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Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations[1]

Mariangela Girlanda[2,5], Rossana Segreto[2], Donata Cafasso[3], Heiko Tobias Liebel[4], Michele Rodda[2], Enrico Ercole[2], Salvatore Cozzolino[3], Gerhard Gebauer[4] and Silvia Perotto[2]

+ Author Affiliations

[2]*Dipartimento di Biologia Vegetale dell'Università di Torino e Istituto per la Protezione delle Piante del CNR, Viale Mattioli 25 10125 Torino, Italy*
[3]*Dipartimento di Biologia Strutturale e Funzionale dell'Università di Napoli Federico II, Complesso Universitario di Monte Sant'Angelo, Via Cintia 21 80126 Napoli, Italy*
[4]*Laboratory of Isotope Biogeochemistry, Center of Ecology and Environmental Research (BayCEER), University of Bayreuth 95440 Bayreuth, Germany*

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Abstract

• *Premise of the study:* We investigated whether four widespread, photosynthetic Mediterranean meadow orchids (*Ophrys fuciflora*, *Anacamptis laxiflora*, *Orchis purpurea*, and *Serapias vomeracea*) had either nutritional dependency on mycobionts or mycorrhizal fungal specificity. Nonphotosynthetic orchids generally engage in highly specific interactions with fungal symbionts that provide them with organic carbon. By contrast, fully photosynthetic orchids in sunny, meadow habitats have been considered to lack mycorrhizal specificity.

• *Methods:* We performed both culture-dependent and culture-independent ITS sequence analysis to identify fungi from orchid roots. By analyzing stable isotope (^{13}C and ^{15}N) natural abundances, we also determined the degree of autotrophy and mycoheterotrophy in the four orchid species.

• *Key results:* Phylogenetic and multivariate comparisons indicated that *Or. purpurea* and *Oph. fuciflora* featured lower fungal diversity and more specific mycobiont spectra than *A. laxiflora* and *S. vomeracea*. All orchid species were significantly enriched in ^{15}N compared with neighboring non-orchid plants. *Orchis purpurea* had the most pronounced N gain from fungi and differed from the other orchids in also obtaining C from fungi.

• *Conclusions:* These results indicated that even in sunny Mediterranean meadows, orchids may be mycoheterotrophic, with correlated mycorrhizal fungal specificity.

Key words:

- *Ceratobasidium*
- fungal diversity
- mycoheterotrophy
- orchid mycorrhiza
- Orchidaceae

- *Sebacina*
- *Tulasnella*

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5 Author for correspondence (e-mail: mariangela.girlanda@unito.it)

Many terrestrial plants benefit from symbiotic microorganisms, but the effectiveness of the symbiosis (that is the amount of benefit that the plant host derives from the microbial symbiont) varies among symbiont genotypes in natural populations. Specificity therefore may be a significant trait of symbiotic relationships established between plants and their microbial partners. Compared to other plant–microbe interactions, the mycorrhizal symbioses formed by the interaction of plant roots with some soil fungi are considered to be mainly nonspecific (Bruns et al., 2002; Roy et al., 2008; Smith and Read, 2008) because the plant is typically generalist toward the mycorrhizal partner. In these associations, the host plant exchanges photosynthetically derived organic carbon with mineral nutrients and water taken up by the extraradical fungal mycelium (Smith and Read, 2008).

Specificity in mycorrhizal symbioses has been found to sharply increase in specialized situations. For example, obligate mycoheterotrophic (OMH) plants are nonphotosynthetic and depend on their mycorrhizal fungal partners for organic carbon supply (Leake, 1994). Most OMH plants associate specifically with very narrow clades of fungi (Leake, 2004; Bidartondo, 2005; Merckx et al., 2009; Hynson and Bruns, 2010). Mycorrhizal specificity has been particularly well documented in OMH orchids, where molecular studies (e.g., Merckx et al., 2009; Hynson and Bruns, 2010) have revealed highly specific interactions either with mycobionts that mostly form ectomycorrhiza (ECM) on neighboring trees or with saprotrophic fungi, which breakdown and assimilate complex organic substrates (Ogura-Tsujita et al., 2009; Dearnaley and Bougoure, 2010).

Many green terrestrial orchids adapted to shady forest habitats still depend, at least in part, on their mycorrhizal symbiont for organic carbon, light availability being a major determinant of the degree of mycoheterotrophy (Preiss et al., 2010). This dual (photosynthetic and mycoheterotrophic) nutrition has been named partial mycoheterotrophy (Gebauer and Meyer, 2003) or mixotrophy (Selosse et al., 2004; Selosse and Roy, 2009). In partially mycoheterotrophic orchids, there is in most cases a wider range of fungal symbionts than in OMH plants, mostly comprising ectomycorrhizal (ECM) fungi (Bidartondo et al., 2004; McCormick et al., 2004, 2006; Shefferson et al., 2005; Girlanda et al., 2006).

Fully photosynthetic orchids in sunny meadow habitats have usually been considered to lack mycorrhizal specificity because of the common isolation of a variety of fungi referred to the form-genus *Rhizoctonia* (Warcup, 1971, 1981; Taylor et al., 2002; Rasmussen, 2002). However, morphological observations and rDNA sequencing have indicated that “rhizoctonias” from orchids are better regarded as a polyphyletic assemblage of teleomorphic genera belonging to three families of basidiomycetes (Sebacinaceae, Ceratobasidiaceae, and Tulasnellaceae) ascribed to distinct orders (Roberts, 1999; Taylor et al., 2002; Weiß et al., 2004). Except for the few Australian orchids’ symbionts, whose teleomorphic state was obtained in culture (Warcup, 1971, 1981), identification

of mycorrhizal fungi in photosynthetic meadow orchids and consequent assessment of mycorrhizal specificity have been limited until the advent of fungal molecular systematics and ecology (Waterman and Bidartondo, 2008). Such a recent application has suggested a preference of some Australian orchids toward a narrow range of mycorrhizal fungi (Bougoure et al., 2005; Bonnardeaux et al., 2007; Roche et al., 2010). These results are apparently in contrast with the situation in *Gymnadenia conopsea*, an orchid widely distributed in Eurasia, which feature a very wide spectrum of potential fungal partners (Stark et al., 2009). These apparently contrasting data may derive, at least in part, from the different approaches used to investigate mycorrhizal specificity in these orchids, for which the mycorrhizal partners have been molecularly identified after the fungi were isolated with a culture-dependent approach (Bougoure et al., 2005; Bonnardeaux et al., 2007; Roche et al., 2010) or after direct amplification of root DNA (Stark et al., 2009). The two approaches may yield different results because analysis of fungal isolates would overlook the occurrence of uncultivable fungi. On the other hand, fungal isolation allows verification of the mycorrhizal status by seed germination assays, even though physiological compatibility under laboratory conditions may differ from that in nature (Rasmussen, 2002).

Knowledge about the identity of mycorrhizal fungi in Mediterranean orchids is so far mostly restricted to a few *Orchis* species, as based on either in vitro cultivation of the fungi (Currah and Sherburne, 1992; Rasmussen, 1995) or recent molecular work (Shefferson et al., 2008; Liebel et al., 2010; Lievens et al., 2010; Schatz et al., 2010). We have used culture-independent PCR-based and culture-dependent methods to assess mycorrhizal specificity in four widespread, photosynthetic Mediterranean meadow orchid species (*Ophrys fuciflora*, *Anacamptis laxiflora*, *Orchis purpurea*, and *Serapias vomeracea*). Meadows where the four species occurred in different combinations provided an interesting scenario to compare the fungal community composition in roots of sympatric and allopatric plants. Sequence-based operational taxonomic units (OTUs) were defined, and phylogenetic trees were built with fungal ITS sequences obtained from either fungal isolates or direct mycorrhizal root DNA amplification, as well as reference sequences. Patterns of occurrence of fungal sequence types were compared statistically by multivariate analysis. We also investigated the autotrophic status of the four orchid species by analysis of stable isotope (^{13}C and ^{15}N) natural abundance. The latter analysis has become established as a convenient tool to assess acquisition of fungus-derived organic C and N based on stable isotope abundances in plant leaf tissue and hence to characterize the mycoheterotrophic lifestyle (Gebauer and Meyer, 2003). The principle of this analysis is based on the observation that N and C in fungi are isotopically distinguishable from N and C of accompanying non-orchid vegetation. The incorporation of fungus-derived carbon, for example, is reflected by the plants' leaf isotope signature since fungal tissues are enriched in the stable isotope ^{13}C relative to accompanying fully autotrophic plants (Högberg et al., 1999).

MATERIALS AND METHODS

Sampling of orchids and neighboring plants

Anacamptis laxiflora (Lam.) R. M. Bateman, Pridgeon & M. W. Chase, *Ophrys fuciflora* (F. W Schmidt) Moench, *Orchis purpurea* Huds., and *Serapias vomeracea* (N. L. Burman) Briquet all belong to the Orchidinae subtribe in the Orchidoideae subfamily of Orchidaceae (Bateman et al., 2003). These species are rather widespread in Mediterranean meadows: *A. laxiflora*, *Oph. fuciflora*, and *S. vomeracea* mainly grow in open habitats, whereas *Or. purpurea* can also be found in forests (Kretzschmar et al., 2007; Rossi, 2002).

Orchid roots (Table 1) were sampled mainly in four meadows in northern Italy (at 410–450 m a.s.l.): two meadows on partly abandoned agricultural terraces, ca. 100 m^2 each (meadows 2 and 3) and two neighboring plots within a lower meadow, again about 100 m^2 wide (meadows 1a and 1b).

In meadows 1a and 1b, agricultural practices (regular grass cutting and patchy treatment with liquid manure) are still performed. The vegetation at the site features dry grasslands (*Festuco-Brometalia*), partly colonized on the terraces by shrubs (*Rosa* sp., *Spartium junceum*, *Ligustrum vulgare*, and *Cornus sanguinea*) and trees (*Quercus pubescens*, *Acer campestre*, and *Ulmus minor*). The soil is alkaline (pH 7.98 ± 0.16, $N = 40$) and originates from Oligocene marls with silt and sand of the Rocchetta Formation (Tertiary; Geological Survey of Italy, 2007). The C/N ratio of the soil is 16 ± 4 ($N = 35$), a typical value for rather N-poor grassland soils (Scheffer, 2002). In addition to the main sampling site, some root samples were also collected in meadows located incentral and southern Italy (Tuscany and Campania; Table 1).

Table 1. Origin and number of plant samples.

Orchid	Site	No. plants analyzed	Species (No. of neighboring herbaceous plants analyzed)
Anacamptis laxiflora	Northern Italy (meadows 1a, 1b)	10	*Bromus erectus* (2), *Carex hirta* (1)
	Campania, Italy	5	—
Ophrys fuciflora	Northern Italy (meadow 3)	8	*Bromus erectus* (2), *Festuca rubra* (1)
	Campania, Italy	2	—
Orchis purpurea	Northern Italy (meadows 2, 3)	13	*Bromus erectus* (2), *Festuca rubra* (1)
	Campania, Italy	2	—
Serapias vomeracea	Northern Italy (meadows 1a, 1b, 3)	14	*Bromus erectus* (3)
	Campania, Italy	4	—
	Tuscany, Italy	3	—

Root samples were collected in early summer during the flowering season from 2005 to 2007. Roots of herbaceous plants surrounding the sampled orchids were also harvested for a limited number of orchid plants at the main study site (Table 1), from the soil core containing orchid roots. Roots were thoroughly washed with tap water, gently brushed, then sonicated in an ultrasonic bath (three cycles of 30 s each) to remove adhering soil particles and microorganisms. Roots were then surface-sterilized with sodium hypochlorite (1:5 active chlorine) for 30 s and rinsed three times with sterile water. Sections from fresh root fragments were observed by light microscopy, and highly colonized root fragments were chosen for further analyses. Root samples were either processed immediately for fungal isolation and light microscopy or frozen in liquid nitrogen and stored at −80° for subsequent molecular analysis.

Fungal isolation

Fungi were isolated from all collected orchids as well as from specimens of neighboring herbaceous plants (Table 1). Following surface-sterilization, at least eight root sections 3–5 mm long were excised from one or two roots per plant and plated onto malt extract agar (MEA) amended with 40 mg gentamicin/L. Petri dishes were incubated at room temperature for up to 2 months to allow any slow-growing mycelia to develop. Nuclei within hyphal compartments of *Rhizoctonia* isolates were counted under UV light after 4′,6-diamidino-2-phenylindole (DAPI) staining [5 µg/mL in a 1:1 (v:v) water:glycerol solution].

DNA extraction and PCR amplification

Genomic DNA from frozen roots (about 0.5–1 g fresh mass) of both orchid and neighboring non-orchid plants was extracted using the cetyltrimethyl ammonium bromide (CTAB) method modified from Doyle and Doyle (1990). A DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used to extract DNA from the fungal isolates obtained from orchid and non-orchid roots. To increase the diversity of orchid fungal endophytes detected, DNA from either orchid roots or fungal isolates was used as a template for three PCR reactions with the primer combinations ITS1F/ITS4 (generic fungal nuclear internal transcribed spacer, nrITS) (White et al., 1990), ITS1F/ITS4B (for basidiomycete nrITS) (Gardes and Bruns, 1993), or ITS1/ITS4-Tul (for tulasnelloid nrITS) (Taylor and McCormick, 2008). Given the low efficiency of direct amplification from neighboring non-orchid roots, genomic DNA was amplified by nested polymerase chain reaction (PCR), using ITS1F/ITS4, ITS1F/ITS4B, or ITS1F/ITS4-Tul as the first set of primers, followed by primers ITS1/ITS4, ITS1/ITS4B, or ITS1/ITS4-Tul, respectively. PCR for all primers combinations was carried out in a final volume of 50 µL, containing 3 µL of extracted genomic DNA at the appropriate dilution and 1.5 U of RED *Taq*TM DNA polymerase (Sigma, St. Louis, Missouri, USA), with the following concentrations: 1× buffer (Sigma), 0.5 µmol/L of each primer, 0.1 mmol/L dNTP. Nested PCR was carried out with the same reagents as described before, using 2 µL of the product from the first reaction as a template. PCRs were performed in a T3000 Thermocycler (Biometra, Goettingen, Germany) using the following temperature profile: 95°C for 4 min (1 cycle); 94°C for 35 s, 53°C for 55 s, 72°C for 55 s (35 cycles); 72°C for 7 min (1 cycle). Nested PCRs were carried out with the same temperature profile, with the exception of the annealing temperature (65°C for the primer combination ITS1/ITS4 and ITS1/ITS4B; 60°C for the primer combination ITS1/ITS4-Tul). Negative control reactions without template DNA were performed with each set of primers.

Cloning and ITS-RFLP analysis

PCR amplicons were purified with QIAquick PCR Purification Kit (Qiagen) and cloned into pGEM-T (Promega, Madison, Wisconsin, USA) vectors; the vectors were used to transform Stratagene XL-2 Blue ultracompetent cells (Agilent, Santa Clara, California, USA). White colonies were randomly picked and plasmid inserts were amplified using the T7 and SP6 vector primers under the following conditions: 95°C for 3 min (1 cycle); 92°C for 1 min, 55°C for 1 min, 72°C for 1 min (35 cycles); 72°C for 7 min (1 cycle). Twenty clones per plant were randomly chosen for RFLP (restriction fragment length polymorphism) analysis of ITS PCR product, using the restriction enzymes *Alu*I and *Hha*I.

DNA sequencing and sequence analysis

Cloned ITS inserts representative of the different RFLP profiles were sequenced with the same primer pair as used for amplification. Dye sequencing was carried out on ABI Prism 310 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA). Sequences were edited and assembled using the program Sequencher 4.1 for MacOS 9, and sequence identity was determined using the BLASTn algorithm available through the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) against the NCBI nucleotide collection. Sequences chosen for inclusion in the phylogenetic analyses comprised best BLAST hits as well as fungal sequences from a variety of terrestrial and epiphytic orchids from different continents and environments, as well as from non-orchid plants, fungal strains and fruitbodies. Due to the phylogenetic distance between the fungi identified (Roberts, 1999), distinct phylogenetic analyses were carried out.

Sequences were aligned using the program Clustal X 2.0 (Larkin et al., 2007) with default conditions for gap opening and gap extension penalty. Alignments were then imported into program MEGA 4.0 (Tamura et al., 2007) for manual adjustment. Methods of phylogenetic reconstruction included Bayesian Markov chain Monte Carlo (MCMC) inference (BI) and maximum likelihood estimation (ML), using the programs MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) and RAxML v.7.0.4 (Stamatakis, 2006), respectively. For the Baysian inference analyses, substitution models suggested as best-fit to the data under both the corrected Akaike information criterion (AIC) and the Bayesian information criterion (BIC) were estimated for each data set using the program jModelTest version 0.1.1 (Posada, 2008) to provide independent substitution models for each alignment. For each alignment, four incrementally heated simultaneous MCMC were run over 10000000 generations, under model assumption, using random starting trees and default starting values of the models. Trees were sampled every 1000 generations resulting in an overall sampling of 10001 trees. The first 2500 trees were discarded as "burn-in" (25%). For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian posterior probabilities (BPP). Branch lengths were estimated as mean values over the sampled trees. Such a Bayesian analysis was repeated three times, always using random starting trees and random starting values for model parameters to test the independence of the results from the revisiting of the prior topologies during chain growth (Huelsenbeck et al., 2002). ML estimation was performed with RAxML v.7.0.4 through 1000 bootstrap replicates (Felsenstein, 1985) using the GTRGAMMA algorithm to perform a tree inference and search for a good topology. Support values from bootstrapping runs were mapped on the globally best tree using the –f option of RAxML and −x 12345 as a random seed to invoke the novel rapid bootstrapping algorithm. Nodes receiving a bootstrap support of <70% in the ML analyses (MLB), or a BPP of <95% in the BI analyses, were not considered as well supported.

Alignments and tree topologies are archived in the database TreeBASE (http://www.treebase.org; submission ID 11297). *Tulasnella*, *Ceratobasidium*, and *Sebacina* sequences were deposited in GenBank (accession numbers JF926459–JF926519, JF912458–JF912491 and JF912454–JF912457, respectively).

Comparison of Rhizoctonia spectra in orchid roots

Sequencing yielded diverse spectra of rhizoctonias that could be ascribed to the teleomorphic genera *Tulasnella*, *Ceratobasidium*, and *Sebacina* (see Results section). These spectra were investigated by multivariate analysis (discriminant analysis, DA). This method is robust against the violation of linear data structures and can be used without knowing any property of the data set (Podani 1994). Since none of the ITS sequence types obtained had 100% identity with GenBank sequences of identified *Tulasnella*, *Ceratobasidium*, and *Sebacina* species, OTUs were determined. Based on the final alignment, a distance matrix was constructed using DNAdist from the PHYLIP suite of programs version 3.6 with default parameters (Felsenstein, 1989, 2005; http://evolution.genetics.washington.edu/phylip.html). These pairwise distances served as input to the program mothur v.1.17.3 (Schloss et al., 2009; http://www.mothur.org/) to assign sequences to OTUs at different distance (sequence identity) levels. DOTUR OTUs at the 97% sequence identity threshold were compared to terminal clusters, receiving high support (≥95% BPP, ≥70% MLB) in phylogenetic analyses. Binary data (occurrence/absence of each OTU in individual orchid plants) were used in DA analysis. The analysis was performed using the SYN-TAX 2000 package subroutine "canonical variates" with the "Spherized scores of objects" (normalization of eigenvectors) option. Correlations with the original variables were also analyzed.

For *Serapias vomeracea*, it was also possible to use DA to compare the *Rhizoctonia* spectra in plants from different meadows at the main study site, namely, meadows 1a and 1b, and meadows at other Italian sites.

Isotope analysis

Following the sampling methodology of Gebauer and Meyer (2003), plants for isotope ratio mass-spectrometry (IRMS) were collected at the main study site in April 2007. Five 1-m^2 plots were identified for each orchid species, and the same three neighboring reference species were selected within each plot. Leaf samples of both orchid and reference species were collected, for a total of 20 samples from four orchid species and 60 samples from 12 non-orchids. Leaf samples were dried at 105°C, ground in a ball mill (Retsch Schwingmühle MM2, Haan, Germany) and stored dry in a desiccator until analyzed. Relative nitrogen and carbon isotope abundances of the leaf samples were analyzed in dual element mode with an elemental analyzer coupled to a continuous flow isotope ratio mass spectrometer as described in Bidartondo et al. (2004). The isotope abundances are denoted as δ-values, where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard.

The $\delta^{15}N$ and $\delta^{13}C$ values were tested for differences between an orchid species and its autotrophic references using the Kruskal–Wallis nonparametric test and Bonferroni-corrected Mann–Whitney *U* tests for post hoc comparisons. When a significant difference between the orchid and its references was found, a linear two-source isotopic mixing model was applied as described in Gebauer and Meyer (2003) to estimate the carbon and nitrogen in the orchid leaves possibly derived from mycorrhizal fungal partners. Because no mycoheterotrophic reference species were present at this site, a mean enrichment factor ε for ^{15}N (12.8 ‰) and ^{13}C (7.2 ‰) was used, as suggested by Liebel and Gebauer (2010).

RESULTS

Fungal associates of Oph. fuciflora, A. laxiflora, Or. purpurea, and S. vomeracea

Whereas tubers of *A. laxiflora*, *Oph. fuciflora*, *Or. purpurea*, and *S. vomeracea* were nonmycorrhizal, cortical cells of the thinner, emerging fleshy roots were extensively colonized by hyaline, septate hyphae (5–9[−16] µm in diameter) forming typical intracellular coils (Fig. 1).

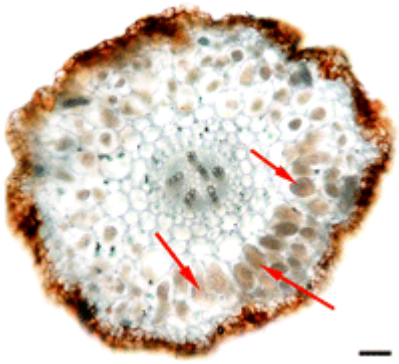

Fig. 1. Light micrograph of unstained, hand section from *Serapias vomeracea*, showing colonization of the cortical cells by fungal hyphae forming pelotons (arrows). Bar = 50 µm.

A diverse spectrum of endophytic fungi was obtained by in vitro isolation from mycorrhizal roots, including mycelia exhibiting typical *Rhizoctonia* features (Fig. 2), sporulating fungi, and hyaline or dematiaceous sterile mycelia. Non-*Rhizoctonia* basidiomycetous and ascomycetous fungal

endophytes, including ECM species (i.e., *Macowanites vinaceodorus*, *Terfezia* sp., *Choiromyces echinulatus*), were also identified following direct amplification of mycorrhizal root DNA with the fungal universal fungal primers pair ITS1F/ITS4 (Appendix S1, see Supplemental Data online at http://www.amjbot.org/content/98/7/1150/suppl/DC1).

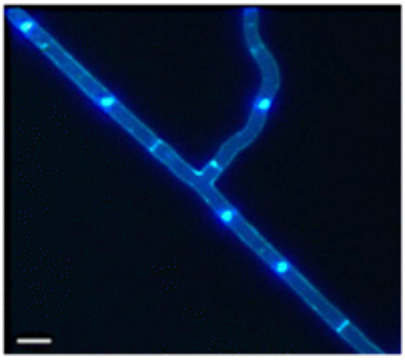

Fig. 2. Fluorescence micrograph of DAPI-stained fungal isolate from mycorrhizal roots of *Serapias vomeracea*, showing typical *Rhizoctonia* features, such as 90° branching of hyphae. Cells are binucleate. Bar =10 µm. *Rhizoctonia* isolates were obtained in culture from 43% of *S. vomeracea*, 40% of *Oph. fuciflora*, 27% of *A. laxiflora* and 20% of *Or. purpurea* individuals (Table 2). All isolates were binucleate (Fig. 2), and their symbiotic potential was confirmed by germination and protocorm development assays with orchid seeds (data not shown). Consistent with their binucleate status (Sharon et al., 2006), ITS sequencing identified such isolates as belonging to the teleomorphic genera *Tulasnella* and *Ceratobasidium*.

Table 2. Occurrence of *Tulasnella*, *Ceratobasidium*, and *Sebacina*fungal endophytes in the roots of the four orchid species, as assessed by either direct mycorrhizal root DNA extraction or fungal isolation.

	Percentage of analyzed plants harboring		
Orchid	*Tulasnella*	*Ceratobasidium*	*Sebacina*
Anacamptis laxiflora	69	50	7
Ophrys fuciflora	100	40	—
Orchis purpurea	47	44	—
Serapias vomeracea	95	33	24

Fungal ITS sequencing following direct amplification of mycorrhizal root DNA also indicated that *Rhizoctonia* species were associated with most (55 of 61) orchid plants. In the remaining six plants (five of *Or. purpurea* and one of *A. laxiflora*), *Rhizoctonia* species could not be identified by direct root amplification or isolation in pure culture. In such plants, direct root DNA amplification yielded fungal ITS sequences that matched sequences of ascomycetes belonging to different genera and higher taxa (e.g., *Scutellinia* sp., *Cadophora luteo-olivacea*, *Fusarium* sp., *Tetracladium* sp., uncultured *Pezizomycotina*), as well as one sequence of an unidentified basidiomycetous mycorrhizal fungus (online Appendix S1).

Rhizoctonias identified in the four orchid species were found to belong to *Tulasnella*, *Ceratobasidium*, and *Sebacina* (Table 2). The dominant teleomorphic genus was *Tulasnella* (64% sequences), followed by *Ceratobasidium* (30% sequences), whereas *Sebacina* (6% sequences) was sporadically identified only in *S. vomeracea* and *A. laxiflora* (Table 2). Several different teleomorphic genera of *Rhizoctonia* were often observed in the same orchid plant, with two genera co-occurring in 38% of *S. vomeracea*, 31% of *A. laxiflora*, 30% of *Oph. fuciflora* and 13% of *Or. purpurea* plants, and all three genera co-occurring in 14% of *S. vomeracea* plants.

Rhizoctonia was also identified in neighboring, non-orchid plants (*Bromus erectus* and *Carex hirta*) by in vitro isolation and direct root DNA amplification. A fungal isolate obtained from *Bromus erectus* (BR_OP8) collected close to *Or. purpurea* (plant OP8) was found to belong to *Tulasnella* (Appendix S1). Direct amplification of root DNA with the generic primers ITS1F/ITS4 was successful for all neighboring plants examined, whereas amplification with the specific primers ITS1F/ITS4B and ITS1F/ITS4tul was successful only for four of the 12 plants analyzed. Two sequences from a *B. erectus* plant (BR_AL9) growing close to *A. laxiflora* (plant AL9) and one sequence from a *Carex hirta* plant (CA_AL8) growing close to another *A. laxiflora* (plant AL8) matched *Ceratobasidium* sequences.

Phylogenetic analysis of the Rhizoctonia endophytes

The *Tulasnella*, *Ceratobasidium*, and *Sebacina* sequences obtained from roots and from fungal isolates of the four Mediterranean species were aligned with GenBank fungal sequences from a variety of terrestrial and epiphytic orchids from different continents and environments, as well as from non-orchid plants, fungal strains, and fruitbodies. Due to the phylogenetic distance between the teleomorphs (Roberts, 1999; Moncalvo et al., 2006; Hibbett et al., 2007), separate phylogenetic (BI and ML) analyses were carried out (Figs. 3–7). In all instances for the fungal sequences from the Mediterranean orchids investigated, clustering and nodal support were consistent between the two methods of phylogenetic reconstruction.

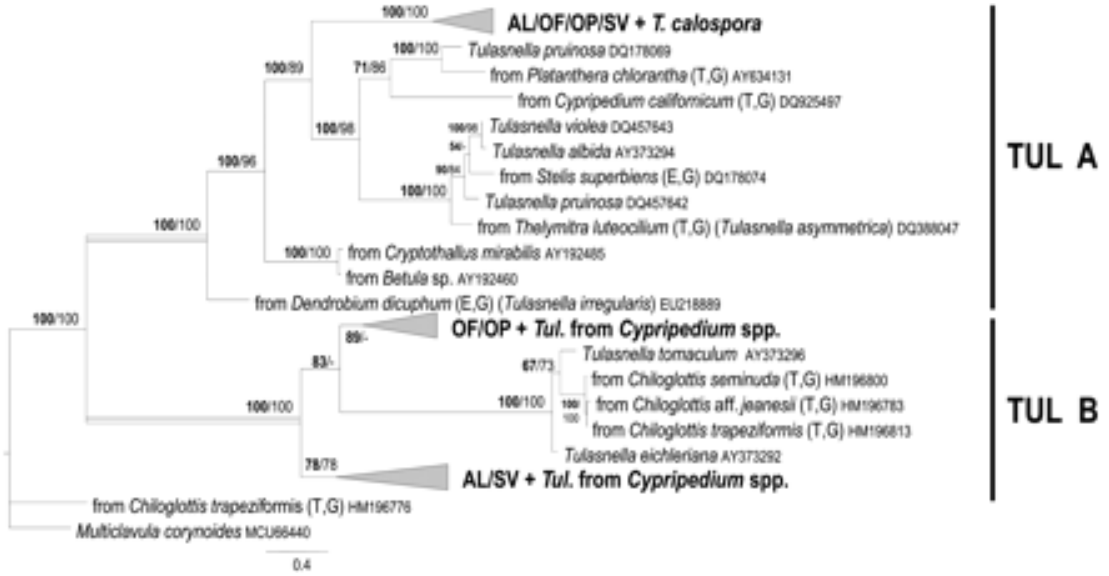

Fig. 3. Bayesian tree obtained from the ITS sequence alignment of tulasnelloid fungi. The GTR+G+I substitution model was used. Sequences from *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), *Orchis purpurea* (OP), *Serapias vomeracea* (SV), and from neighboring non-orchid species were obtained either with the universal fungal primer pair ITS1F/ITS4 or with the primer combination ITS1/ITS4-Tul specific for tulasnelloid fungi. The data set includes representatives of European, American, and Australian meadow and forest photosynthetic orchids, tropical terrestrial and epiphytic orchids, non-orchid species and fungal strains and fruitbodies. *Multiclavula corynoides* (MCU66440) was used as an outgroup taxon. Clade support as determined using both Bayesian posterior probabilities (BPP) and nonparametric bootstrapping from 1000 maximum likelihood replicates (MLB) is reported alongside each node (BPP /MLB). A hyphen (-) indicates <50% MLB. T, terrestrial; G, green.

Fig. 4. Detail of the "TUL A" clade in Fig. 3. Sequences from *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), and *Serapias vomeracea* (SV) were obtained either with the universal fungal primer pair ITS1F/ITS4 (open squares) or with the primer combination ITS1/ITS4-Tul specific for tulasnelloid fungi (closed circles). Clade support as determined using both Bayesian posterior

probabilities (BPP) and nonparametric bootstrapping from 1000 maximum likelihood replicates (MLB) is reported alongside each node (BPP /MLB). A hyphen (-) indicates <50% MLB. Operational taxonomic units (OTUs) recognized on the basis of sequence identity and tree topology are reported. Percentage of *Tulasnella* sequences and orchid plants are also indicated for the main OTUs. T, terrestrial; E, epiphytic; G, green.

Fig. 5. Detail of the "TUL B" clade in Fig. 3. Sequences from *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), *Orchis purpurea* (OP), *Serapias vomeracea* (SV), and from neighboring non-orchid species (BR_OP: *Bromus erectus* collected close to *O. purpurea*) were obtained with the universal fungal primer pair ITS1F/ITS4 (open squares). Clade support as determined using both Bayesian

posterior probabilities (BPP) and nonparametric bootstrapping from 1000 maximum likelihood replicates (MLB) is reported alongside each node (BPP /MLB). A hyphen (-) indicates <50% MLB. Operational taxonomic units (OTUs) recognized on the basis of sequence identity and tree topology are reported. Percentage of *Tulasnella* sequences and orchid plants are also indicated for the main OTUs. T, terrestrial; G, green.

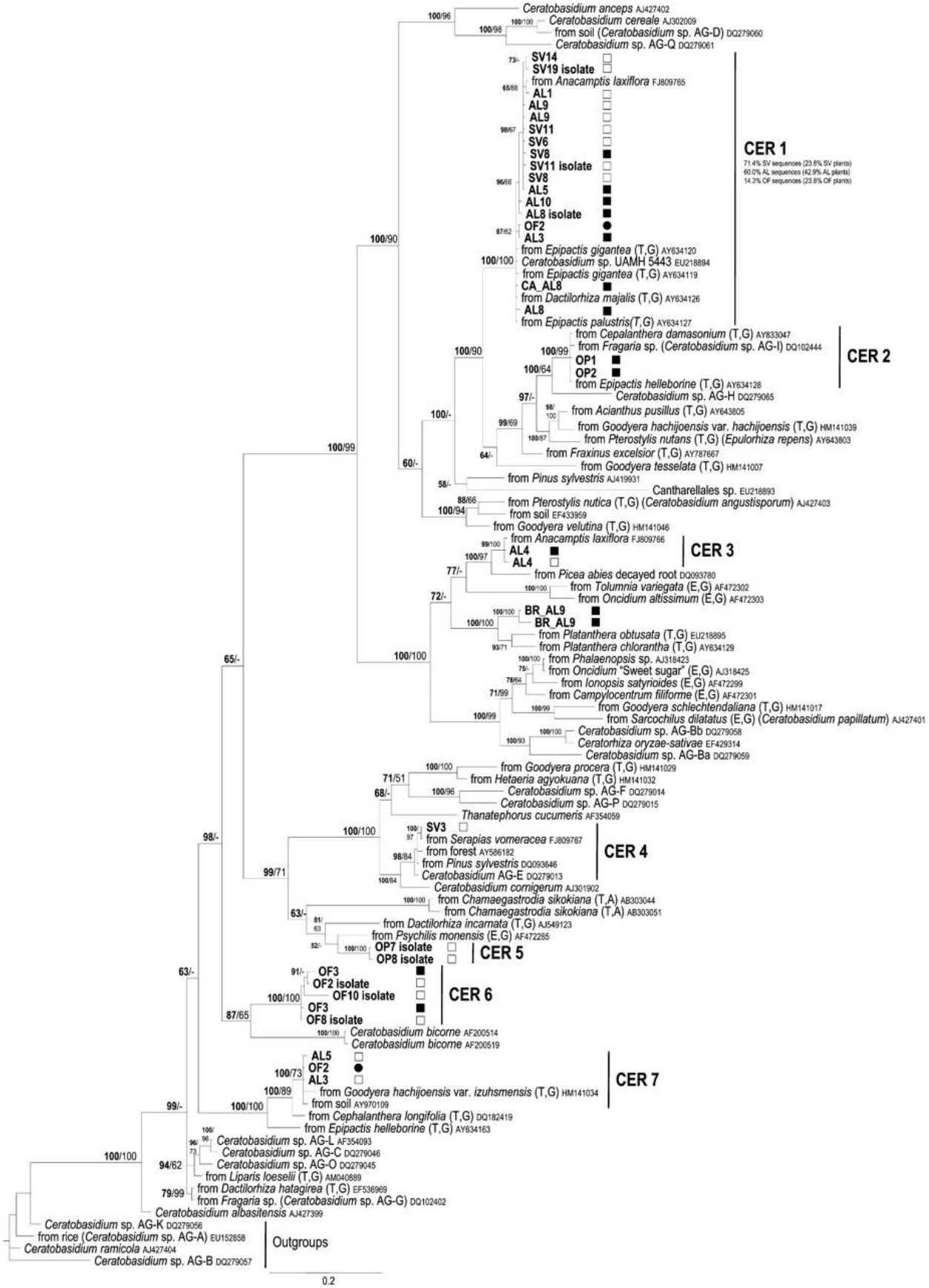

Fig. 6. Bayesian tree obtained from the ITS (ITS1-5.8S-ITS2) sequence alignment of ceratobasidioid fungi. The HKY+G substitution model was used. Sequences from *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), *Orchis purpurea* (OP), and *Serapias vomeracea* (SV) were obtained either with the universal fungal primer pair ITS1F/ITS4 (open squares), the primer

combination ITS1/ITS4-Tul specific for tulasnelloid fungi (closed circles), or the ITS1F/ITS4B primer pair specific for basidiomycete fungi (closed squares). Sequences of distantly related *Ceratobasidium* species were used as outgroup taxa. Clade support as determined using both Bayesian posterior probabilities (BPP) and nonparametric bootstrapping from 1000 maximum likelihood replicates (MLB) is reported alongside each node (BPP/MLB). A hyphen (-) indicates <50% MLB. Operational taxonomic units (OTUs) recognized on the basis of sequence identity and tree topology are reported. Percentage of *Ceratobasidium* sequences and orchid plants are also indicated for the main OTUs. T, terrestrial; E, epiphytic; G, green.

Fig. 7. Bayesian tree obtained from the ITS (ITS1-5.8S-ITS2) sequence alignment of sebacinoid fungi. The HKY+G substitution model was used. Sequences from *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), *Orchis purpurea* (OP), and *Serapias vomeracea* (SV) were obtained with the universal fungal primer pair ITS1F/ITS4 (open squares). *Geastrum schmidelii* (EU784247) was used as an outgroup taxon. Clade support as determined using both Bayesian posterior probabilities (BPP) and nonparametric bootstrapping from 1000 maximum likelihood replicates (MLB) is reported alongside each node (BPP /MLB). A hyphen (-) indicates <50% MLB. Operational taxonomic units (OTUs) recognized on the basis of sequence identity and tree topology are reported. T, terrestrial; G, green; A, achlorophyllic.

In the phylogenetic trees of the *Tulasnella* data set (Fig. 3), sequences from the four orchid species segregated into two main clades. In particular, most sequences from *S. vomeracea* and *A. laxiflora* (>80% and 70% of total sequences, respectively) co-segregated in a clade receiving 100% BPP and MLB support (indicated as "TUL A" in Fig. 3, magnified in Fig. 4). This group (Fig. 4) included most sequences from *S. vomeracea* and *A. laxiflora* (90% and 64% of plants yielding *Rhizoctonia* sequences, respectively), a few sequences from *Oph. fuciflora* (40% plants) mostly obtained with the ITS1F/ITS4-Tul primer pair, GenBank fungal sequences from different green meadow and forest orchids, tropical terrestrial and epiphytic orchids, and fungal strains or fruitbodies (mainly from *Tulasnella calospora*). In the other tulasnelloid main clade (indicated as "TUL B" in Fig. 3, magnified in Fig. 5), *Oph. fuciflora* and *Or. purpurea* sequences clustered either independently, with sequences from the same orchid species (sequences from *Or. purpurea* plants in Belgium), neighboring plants (sequence BR_OP8, from a *Bromus erectus* plant) or sequences from the forest orchid genus *Cypripedium* (Shefferson et al., 2007). No *Tulasnella* sequence was obtained with the ITS1F/ITS4B primer pair.

Ceratobasidium ITS sequences from the four Mediterranean orchid species were obtained with all primer pairs and could be aligned with GenBank fungal sequences from green meadow and forest orchids, from an epiphytic tropical orchid (*Psychilis monensis*), from ectomycorrhizal plants and other sources (Fig. 6). *Sebacina* sequences from *S. vomeracea* and *A. laxiflora* were obtained with the universal fungal primer pair ITS1F/ITS4 and could be aligned with sequences from green and achlorophyllous forest and meadow orchids, from ectomycorrhizal and other non-orchid plants, and from fungal fruitbodies (Fig. 7).

In the phylogenetic analyses, terminal clusters (supported by ≥95% BPP, ≥70% MLB) comprising sequences from the four orchid species were consistent with 97% sequence identity groups recognized by mothur (data not shown) and were therefore regarded as distinct operational taxonomic units (OTUs). The only exceptions were the large group TUL A1 (exhibiting 100–96% intra-OTU sequence identities), and the TUL B3 group, which received low nodal support (78% BPP, <50% MLB). Ten OTUs were thus recognized for *Tulasnella*, seven for *Ceratobasidium* and two for *Sebacina* (Figs. 4–7). Although direct root DNA amplification yielded a much higher number of OTUs than fungal isolation, some OTUs (e.g., CER 5) were obtained exclusively by the latter approach (Fig. 6).

OTUs did not include reference sequences of identified fungal species, the only exception being OTU TUL A1, which comprised sequences from *T. deliquescens* and *T. calospora* (syn. *T. deliquescens* sensu Warcup and Talbot, 1967, fide Roberts, 1999).

Several *Tulasnella* (Figs. 4, 5) and *Ceratobasidium* (Fig. 6) OTUs included GenBank sequences from different orchids, mainly European and American green orchids of either open habitats (*A. laxiflora* in Hungary, *Dactylorhiza* spp., *Epipactis* spp., *Orchis* spp., *Platanthera praeclara*) or forest (*Cypripedium* spp., *Cephalanthera* spp., *Liparis* spp., *Epipactis helleborine*, *Goodyera repens*, *Tipularia discolor*). Some *Ceratobasidium* OTUs included sequences from diverse sources, such as seedlings in forest nurseries, soil, or isolates from non-orchid plants belonging to different anastomosis groups. S*ebacina* OTU SEBA 2 (Fig. 7) comprised sequences from non-orchid plants. The remaining OTUs (comprising unique sequences from *A. laxiflora*, *Oph. fuciflora*, *Or. purpurea*, and *S. vomeracea*) either bore a sister-group relationship with other OTUs from the same orchids, or to GenBank fungal sequences from different European and North American terrestrial orchids, or were distantly related to both groups. Interestingly, many *Tulasnella* sequences (amplified with the universal fungal primers ITS1F/ITS4) clustered in OTUs that were sister groups to fungal endophytes of *Cypripedium* species (Fig. 5). Fungal sequences from tropical (terrestrial or

epiphytic) or Australian orchids were generally more distantly related, the main exception being sequences from Australian orchids related to *T. calospora* (Fig. 4).

Mycorrhizal specificity

Alignments of the tulasnelloid sequences obtained from *Or. purpurea*, *A. laxiflora*, *S. vomeracea* and *Oph. fuciflora* indicated a wide spectrum of OTUs for the first three species, with a high evenness (the most frequent OTUs occurring in 50.0–55.6% and the second most frequent OTUs in 22.2–42.9% of the plants). By contrast, a less diverse OTU spectrum was observed in *Oph. fuciflora* (where OTU TUL B5 occurred in 70.0%, and the other OTUs in ≤20.0% of the plants).

Diversity of fungi from *Or. purpurea*, *Oph. fuciflora*, *A. laxiflora*, and *S. vomeracea* was similar as these species yielded sequences belonging to 3, 4, 4, and 5 tulasnelloid OTUs, respectively, but fungal sequences from 90.0% of *Or. purpurea* and 77.8% of *Oph. fuciflora* plants belonged to *Tulasnella* OTUs that were not shared with other orchid species. Fungal sequences from 64.3% of *A. laxiflora* and 52.4% of *S. vomeracea* plants belonged to *Tulasnella* OTUs shared between these two plant species.

Association of the four orchids with ceratobasidioid fungi also featured different degrees of specificity. For example, OTU CER 6 (grouping 14.8% of *Ceratobasidium* sequences) was exclusively found in *Oph. fuciflora* (40.0% plants). By contrast, OTU CER 1, that contained most *Ceratobasidium* sequences (44.4%), encompassed 60.0% and 71.4% *Ceratobasidium* sequences from *A. laxiflora* and *S. vomeracea*, respectively.

Sebacina sequences were only obtained from *A. laxiflora* and *S. vomeracea*.

Discriminant analysis was carried out to compare *Rhizoctonia* spectra in the four orchid species (Fig. 8A). The first axis (67.1% of the total variation) discriminated between *Orchis purpurea* and the other orchid species. Discrimination between *Or. purpurea* and the other orchids was mainly due to OTUs TUL B3, TUL B2, CER 2, and CER 5 (associated exclusively with *Or. purpurea*) and OTUs TUL A1, TUL B5, and CER 1 (associated exclusively with the other orchid species), as indicated by correlations with the first axis. The second axis (30.6% of the total variation) distinguished instead *Oph. fuciflora* from the other species, mainly due to OTUs TUL B5 and CER 6 (associated exclusively with *Oph. fuciflora*). Within the ordination space, no geographical patterning was found for samples from different plots.

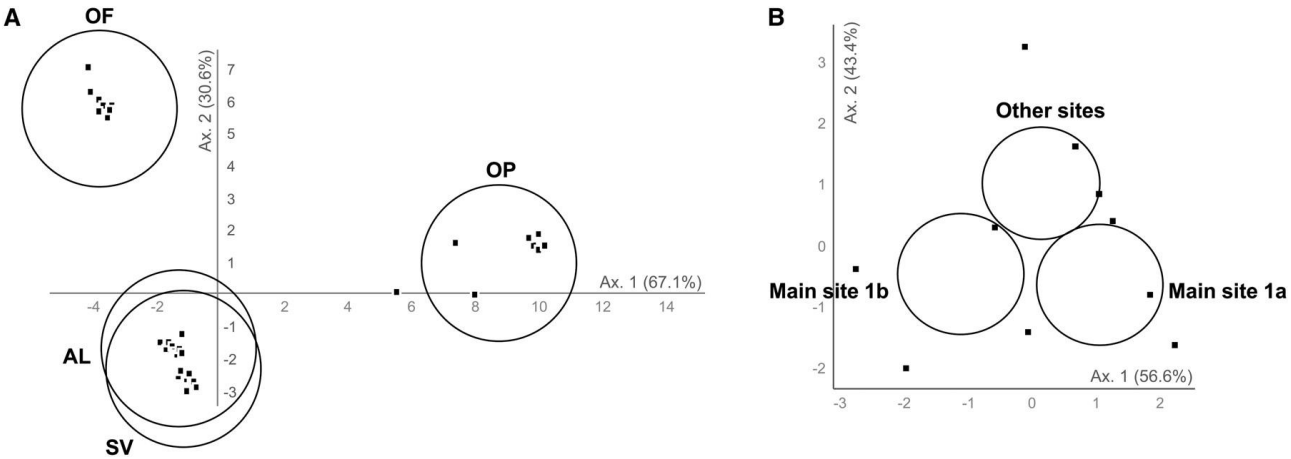

Fig. 8. Discriminant analysis (DA) plots comparing *Rhizoctonia* spectra in (A) *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), *Orchis purpurea* (OP), and *Serapias vomeracea* (SV) roots collected at all sampling sites, and (B) *S. vomeracea* plants collected at the main study site

meadows 1a and 1b and other sites. Isodensity circles representing each group (circles drawn around group centroids and expected to contain 95% of the observations within each group) are shown. Percentage of the total variation accounted for by each DA axis is reported.

Rhizoctonia spectra in plants growing in different meadows and at distant geographic sites were compared with a separate analysis for *S. vomeracea* (Fig. 8B). When *Rhizoctonia* spectra in *S. vomeracea* plants collected in meadows 1a and 1b at the main study site and in other sites were compared (5, 2, and 3, *Tulasnella*, *Ceratobasidium*, and *Sebacina* OTUs, respectively), meadow 1a was separated both from meadow 1b and from other sites along the first axis (56.6% of the total variation). Discrimination between the main and the other study sites could only be appreciated along the second axis (43.4% of the total variation). OTUs TUL A4, TUL A3, and TUL A2 were mainly responsible for the separation along axis 1, whereas discrimination along axis 2 was mainly due to TUL A3 and TUL A2.

Nutrient acquisition

The four orchids analyzed were all significantly enriched in ^{15}N compared to nearby reference plants (Fig. 9). The results of the linear two-source mixing model showed the highest N gain from fungi in *Orchis purpurea*, which was also the only orchid with a significant C gain (Table 3). Comparatively high $\delta^{15}N$ values were found in both *Anacamptis laxiflora* and its autotrophic references (Fig. 9).

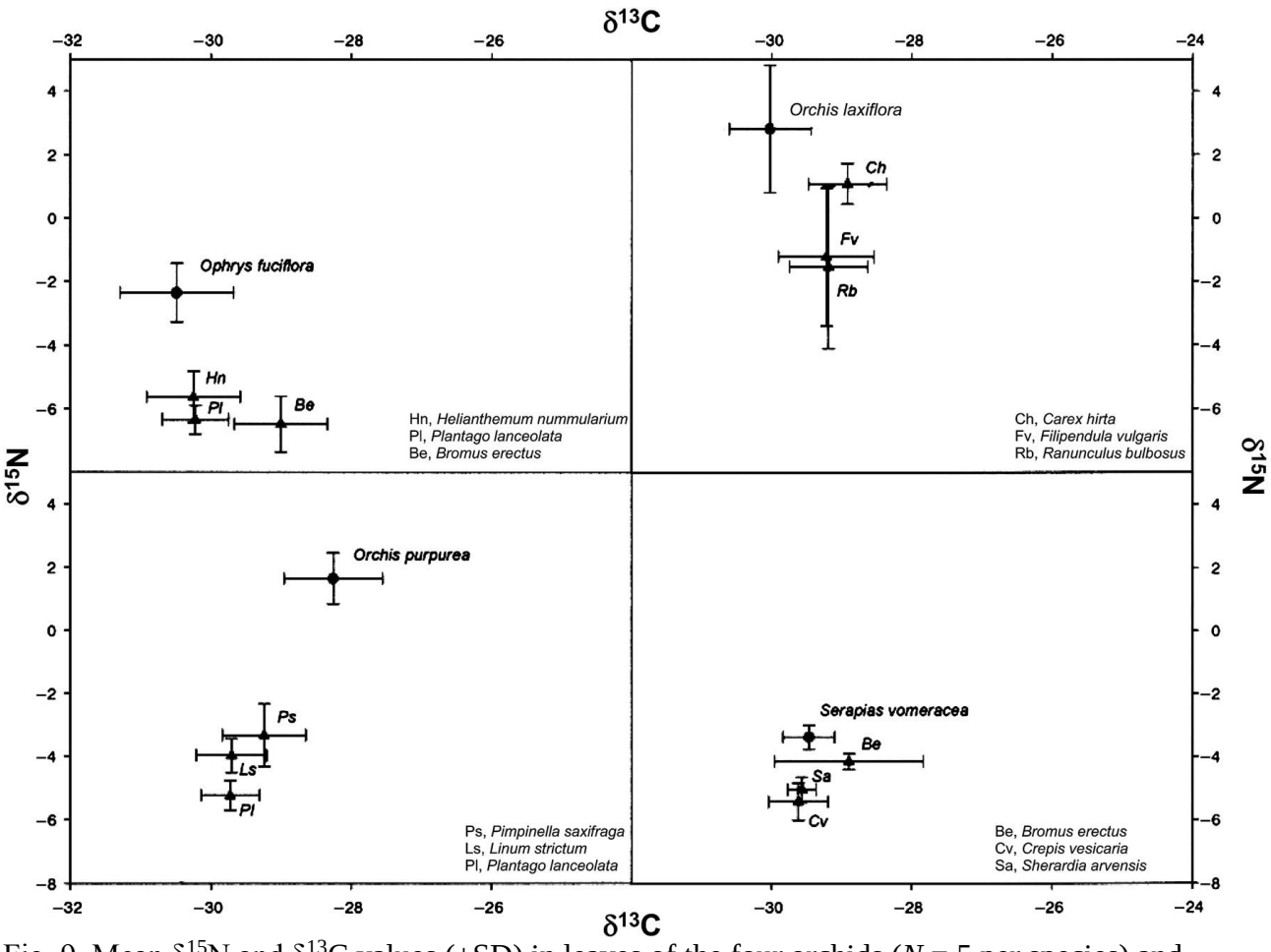

Fig. 9. Mean $\delta^{15}N$ and $\delta^{13}C$ values (±SD) in leaves of the four orchids (N = 5 per species) and neighboring autotrophic non-orchid species (N = 5; Be, *Bromus erectus*; Ch, *Carex hirta*; Cv,

Crepis vesicaria; Fv, *Filipendula vulgaris*; Hn, *Helianthemum nummularium*; Ls, *Linum strictum*; Pl, *Plantago lanceolata*; Ps, *Pimpinella saxifraga*; Rb, *Ranunculus bulbosus*; Sa, *Sherardia arvensis*).

Table 3. Percentages of N (mean % N ±SD, *N*= 5) and C (mean % C ±SD, *N*= 5) derived from fungi in the leaves of four green orchids that differed significantly in their $\delta^{15}N$ values from the surrounding autotrophic references. The data were calculated based on a linear two-source isotopic-mixing model.

Orchid	% N	% C
Anacamptis laxiflora	26 ± 9 **	−13 ± 1 *
Ophrys fuciflora	30 ± 7 ***	—
Orchis purpurea	46 ± 7 ***	18 ± 1 **
Serapias vomeracea	12 ± 4 ***	—

DISCUSSION

Mediterranean meadow orchids can feature fungal specificity

Considerable diversity of *Rhizoctonia* fungi has been uncovered in the four Mediterranean meadow orchid species. In spite of the limitations of the culture-dependent approach, the fungal isolates yielded ITS sequences that were not found by culture-independent, direct mycorrhizal root DNA amplification, and therefore complemented the latter approach. As observed by other authors, the three primer combinations used in this study resulted in a wide taxonomic coverage of orchid mycobionts, and a new combination of three ITS primers has been designed to cover a similar spectrum of mycorrhizal associates in a single amplification step (Taylor and McCormick, 2008).

Tulasnelloid fungi were found to dominate among the rhizoctonias identified, co-occurring with *Ceratobasidium* and, only in few *A. laxiflora* and *S. vomeracea* plants, *Sebacina* species. Fungi related to either *Tulasnella*, *Ceratobasidium*, and *Sebacina* have been found associated with terrestrial orchids of forests and open habitats, as well as epiphytic orchids (Taylor et al., 2002; Dearnaley, 2007; Shefferson et al., 2005, 2007, 2008, 2010; Suárez et al., 2008; Yagame et al., 2008; Waterman and Bidartondo, 2008; Cruz et al., 2010; Roche et al., 2010; Schatz et al., 2010; Swarts et al., 2010; Wright et al., 2010).

Species remain hard to define within tulasnelloid, ceratobasidioid, and sebacinoid fungi, because teleomorphic states remain unknown for orchid symbionts, the main exception being *Tulasnella calospora*. This species has been obtained from terrestrial orchids from a wide range of sites (Roberts, 1999) and qualifies as a generalist orchid symbiont. However, *T. calospora* has been suggested to comprise several distinct species (Suárez et al., 2006), and this taxonomic problem may obscure the actual host ranges of this fungus.

Different methods of molecular species identification have been proposed based on single-gene sequence similarity (BLAST and genetic distance) and tree topology (Ross et al., 2008). Bayesian analyses of tulasnelloid, ceratobasidioid, and sebacinoid ITS sequences obtained from *A. laxiflora*, *Oph. fuciflora*, *Or. purpurea*, and *S. vomeracea* in this study indicated that terminal clusters, supported by ≥70% posterior probabilities, corresponded to ≥97% sequence identity groups. Taking

the 97% sequence identity OTUs as a proxy for fungal species (Nilsson et al., 2008), several fungi associated with our four orchid species were found to be possibly conspecific with mycorrhizal partners of other European and North-American green orchids of both open and forest habitats (mostly to fungi from *Cypripedium* spp.). Only few fungi of Australian or tropical/epiphytic orchids were found to cluster with fungi from Mediterranean orchid species, suggesting some ecogeographical specificity.

Some OTUs featured fungi reported from non-orchid plants, mostly ECM trees. For instance, sebacinoid associates included fungi from photosynthetic orchids, thought to be saprotrophic, or uncultured sebacinoid mycobionts with ECM potential (Weiß et al., 2004; Selosse et al., 2007). A number of fungi associating with *A. laxiflora*, *Oph. fuciflora*, *Or. purpurea*, and *S. vomeracea* did not cluster with fungi from other plant species, thus confirming the suggestion (Shefferson et al., 2007) that a great deal of unassessed phylogenetic diversity still exists within *Rhizoctonia*, further broadening the taxonomic range of fungi forming orchid mycorrhiza.

Multivariate comparison of *Rhizoctonia* patterns in sympatric and allopatric orchid plants, together with the genetic and phylogenetic range of fungal symbionts in the same plants, indicated for *Or. purpurea* and *Oph. fuciflora* a lower diversity of mycobionts, that were mostly specific to the two orchid species, than *A. laxiflora* and *S. vomeracea*. In phylogenetic analyses, sequences from *Oph. fuciflora* or *Or. purpurea* generally segregated from sequences from *S. vomeracea* and *A. laxiflora*, which instead mostly cosegregated. Similarly, a wide range of levels of mycorrhizal fidelity, including high specificity, has been reported in Australian open habitat orchids (Warcup, 1971, 1981; Bougoure et al., 2005; Bonnardeaux et al., 2007). For orchids associating frequently with fungi from different families or orders, it remains to be assessed whether mycorrhizal specificity is enforced by the plant or rather due to fungal preference.

Comparison of *Rhizoctonia* spectra in *S. vomeracea* plants growing in different meadows indicated very limited site effects. In contrast to the hypothesis of a lack of mycorrhizal specificity in photosynthetic meadow orchids, discriminant analysis indicated that host preference can be observed both in co-occurring orchid species and in plants collected at distant geographic sites. This suggests, at least for this species, that individual plants from different populations are not locally adapted to the fungi present at that location, but they associate specifically with fungi that are present throughout their range in similar habitats. These results are apparently in contrast with the situation in *Gymnadenia conopsea* (Stark et al., 2009), where taxon composition of the fungal partners associated with plants at different sites showed little overlap. However, only rhizoctonias were considered in our multivariate analysis, whereas the full range of fungal associates (including known mycorrhizal fungi as well as plant endophytes and potential pathogens) was taken into account by Stark et al. (2009).

A partial mycoheterotrophic strategy in Mediterranean meadow orchids

Stable isotope analysis showed that all four orchid species were significantly enriched in ^{15}N compared with non-orchid reference species. The comparatively high ^{15}N values of both *A. laxiflora* and its autotrophic references were possibly due to fertilization with manure. This finding is consistent with other studies on meadow, forest photosynthetic and achlorophyllous orchids (Gebauer and Meyer, 2003; Bidartondo et al., 2004; Zimmer et al., 2007; Liebel et al., 2010), although N gain in photosynthetic and achlorophyllous forest orchids appears to be greater than gains in *A. laxiflora*, *Oph. fuciflora*, *Or. purpurea*, and *S. vomeracea*. Among the orchids investigated, *Or. purpurea* had the highest N gain from fungi and was the only species also with a significant C gain. *Orchis purpurea* was our only nonwintergreen species, sprouting in spring, which may explain its higher dependency on fungal nutrient supply (Rasmussen, 1995).

In *Or. purpurea*, the spatial patterns of seedling recruitment indicate limited seed dispersal (a few meters from the mother plant); seed germination may be thus confined to particular microsites where both adults and seedlings are clustered (Jacquemyn et al., 2007). Although the mycorrhizal fungi are likely to be distributed independently of the orchids (Feuerherdt et al., 2005), higher abundance of fungal symbionts has been found close to adult plants (Batty et al., 2001; Diez, 2007). Thus, spatial aspects of *Or. purpurea* seedling recruitment also suggest dependency on locally distributed mycorrhizal fungi for protocorm establishment and development.

Earlier studies in orchids growing in open habitat report smaller carbon gains from fungi in comparison with the more pronounced carbon gain in forest orchids (Gebauer and Meyer, 2003; Bidartondo et al., 2004; Liebel et al., 2010). Nevertheless, a C gain from fungi might go undetected due to respiratory carbon loss or be masked by photosynthesis. Stable isotope analyses in our study were carried out during flowering time; the possibility of (higher) carbon gains from fungi, in either *Or. purpurea* or the other Mediterranean species, in different seasons cannot be excluded.

The significance of, and the process leading to the high mycorrhizal specificity of mycoheterotrophic plants is a matter of ongoing debate, and it remains therefore unresolved whether such a specificity may be related to fine-tuning of the physiology of the plant–fungal interactions (Merckx et al., 2009; Hynson and Bruns, 2010; Leake and Cameron, 2010). Where evolution of mycoheterotrophy concurs with a switch in fungal functional types (e.g., from saprotrophic to ectomycorrhizal fungi), the question is open whether the new fungal partners are selected to provide larger and/or more easily available sources of C and nutrients (Leake and Cameron, 2010). The ultimate source of nutrients delivered by fungal symbionts to their orchid hosts remains uncertain. Differences in isotopic signatures can be related to the nutrient sources used by the symbiotic fungi (Gebauer and Taylor, 1999; Leake and Cameron, 2010). Recent molecular analyses have shown that *Rhizoctonia* ecology is much more complex than previously thought. Traditionally, rhizoctonias have been considered saprotrophic and pathogenic fungi. However, some rhizoctonias were recently shown to be ectomycorrhizal. Sebacinoid fungi have been recognized among the most common ECM species in temperate and Mediterranean forests (Glen et al., 2002; Avis et al., 2003; Kennedy et al., 2003; Walker et al., 2004; Richard et al., 2005; Tedersoo et al., 2006). Some *Tulasnella* species have also been reported to exhibit ectomycorrhizal potential (Warcup and Talbot, 1967; Bidartondo et al., 2003). Ceratobasidioid fungi have been shown to include strains that are endophytes of *Pinus sylvestris* (Sen et al., 1999), root-growth promoters (Grönberg et al., 2006), and ECM (Yagame et al., 2008; Bougoure et al., 2009).

Bidirectional transfer of carbon between a green orchid (*Goodyera repens*) and *Ceratobasidium cornigerum* was demonstrated by Cameron et al. (2006, 2008). Characteristic stable isotope abundance data (^{13}C depletion compared to accompanying plants) indicate that this bidirectional carbon transfer is more widespread among the tribes Cranichideae and Orchideae (Hynson et al., 2009; Liebel et al., 2010). Despite the limited sampling, we have shown that some fungi found in the roots of orchids and of neighboring herbaceous plants clustered in the same OTU. Although there is currently no evidence of a nutritional link between orchid and non-orchid plants in meadow habitats, this finding may indicate at least a potential hyphal link.

In conclusion, statistical analysis of mycorrhizal fungal diversity in four photosynthetic Mediterranean meadow orchids indicates that, as in other mycorrhizal associations (Sanders 2003), meadow orchids may prefer specific fungal partners in natural conditions. The species showing the most distinct fungal spectrum (*Or. purpurea*) was also partly dependant on the fungal partner(s) for organic carbon, suggesting that specific requirements of the symbiosis (e.g., carbon supply to the plant) may increase mycorrhizal specificity.

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