *C. longifolia* adult plants in Liebel et al. (2010)), from a single sampling site. Very different environmental conditions have been described for the sampling sites investigated in the above mentioned works. These conditions, together with the geographical distance between studied areas, may influence the fungal community in each site (Gale et al., 2010; McCormick & Jacquemyn, 2014), and hence make difficult to understand whether the mycorrhizal diversity up to now reported in *C. damasonium* and *C. longifolia* reflects the ecological preference of the two orchid species or is mainly a consequence of local factors. Therefore, the degree of mycorrhizal specificity in *C. damasonium* and *C. longifolia* is still uncertain. The extent of specificity towards fungi shown by these two orchid species has been previously investigated just in a single work, carried out by Bidartondo & Read (2008). Specificity of orchid-fungus relationships has an important role in orchid biology and conservation (Jacquemyn et al., 2010; Phillips et al., 2011).

Orchid species that establish generalist associations with multiple fungal symbionts may be more adaptive under changing environmental condition or in fragmented habitats than orchids that associate with only a few fungal partners (Swarts & Dixon, 2009). On the other hand, specialist orchid taxa may increase their fitness in specific habitats or under a narrow range of environmental conditions by selecting a specific, highly ecologically competent fungal symbiont (Bonnardeaux et al., 2007), but this high degree of mycorrhizal specificity may be linked to orchid rarity when the fungal partner has a limited distribution (Swarts et al., 2010).

In this study, we analysed fungal diversity and specificity in *C. damasonium* and *C. longifolia* mycorrhizal associations, using morphological and molecular methods. Orchid roots were collected from different sites, both total root DNA and DNA from isolated fungi were extracted and fungal ITS regions were PCR amplified, cloned and sequenced.

First, we investigated for each species whether an association with different fungal partners might occur in different sites. Second, we assessed specificity between the two orchids and their mycobionts by analysing the phylogenetic distance of their fungal associates. Finally, we tested mycorrhizal specificity for the studied orchid species by determining whether they associate with the same fungi when they grow in sympatric populations or they maintain distinct mycorrhizal diversity.

2 Materials and methods

2.1 Study sites and sampling

Cephalanthera damasonium and *C. longifolia* plants were sampled from nine forest edge sites located in three geographically distinct protected areas in Tuscany (Central Italy), “Monte Cetona”, “Monte Penna” (specifically on “Monte Rotondo”), and “Cornate di Gerfalco” Natural Reserves, characterized by the presence of dry calcareous meadow habitats with *Bromus erectus* Hudson dominating among herbaceous plants, surrounded by forests with various broad-lived species such as *Fagus sylvatica* L., *Quercus cerris* L., *Ostrya carpinifolia* Scop., and *Acer* sp.pl. mixed with conifers such as *Pinus nigra* Arnold. The study sites lie on the top of mountains, at an altitude ranging from 755 to 1026 m a.s.l.

The two analysed orchid species were growing together only in one (“Monte Rotondo”) out of the three investigated areas.

During the early summer in 2007 and 2008, at orchid flowering, root samples were collected from a total of eleven individuals for each studied *Cephalanthera* species (six *C. longifolia* individuals in "Cornate di Gerfalco", six *C. damasonium* in "Monte Cetona", and five plants for each species in "Monte Rotondo"). All roots were washed under running water, carefully brushed, and treated in an ultrasonic bath (three cycles of 30 s each) in order to remove soil debris and to minimize the detection of microorganisms adhering to the root surface. Fresh root samples were cut into 1 cm long pieces and their mycorrhizal morphology was observed under a light microscope, on thin cross sections. Root portions exhibiting high fungal colonization were partly processed for fungal isolation and partly frozen in liquid nitrogen and stored at -80°C for molecular analysis.

2.2 Fungal isolation

Fresh orchid root fragments were immediately processed after sampling. Five or six roots per plant were surface-sterilized with consecutive washes of 5% sodium hypochlorite (30 s) and three rinses of sterile water. Ten 3-5 mm long 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.

2.3 Molecular identification of orchid root fungi

Both total DNA from orchid root samples and DNA from isolated fungi were extracted using the cetyltrimethyl ammonium bromide (CTAB) procedure modified from Doyle & Doyle (1990).

Fungal ITS regions were PCR amplified using the primer pair ITS1F/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 $MgCl_2$, 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. 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 that were used to transform XL-2 Blue ultracompetent cells (Stratagene). After transformation, twenty white colonies per plant 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 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 MacOsX from Genes Codes (Ann Arbor, Michigan). 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 allowing taxonomic identification. DNA sequences were deposited in GenBank (Accession Numbers KT1222767–KT1222789).

Phylogenetic analysis was conducted using the software Mega v. 5.0 (Tamura et al., 2011). Sequences were aligned with Clustal X v. 2.0 (Larkin et al., 2007). Both a neighbour-joining tree and a maximum likelihood tree against selected database sequences were constructed using Kimura 2-parameter distances, with bootstrapping of 1000 replicates (Felsenstein, 1985). A *Geastrum* species (*G. schmidelii* Vittad.) was used as outgroup to root the tree, following Weiss et al. (2004).

2.4 Statistical analysis

In order to test the hypothesis that the two orchid species differ in terms of fungal communities, we calculated a community dissimilarity matrix using the Jaccard index on the individual plant-fungal taxon binary matrix and applied a PERMANOVA test to this matrix (McArdle & Anderson, 2001; Anderson, 2001; Legendre & Legendre, 1998; Oksanen et al., 2011). PERMANOVA is a non-parametric equivalent of MANOVA and solves all the issues that may affect the application of MANOVA to ecological matrices, especially binary ones. The main focus of the analysis was the factor "Orchid species": to avoid spurious effects due to sampling sites and spatial positions of samples, we tested for the main factor after statistically removing the effects of all other confounding factors. In any

case, factors such as “sampling site” did not have statistically significant effects on the fungal assemblage.

We compared fungal taxa richness between different orchid species and sites using rarefaction curves to “standardize” comparisons. We used various richness estimators (only Chao estimator is reported) to generate sample-based rarefaction curves of species richness and associated 95% CI.

All analyses were conducted with R version 3.0.2 (R Development Core Team, 2011) and the vegan package (Oksanen et al., 2011).

3 Results

3.1 Microscopical features of Cephalanthera mycorrhizal roots and fungal isolation

All *Cephalanthera* individuals collected in the three studied areas were dissected and examined. Microscopical observation clearly showed that both *C. damasonium* and *C. longifolia* roots were extensively colonized by fungi forming pelotons, dense intracellular hyphal coils, predominantly confined to the cortical cells and just marginally approaching the central stele (Fig. 1a, b, c). Fungal hyphae emanating from pelotons mostly appeared to be dark, septate and clamped, measuring 10-12 μm in diameter (Fig. 1d). Given the paucity of distinctive morphological characters, taxonomic delimitation of the orchid root associated fungi, based on microscopy, was extremely limited. These mycobionts were subsequently identified using molecular taxonomy.

Fungal *in vitro* isolation was in most cases unsuccessful or just yielded endophytic ascomycetous fungi, such as *Fusarium* strains, playing an ambiguous role in orchid-fungus interactions (Tondello et al., 2012; Pecoraro et al., 2015). However, mycelia that could be morphologically assigned to basidiomycetes by the presence of clamp connections were isolated from two *C. longifolia* plants, MR8 and CG1, respectively collected in “Monte Rotondo” and “Cornate di Gerfalco”. Ribosomal gene sequence analysis of these isolated fungi was further performed in order to assess their identity.

3.2 Molecular assessment of fungal diversity in C. damasonium and C. longifolia

Molecular approach revealed several fungal taxa colonizing *C. damasonium* and *C. longifolia* roots. Total DNA was extracted both from root tissues and from fungal cultures. PCR products with the ITS1F/ITS4 primer combination were obtained from 8 out of 11 analysed plants for each orchid

species. Fungal ITS sequencing following direct amplification of total orchid root DNA indicated that the two analysed orchid species were mostly associated with different fungal types (Table 1), even when sampled in sympatric populations, with only one exception represented by *Sebacina* species found both in *C. damasonium* (samples MC1 and MC4) and *C. longifolia* (sample CG4) from different areas. *Sebacina* sequences from the investigated orchids could be aligned with sequences from a variety of orchid and non-orchid plant species, as well as from fungal strains and fruitbodies. Both the neighbour-joining and the maximum likelihood trees showed that the *Sebacina*-like fungi identified in this work are phylogenetically close to other Sebacinaceae including *Sebacina incrustans* (Pers.) Tul. & C. Tul. and an uncultured ectomycorrhizal fungus associated with *Tuber magnatum* Picco in a natural truffle-ground from Italy (AJ879657), that formed a clade with the sequence from *C. longifolia* sample CG4, while Sebacinaceae sequences amplified from *C. damasonium* sample MC4 (clones a and b) and MC1 (clone e) respectively clustered with a sebacinoid fungus previously found in ectomycorrhizal root tip from *Tilia* sp. in Austria (AF509964) and with a mycobiont (AF440653) from roots of *Neottia nidus-avis* (L.) L.C. Rich. collected in France (100% bootstrap support, Figs. 2, 3).

As regards other fungal taxa collected from the roots of the studied orchids, results showed that *C. damasonium* associated with fungi belonging to Hymenogastraceae family, both in “Monte Cetona” and “Monte Rotondo” sampling areas (Table 1). Sequence from *C. damasonium* sample MC3 matched instead a sequence from *Ceratobasidium* sp. found in *Fragaria ananassa* Duch. The closest match for sample MC1 clone a was with *Cenococcum geophilum* Fr. from *F. sylvatica* in France, whereas clone d from sample MC4 shared 96% (over 881 bp) of similarity with an ascomycete detected on ectomycorrhizal root tips from an oak-dominated tropical montane cloud forest in Mexico. In “Monte Rotondo” Natural Reserve, the main fungal root associate of *C. damasonium* sample MR2 was a fungus with identity to Pezizomycetes found in *C. damasonium* roots in France.

By contrast, *C. longifolia* was found to be associated with *Tomentella* species in “Monte Rotondo” (Table 1). ITS sequencing of DNA extracted from basidiomycetous isolates obtained from *C. longifolia* showed that the studied orchid was associated with fungi belonging to Meruliaceae, *Bjerkandera adusta* (Willd.) P. Karst. from sample MR8 and *Phlebia acerina* Peck from sample CG1, in the two investigated areas.

Ascomycetes such as *Davidiella macrospora* (Kleb.) Crous & U. Braun, *Exophiala salmonis* J.W. Carmich., *Neonectria radicicola* (Gerlach & L. Nilsson) Mantiri & Samuels, *Tetracladium* sp. and *Fusarium* sp. were sporadically found in the two analysed orchid species.

Statistical results

There was not statistical difference between the fungal communities associated with the two orchid species. This negative result is due to the fact that each and every individual plant possess one or two fungal associates but the identity of the fungi changes from plant to plant, and so between the two orchid species: there is not a set of fungi uniquely associated with an orchid species. Overall, this result implies a thorough fungal compositional turnover from plant to plant and high number of fungal taxa retrieved on a collection of plants belonging to the same orchid species, with no difference in richness between orchid species. For example, the estimated mean (±95% CI) number of fungal taxa was 26 ± 14 for *C. damasonium* and 30 ± 22 for *C. longifolia*.

4 Discussion

The accurate analysis of fungal diversity associated with *C. damasonium* and *C. longifolia* performed in the present work, using fungal isolation, microscopy, and DNA sequencing, uncovered a range of fungi in the roots of the investigated orchids.

The first evidence of the establishment of orchid-fungus associations in the studied orchid species was provided by microscopic observations, showing abundant pelotons in the sampled root cells, which represent the main morphological aspect of orchid mycorrhiza (Rasmussen, 1995; Kristiansen et al., 2001).

In spite of the poor success in the isolation of fungal associates from the analysed *Cephalanthera* plant roots, culture-dependent methods complemented PCR-based approach, as the basidiomycetes isolated from *C. longifolia* yielded ITS sequences that were not amplified using direct total orchid root DNA amplification. These fungal isolates from samples MR8 and CG1 respectively showed close identity to *B. adusta* and *P. acerina*, two species of wood-decaying fungi, in the Meruliaceae family, that colonize both hardwood and conifer wood, and are capable of degrading a variety of substrates by secreting specific enzymes (Nakasone & Sytsma, 1993; Romero et al., 2007). Wood-inhabiting fungal species have been previously identified in orchid mycorrhizal associations.

Erythromyces crocicreas (Berk. & Broome) Hjortstam & Ryvarden has been found to support seed germination and to be effective for further development of the orchid *Galeola altissima* (Bl.) Reichb. f. (Umata, 1995), while *Armillaria mellea* (Vahl) P. Kumm. has been shown to associate with *Gastrodia elata* Blume (Kusano, 1911; Kikuchi et al., 2008). *Resinicium* sp., that is a member of Meruliaceae, has been identified by Martos et al., (2009) in *Gastrodia similis* Bosser mycorrhizal roots, using molecular methods. Our findings of Meruliaceae fungi in the roots of *C. longifolia* could support the hypothesis of a trophic relationship between the studied orchid and wood-inhabiting fungal taxa, although further analyses are needed to verify this hypothesis.

A range of fungi was identified through fungal ITS sequencing from amplified total orchid root DNA. Neither *C. damasonium* nor *C. longifolia* were associated with a dominant fungal taxon, thus showing a low level of mycorrhizal specificity that is consistent with their mixotrophic life style (Selosse et al., 2004; Dearnaley et al., 2012). To date, specificity in associations between *Cephalanthera* species and fungal symbionts has been controversial. Previous studies have been mostly based on a very limited number of *Cephalanthera* plant samples collected in a single site. As a consequence, results obtained from these studies could not provide a clear picture of fungal diversity associated with *C. damasonium* and *C. longifolia*, and therefore, exhaustive information on their mycorrhizal specificity has been hardly available. Bidartondo et al. (2004) and Liebel et al. (2010) have found low degree of mycorrhizal specificity in *C. damasonium* and *C. longifolia* respectively, the fungal associates being *Cortinarius*, *Hymenogaster*, *Inocybe*, *Thelephora*, and *Tomentella* in *C. damasonium*, while *C. longifolia* has been found to associate with *Hebeloma*, *Russula*, and *Tomentella*. On the contrary, Abadie et al. (2006) have shown a very specific association between *C. longifolia* and Thelephoraceae considering that all ITS, in a typing of fungal ITS on 60 orchid root pieces (from 7 plants) harbouring pelotons, were from thelephoroid fungi, with the exception of two root pieces exhibiting ITS from a Sebacinaceae and an ascomycete belonging to *Wilcoxina*. Interesting information about mycorrhizal associations in *C. damasonium* has been provided by Julou et al. (2005). These authors have amplified a number of asco- and basidiomycetes sequences from 4 *C. damasonium* samples collected from a single population in France, using ITS sequencing. Among these sequenced fungi, Thelephoraceae, Cortinariaceae (including *Hymenogaster*), and Pezizomycetes (including Helotiales, Pezizales, *Tuber*, *Phialophora*, and *Leptodontidium*) were over-represented. One *Sebacina* and one *Ceratobasidium* sequences were also amplified from the same orchid plants. Julou and co-

authors, in the analysis of data collected during their investigation, have proposed a quite specific mycorrhizal association between *C. damasonium*, Thelephoraceae and Cortinariaceae, but at the same time, they have not excluded a symbiotic role for the other fungal taxa identified from the studied orchid roots. The only one previous work, in which *C. damasonium* and *C. longifolia* have been investigated together, for their mycorrhizal associations, has reported on a high degree of specificity in the relationship between the two investigated orchids and Thelephoraceae fungi during the seedling stage, while mycorrhizal specificity was lower in different plant life stages (Bidartondo & Read, 2008). Indeed, these authors have found seedlings of both *Cephalanthera* species to associate with only a subset of the thelephoroid fungi capable of stimulating seed germination, and to lack completely Cortinariaceae and Sebacinaceae that have been collected from germinating seeds and mature plants. The present study, based on the analysis of 22 orchid samples from three geographically distinct protected areas to allow statistical validation, revealed at least 11 fungal taxa colonizing *C. damasonium* roots, while about 9 endophytic fungal types were found to associate with *C. longifolia* (Table 1). Such a large diversity of fungal associates strongly suggests that both *C. damasonium* and *C. longifolia* are generalist in their mycorrhizal associations. Statistical analyses clearly show distinct fungal communities associated with each and every orchid plant individual, irrespective of the orchid species and the site of origin.

We mainly found different fungal associates in the two investigated orchids (Table 1). *Sebacina* represents the only putative mycorrhizal fungus that was associated with both *C. damasonium* (two plants on “Monte Cetona”) and *C. longifolia* (one plant on “Cornate di Gerfalco”). Phylogenetic analysis suggests that the sequences of sebacinoid fungi amplified from the studied orchids are sufficiently different to include at least three related species (Figs. 2, 3). *C. longifolia* mycobiont has close affinity with *S. incrustans* previously found in symbiotic relationship with the achlorophyllous orchid *N. nidus-avis* (Selosse et al., 2002) and with Sebacinaceae playing a symbiotic role in unidentified morphotypes of ectomycorrhizal tips collected in a truffle-ground by Murat et al. (2005). *C. damasonium* sebacinoid associates are instead phylogenetically close to Sebacinaceae that have been previously found to establish mycorrhizal associations with orchid (Selosse et al., 2002) and non-orchid plants (Urban et al., 2003). Sebacinoid basidiomycetes show a remarkable diversity of mycorrhizal types (Weiss et al., 2004). Moreover, Sebacinoid mycobionts can support the myco-heterotrophic growth of achlorophyllous orchids (McKendrick et al., 2002; Selosse et al., 2002; Taylor et al., 2003)

as well as they can play a functional role in association with chlorophyllous orchid species (Bonnardeaux et al., 2007; Wright et al., 2010). Considering the puzzling variety of symbiotic plant-fungus associations in which Sebacinaceae are involved, including orchid mycorrhizas, we speculate that sebacinoid fungi found in the present study from the roots of *C. damasonium* and *C. longifolia*, may play a trophic role in their relationship with the analysed orchids. Much further work is required to provide an integrated view of *Sebacina-Cephalanthera* interactions based on morphological, molecular, and physiological data.

Together with *Sebacina*, another fungal taxon belonging to the anamorphic form genus *Rhizoctonia* was associated with only one of the two analysed orchids, the best match for the sequence amplified from *C. damasonium* sample MC3 being with *Ceratobasidium* sp. collected by Sharon et al. (2007) from *F. ananassa* in Israel. Ceratobasidioid fungi have been found in symbiotic associations with terrestrial orchids from both forests and meadows, as well as epiphytic orchids (Shefferson et al., 2005; Otero et al., 2005; Otero et al., 2007, 2011; Irwin et al., 2007; Yagame et al., 2008, 2012; Girlanda et al., 2011; Jacquemyn et al., 2011a, b; Pecoraro et al., 2012a; Tondello et al., 2012). Moreover, the trophic relationship between a *Ceratobasidium* species *C. cornigerum* (Bourdot) D.P. Rogers and the green forest orchid *Goodyera repens* (L.) R. Br. represents the first known example of mutualistic mycorrhiza in orchids (Cameron et al., 2006). The second best match for the *Ceratobasidium* sequence that was amplified from *C. damasonium* in the present work is with *C. cornigerum* (isolate XSD-44, Jiang et al., unpublished, GenBank record EU273525). We propose the role of symbiont for this *Ceratobasidium* taxon associated with the roots of *C. damasonium*, although the confirmation of its exact physiological role would require detailed analyses.

Ectomycorrhizal (ECM) fungi were also found to colonize the roots of the two investigated *Cephalanthera* species. Hymenogastraceae were collected from 37,5 % of *C. damasonium* analysed plants, being the only fungal partners associated with the studied orchid in both of the two geographical distinct sampling areas ("Monte Cetona" and "Monte Rotondo", see Table 1). All the Hymenogastraceae sequences amplified in the present work had the same BLAST search closest match with a *C. damasonium* mycorrhizal symbiont (GenBank accession code AY634136) collected by Bidartondo et al. (2004) from orchid root samples, in a German forest site. Identification of *C. damasonium* mycobiont as a member of Hymenogastraceae family is supported by the high similarity that the sequences collected in the present study also showed, as their second best match, with

sequences amplified from *Hymenogaster bulliardii* Vittad. (Peintner et al., 2001) and *H. citrinus* Vittad. (Brock et al., 2009) specimens deposited in herbaria. ECM thelephoroid basidiomycetes belonging to *Tomentella* were instead associated with *C. longifolia* in "Monte Rotondo" Natural Reserve. Tomentelloid fungi found in the roots of the studied orchid shared similarity with *Tomentella* species involved in ectomycorrhizal associations with several tree species growing in boreal forests (Tedersoo et al., 2003; Kjøller, 2006; Bidartondo & Read, 2008). In particular, *Tomentella* taxa detected in tree ectomycorrhizas in England by Bidartondo & Read (2008) have been shown to support both *C. damasonium* and *C. longifolia* seed germination, seedling development and adult plant growth. Symbiotic associations with thelephoroid fungi have previously been reported in several *Cephalanthera* species including *C. austiniae* (Taylor & Bruns, 1997), *C. damasonium* (Bidartondo et al., 2004; Julou et al., 2005; Bidartondo & Read, 2008), *C. erecta* Blume (Matsuda et al., 2009), *C. falcata* Blume (Yamato & Iwase, 2008; Matsuda et al., 2009), *C. longifolia* (Abadie et al., 2006; Bidartondo & Read, 2008; Liebel et al., 2010), and *C. rubra* Rich. (Bidartondo et al., 2004). Yagame & Yamato (2012) demonstrated the establishment of tripartite symbioses between the mycoheterothrophic orchid *C. falcata*, Thelephoraceae fungi, and *Quercus serrata* Murray (Fagaceae), in culture condition.

The simultaneous association with rhizoctonia-forming fungi and ECM fungal groups is a noteworthy aspect of mycorrhizal diversity in the analysed *Cephalanthera* species. This feature suggests that *C. damasonium* and *C. longifolia* represent an intermediate step in the evolution from fully photosynthetic orchids, mostly associated with *Rhizoctonia*-like fungi, to fully mycoheterotrophic orchids, often found in symbiosis with ECM fungal associates (Bidartondo et al., 2004; Motomura et al., 2010; Dearnaley et al., 2012). It has been previously suggested by Taylor and Bruns (1997) that the achlorophyllous mycoheterotrophic *Cephalanthera* species *C. austinae* evolved from autotrophic ancestors by switching from *Rhizoctonia* to thelephoroid fungal partners, as a probable adaptation for colonizing dark, understory habitats.

Other fungal ITS sequences sporadically collected from the roots of the studied orchid species deserve to be discussed. Several mycorrhizal fungi previously found in a variety of environmental sources, such as *C. geophilum* from *F. sylvatica* ECM root tips (Buée et al., 2005), an ectomycorrhizal ascomycete from root tips in a tropical cloud Mexican forest (Morris et al., 2008), and pezizomycetes from orchid roots (Julou et al., 2005) were the best matches for sequences detected in *C. damasonium*. We cannot exclude that these fungal associates may be involved in a trophic relationship with the

analysed orchid. They could represent occasional symbionts for mycorrhizal associations that the investigated orchid explores, looking for the best partners available from the fungal community that characterizes a particular habitat.

A fungal taxon with 99% identity to *Tetracladium* sp. found in orchid roots (Abadie et al., 2006) and mycorrhizal roots of conifer seedlings (Menkis et al., 2005) was identified in *C. longifolia*. The genus *Tetracladium* is a member of the so-called Ingoldian fungi, asexual microfungi commonly occurring on dead plant material, in running freshwater (Bärlocher, 1992). These aquatic hyphomycetes have been recently found as endophytes in several plant tissues, including orchid roots (Abadie et al., 2006; Vendramin et al., 2010). Selosse et al. (2008) proposed that some Ingoldian fungi spend part of their life in plants and use water for dispersion. These authors suggested to investigate the impact of Ingoldian fungi on plant species, during their endophytic life stage, in order to test their protective effects on hosts. Other putative endophytic fungi, with a diverse and mostly unknown ecology, were detected in the studied orchids. Among them, *E. salmonis* was found in the roots of *C. damasonium* sample MR2, showing similarity with a fungal strain collected from *Salmo clarkii* (Untereiner & Naveau, 1999). *Exophiala salmonis* has been reported from the roots of several orchid species, such as *Orchis pauciflora* Tenore (Pecoraro et al., 2012b) and *Himantoglossum adriaticum* H. Baumann (Pecoraro et al., 2013) and has been also detected in the roots of *C. damasonium* adult plants by Julou et al. (2005). Such findings of orchid fungal associates that show a large ecological plasticity and are capable to play different roles in their interactions with several host organisms deserve further attention. More studies on their endophytic phase in orchid hosts are necessary to clarify their real function in colonized plants. These intriguing fungi could represent “unexpected” orchid symbionts with some important nutritional roles. A better understanding of the real diversity of orchid mycorrhizal fungi is a fundamental starting point to support any consideration on orchid-fungus specificity.

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Table 1. Fungal diversity molecularly detected in the analysed orchids. BLAST search closest matches of fungal ITS-DNA sequences amplified from C. damasonium and C. longifolia roots collected in "Monte Cetona" (samples MC1-MC4), "Monte Rotondo" (samples MR1-MR8), and "Cornate di Gerfalco" (samples CG1-CG4). Sample GenBank accession codes, accession codes for the closest GenBank matches, sequence identity, and overlap of each match are reported.

Orchid species	Sample	Clone	Site	GenBank code	Best BLAST match(es)	Accession code	Overlap lenght	% match
C. damasonium	MC1	a	B	KT122776	*Cenococcum geophilum* (from ectom.)	AY299214	610	93%
		c	B	KT122777	*Cryptococcus carnescens*	AB105438	854	98%
		e	B	KT122778	*Sebacina* (from *N. nidus-avis*)	AF440653	1002	96%
					Sebacina aff. *epigaea*	AF490393	841	91%
	MC2	c	B	KT122779	*Hymenogastraceae* (from *C. damasonium*)	AY634136	1146	98%
					Hymenogaster bulliardii	AF325641	1009	95%
	MC3	c	A	KT122780	*Ceratobasidium* sp. (from *F. ananassa*)	DQ102416	765	87%
					Ceratobasidium cornigerum	EU273525	763	87%
	MC4	a	A	KT122781	*Sebacinaceae* (from *E. helleborine*)	AY452676	1003	96%
					Sebacina (from *N. nidus-avis*)	AF440657	806	90%
		b	A	KT122782	*Sebacina* (from *N. nidus-avis*)	AF440647	737	90%
		d	A	KT122783	Ascomycota (from ectomycorrhizal root)	EU624334	881	96%
	MR1	c	D	KT122784	Agaricomycetes (from forest soil)	FJ553950	292	97%
	MR2	c	D	KT122785	Pezizomycetes (from *C. damasonium*)	AY833035	905	93%
					Cadophora luteo-olivacea	DQ404349	898	96%
		e	D	KT122786	*Exophiala salmonis*	AF050274	715	92%
	MR3	c	D	KT122787	*Hymenogastraceae* (from *C. damasonium*)	AY634136	1013	94%
					Hymenogaster citrinus	EU784360	928	94%
		e	D	KT122788	*Tetracladium furcatum*	EU883432	739	90%
	MR4	b	D	KT122789	*Hymenogastraceae* (from *C. damasonium*)	AY634136	1038	97%
					Hymenogaster citrinus	EU784360	942	94%
C. longifolia	MR5	a	D	KT122767	*Tomentella* (from mycorrhizal roots)	EU668200	1170	99%
					Tomentella lilacinogrisea (from *T. cordata*)	AJ534912	989	94%
		e	D	KT122768	*Neonectria radicicola* (from mushroom)	FJ481036	937	98%
	MR6	e	D	KT122769	*Tetracladium* sp. (from *C. longifolia*)	DQ182426	985	99%
					Tetracladium maxilliforme (*P. sylvestris*)	DQ068996	985	99%
	MR7	a	C	KT122770	*Tomentella bryophila* (from beech root)	AM161534	1147	98%
					Tomentella bryophila (from sporocarp)	AJ889981	1147	98%
	MR8	Isolated	C	KT122771	Uncultured basidiomycete (from dust)	AM901992	1092	98%
					Bjerkandera adusta	AJ006672	1081	98%
	CG1	Isolated	A	KT122772	*Phlebia acerina*	AB210083	1086	98%
	CG2	b	B	KT122773	*Davidiella macrospora*	EU167591	886	98%
	CG3	a	B	KT122774	*Fusarium* sp.	EU750682	957	99%
	CG4	a	B	KT122775	*Sebacinaceae* (from mycorrhizal root)	AJ879657	1040	98%
					Sebacina incrustans	DQ520095	1037	98%

Fig. 1. Microscopical features of *C. longifolia* mycorrhizal roots: **(a)** cross-section showing intracellular hyphal coils (pelotons). **(b-c)** Details of fungal pelotons in orchid root cells. **(d)** Details of dark, septate and clamped hyphae emanating from pelotons. Scale bars: 500 µm (a), 200 µm (b), 30 µm (c), 20 µm (d).

Fig. 2. Neighbour-joining phylogenetic tree showing the relationship between the Sebacinaceae sequences obtained from the two analysed *Cephalanthera* species (*) and selected database relatives. Kimura 2-parameter distances were used. Bootstrap values are based on percentages of 1000 replicates. The tree was rooted with *Geastrum schmidelii* as outgroup

Fig. 3. Maximum likelihood phylogenetic tree showing the relationship between the Sebacinaceae sequences obtained from the two analysed *Cephalanthera* species (*) and selected database relatives. Kimura 2-parameter distances were used. Bootstrap values are based on percentages of 1000 replicates. The tree was rooted with *Geastrum schmidelii* as outgroup