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Temporal variation in mycorrhizal diversity and carbon and nitrogen stable isotope abundance in the wintergreen meadow orchid *Anacamptis morio*

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Summary

- Many adult orchids, especially photoautotrophic species, associate with a diverse range of mycorrhizal fungi, but little is known about the temporal changes that might occur in the diversity and functioning of orchid mycorrhiza during vegetative and reproductive plant growth.
- Temporal variations in the spectrum of mycorrhizal fungi and in stable isotope natural abundance were investigated in adult plants of *Anacamptis morio*, a wintergreen meadow orchid.
- *Anacamptis morio* associated with mycorrhizal fungi belonging to *Tulasnella*, *Ceratobasidium* and a clade of Pezizaceae (Ascomycetes). When a complete growing season was investigated, multivariate analyses indicated significant differences in the mycorrhizal fungal community. Among fungi identified from manually isolated pelotons, *Tulasnella* was more common in autumn and winter, the pezizacean clade was very frequent in spring, and *Ceratobasidium* was more frequent in summer. By contrast, relatively small variations were found in carbon (C) and nitrogen (N) stable isotope natural abundance, *A. morio* samples showing similar ^{15}N enrichment and ^{13}C depletion at the different sampling times.
- These observations suggest that, irrespective of differences in the seasonal environmental conditions, the plant phenological stages and the associated fungi, the isotopic content in mycorrhizal *A. morio* remains fairly constant over time.

Introduction

To complete their life cycle, orchids rely on symbiotic relationships with other organisms, namely pollinators for sexual reproduction and mycorrhizal fungi for seed germination and seedling recruitment (Waterman & Bidartondo, 2008; McCormick & Jacquemyn, 2014), although most orchids depend on mycorrhizal fungi throughout their life (Smith & Read, 2008). Whereas interactions with pollinators have shaped orchid floral morphology, interactions with mycorrhizal fungi have deeply influenced their trophism (Hynson *et al*., 2013). A wide range of mycorrhizal fungi, mainly in the basidiomycetes, can associate with orchids (Taylor *et al*., 2002; Selosse, 2014), and their taxonomic position and ecological functions mirror the host trophic behavior, which can range from full heterotrophy to photoautotrophy (Hynson *et al*., 2013).

Plant isotopic studies, together with molecular identification of mycorrhizal partners, have been instrumental in revealing different nutritional types among adult orchids (Gebauer & Meyer, 2003; Trudell *et al*., 2003; Selosse & Roy, 2009; Hynson *et al*., 2013). Stable isotope natural abundance can be used to evaluate nutrient fluxes under field conditions and is particularly useful in mycorrhizal studies, provided that plant-derived carbon (C) and nitrogen (N) are isotopically distinguished from fungal-derived C and N. This is the case for orchids that associate with ectomycorrhizal (ECM) fungi, such as achlorophyllous (McKendrick *et al*., 2000; Taylor *et al*., 2002; Bidartondo *et al*., 2004; Roy *et al*., 2009) and green forest orchids (Julou *et al*., 2005; Girlanda *et al*., 2006; Těšitelová *et al*., 2012; Yagame *et al*., 2012), but also for achlorophyllous orchids associated with saprotrophic wood-decomposers and litter-decaying fungi in forests that are probably devoid of ECM plant species (Martos *et al*., 2009; Ogura-Tsujita *et al*., 2009). ECM and saprotrophic wood-decomposers and litter-decaying fungi are enriched in ^{13}C and ^{15}N isotopes as a result of their specific physiology and/or access to compounds enriched in these heavy isotopes (Mayor *et al*., 2009). When compared with neighboring photoautotrophic plants, achlorophyllous and green forest orchids are enriched in heavy C and N isotopes, thus indicating that their symbiotic fungi cover the orchid carbon and nitrogen demand (Hynson *et al*., 2013). However, whereas achlorophyllous orchids fully depend on fungal-derived C, a type of nutrition called mycoheterotrophy (see references in Hynson & Bruns, 2010; Leake & Cameron, 2010), green forest orchids complement photosynthesis-derived C with fungal-derived organic C (Gebauer & Meyer, 2003; Hynson *et al*., 2013). This mode of nutrition is referred to as partial mycoheterotrophy (Gebauer & Meyer, 2003; Merckx, 2013), a special case of mixotrophy (Selosse & Roy, 2009).

In contrast with mycoheterotrophic orchids, stable isotope natural abundance has not provided a clear picture for photosynthetic orchids, that represent the majority of species. Photosynthetic orchids typically associate with a range of mostly saprotrophic basidiomycetes in the Tulasnellaceae, Ceratobasidiaceae and Sebacina, collectively called ‘rhizoctonias’ (Roberts, 1999). Although photosynthetic rhizoctonia-associated orchids may be significantly enriched in ^{15}N (Gebauer & Meyer, 2003; Liebel *et al*., 2010; Sommer *et al*., 2012), contrasting results are available for C isotope abundance (Hynson *et al*., 2013). Some rhizoctonia-associated orchids exhibit ^{13}C abundance equivalent to photoautotrophic reference species (Gebauer & Meyer, 2003), and may reflect full photosynthetic capabilities. However, ^{13}C enrichment, which would indicate partial mycoheterotrophy, was reported for *Listera ovata* (Gebauer & Meyer, 2003), *Orchis purpurea* (Girlanda *et al*., 2011) and some other species (Liebel *et al*., 2010). By contrast, significant ^{13}C depletion was found in adult rhizoctonia-associated orchids in the tribes Orchideae and Cranichideae (Hynson *et al*., 2009; Liebel *et al*., 2010).

Recent isotope abundance data by Stöckel *et al*. (2014), obtained from fully mycoheterotrophic protocorms of orchids associated with either rhizoctonias or ECM fungi, found a significantly lower ^{13}C and ^{15}N enrichment in the rhizoctonia-associated protocorms. Although the isotopic signature of rhizoctonias is currently unknown, as well as possible isotopic differences among Tulasnellaceae, Ceratobasidiaceae and Sebacinales, this finding indicates that ^{13}C and ^{15}N natural abundance is not suited to unequivocally identifying partial mycoheterotrophy in rhizoctonia-associated orchids. Thus, the mode of nutrition in these orchids currently remains an open question.

With few exceptions (e.g. Roy *et al*., 2013), stable isotope natural abundance in orchids has been investigated in plants collected at single time points, thus neglecting possible seasonal variation in isotopic signature that may reflect changes in plant nutritional requirements during the vegetative and reproductive stages. Such changes could explain, at least in part, the contrasting isotopic data in photosynthetic orchids. Seasonal changes in plant–mycorrhizal fungi combinations could also potentially lead to differences in plant ^{13}C and ^{15}N abundance if individual fungi gain access to different soil nutrient resources (see Johnson *et al*., 2012). Multiple mycorrhizal fungal partners can

be simultaneously found in photosynthetic orchids (Roy *et al*., 2009; Lievens *et al*., 2010; Girlanda *et al*., 2011; Jacquemyn *et al*., 2014), but whether these fungal communities remain stable over time is largely unknown. Most molecular studies have investigated steady-state situations in orchid mycorrhizal roots, apart from sporadic reports (Taylor & Bruns, 1999; Kohout *et al*., 2013). The abundance of fungal colonization in orchid roots and tubers during the growing season, as well as morphological features of the colonizing hyphae have been investigated in some orchid species (Rasmussen & Whigham, 2002; Huynh *et al*., 2004; Roy *et al*., 2013), but although variations were reported in some cases, the symbiotic fungi were not identified in these previous studies.

The aim of this paper was to investigate whether temporal variation occurs in the mycorrhizal fungal assemblage associated with the photosynthetic orchid *Anacamptis morio* and/or in the C and N stable isotope composition in this orchid. This wintergreen species allowed us to investigate possible changes from autumn to summer, through five different phenological stages. Mycorrhizal fungal diversity was assessed by PCR amplification of peloton DNA and molecular taxonomic identification of the internal transcribed spacer (ITS) region, whereas isotopic composition was investigated by assessment of stable isotope natural abundance in *A. morio* leaves.

Materials and Methods

Plant species

Anacamptis morio Bateman, Pridgeon & Chase is widespread in Mediterranean meadows (Rossi, 2002; Kretzschmar *et al*., 2007) and belongs to the Orchidinae subtribe in the Orchidoideae subfamily of Orchidaceae (Bateman *et al*., 2003). Adult plants form mycorrhizal associations with Ceratobasidiaceae and Tulasnellaceae (Bailarote *et al*., 2012). *A. morio* has been described as a photoautotrophic orchid (Liebel *et al*., 2010), and is characterized by the phenological stages described in Table 1.

Table 1. Phenological stages and sampling times of *Anacamptis morio* and reference photoautotrophic plants

Season	*A. morio* phenological stage	Sampling dates	Sample code	*A. morio* individuals	Reference plants species
Autumn	After summer dormancy, the tuber produces some roots and a basal rosette.	October 2009	T1	6	*Salvia pratensis*, *Plantago lanceolata*, *Ranunculus* sp., *Bromus* sp.
Winter	The plant maintains the structures already produced, but it doesn't grow more.	December 2009	T2	6	*Salvia pratensis*, *Plantago lanceolata*, *Ranunculus* sp., *Bromus* sp.
Late winter/early spring	Leaves grow until the flowering size and roots enlarge. A floral stem is produced.	February 2010	T3	6	*Plantago lanceolata*, *Ranunculus* sp., *Sanguisorba* sp., *Potentilla* sp.
Late spring	Flowering, pollination, and seed production.	March 2010	T4	6	*Plantago lanceolata*, *Ranunculus* sp., *Bromus* sp.
Summer	Seed maturation. Leaves and floral stem start drying	May 2010	T5	6	*Salvia pratensis*, *Plantago lanceolata*,

Season	*A. morio* phenological stage	Sampling dates	Sample code	*A. morio* individuals	Reference plants species
	out, the roots loose turgor and nutrients are stored in tuber for dormancy.				*Ranunculus* sp., *Bromus* sp., *Sanguisorba* sp.

Sampling

Plants were sampled from autumn 2009 to summer 2010 in a characteristic Mediterranean meadow in northern Italy. The site was assigned to the association *Festuco-Brometalia* (Braun-Blanquet, 1964) and was located 460 m above sea level (asl), in a transition zone between Mediterranean and sub-Atlantic climates. Root and leaf samples from six different orchid individuals were collected at five time points covering the different phenological stages (Table 1). Target plants were randomly chosen within a large *A. morio* population (> 100 individuals), with a minimum distance of 2 m among sampled individuals. Plants sampled at different seasons were geographically intermixed within the same population. Root samples were stored at 4°C and processed within 24 h. Pelotons were isolated from two to three roots per plant. To confirm possible trends observed in the first year, orchid roots were collected 3 yr later from four individuals in the same population, at two time points (autumn 2012 and spring 2013).

During the 2009–2010 sampling, the most recently formed leaves were collected at each time point. Leaves of nonorchid plants were also collected around each orchid individual, following the sampling scheme described by Gebauer & Meyer (2003). They served as reference plants in order to calibrate the stable isotope data.

Molecular identification of mycorrhizal fungi

Genomic DNA was extracted from fungal pelotons manually isolated from orchid mycorrhizal roots (*c*. 0.5–1 g fresh mass). Roots were rinsed with tap water and sonicated to remove adhering soil and organisms. Each root was cut into 5-cm-long segments and fungal colonization was examined under a stereomicroscope. Highly colonized root segments were teased with a sterile scalpel in a 6 cm Petri dish containing 5 ml of sterile water, in order to release the fungal pelotons (Kristiansen *et al*., 2001). Twelve pelotons per sample were collected with a micropipette and individually transferred in a PCR tube. Before PCR amplification, pelotons were disrupted by heat shock (10 min at 95°C) in 10 μl 1× PCR buffer (Sigma-Aldrich). The nuclear ribosomal ITS was amplified using the primers ITS-1OFa, ITS-1OFb and ITS-4OF, specifically designed for orchid mycorrhizal fungi (Taylor & McCormick, 2008). Linde *et al*. (2014) showed that phylogeny with ITS alone was congruent with multilocus analyses in *Tulasnella*.

Polymerase chain reaction amplification was carried out in a final volume of 25 μl, containing 1.5 μl 10× buffer (Sigma-Aldrich), 0.5 μl of each forward primer (10 μM), 1 μl of reverse primer (10 μM), 2 μl of dNTPs (2.5 mM), and 1.5 U of RED Taq DNA polymerase (Sigma-Aldrich) in a T3000 Thermocycler (Biometra, Goettingen, Germany) with the temperature profile developed by Taylor & McCormick (2008).

To improve yield, a heminested PCR was then performed using the ITS1-ITS-4OF primers. Amplifications were carried out with the same reagents and thermocycler as described before, using 1 μl of the product from the first reaction as a template and with the following temperature profile: initial denaturation for 2 min at 96°C, followed by 35 cycles with denaturation for 30 s at 94°C, annealing for 40 s at 58°C, and extension for 60 s at 72°C, followed by final extension at 72°C for

10 min. Amplicons were visualized on 1% agarose gels after staining with ethidium bromide. Because the ITS is a multicopy gene region and multiple copies were observed in preliminary sequencing analysis, PCR products from each plant were pooled and cloned using pGem-T Easy Vector kit (Promega) according to the manufacturer's instructions. Sequencing of 12 cloned ITS inserts per orchid individual sampled was performed by Macrogen (Seoul, Korea).

Sequence alignment and phylogenetic analysis

Cloned sequences were checked for quality and assembled using Geneious v 5.3 (Drummond *et al*., 2011). Taxonomic affinities were determined using the Blastn algorithm at NCBI (www.ncbi.nlm.nih.gov). For phylogenetic analyses, the ITS sequences were selected using a 'per plant' criterion: from each plant, sequences exhibiting < 99% identity were selected, whereas sequences with ≥ 99% identity were assembled to get a consensus sequence.

Alignments were generated using MAFFT (Katoh *et al*., 2002) with default conditions for gap openings and gap extension penalties. The alignments were then imported into MEGA 5.0 (Tamura *et al*., 2011) for manual adjustments.

Phylogenetic hypotheses were constructed under Bayesian inference (BI) and maximum likelihood (ML) criteria. BI of phylogeny using Monte Carlo Markov chains (MCMCs) was calculated with MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Four incrementally heated simultaneous MCMCs were run over 10 000 000 generations, under model assumption. Trees were sampled every 1000 generations, resulting in a sampling of 10 001 trees. The first 20 000 trees were discarded as 'burn-in'. For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian posterior probabilities (PPs). ML estimation was performed through RAxML v.7.0.4 (Stamatakis, 2006) with 1000 bootstrap replicates. Only PP values > 0.70 and bootstrap support (BS) > 50% are reported in the resulting trees.

Operational taxonomic unit (OTU) definition

As several ITS sequences could not be assigned to identified species, OTUs were determined. Based on the final alignment, a distance matrix was constructed and pairwise distances served as input to the program MOTHUR v.1.17.3 (Schloss *et al*., 2009) to assign sequences to OTUs at different sequence identity levels. OTUs at the 97% sequence identity threshold were compared with terminal clusters in phylogenetic analyses, receiving high support (PP ≥ 0.9% and/or BS ≥ 70%). To avoid redundancy in the phylogenetic comparison of sequences from *A. morio* and from other orchids, only a single sequence for each OTU (either the longest or the most represented sequence) was included in phylogenetic analyses. Representative sequences of each OTU were submitted to GenBank (accession numbers KJ789932–KJ789948).

Analysis of stable isotope abundance and N concentration

Leaf samples were oven-dried at 105°C and ground to fine powder. Relative C and N isotope abundances were measured using a dual element analysis mode with an elemental analyzer coupled to a continuous flow isotope ratio mass spectrometer, as described in Bidartondo *et al*. (2004). Relative isotope abundances are denoted as δ values, which were calculated according to the following equation:

$$\delta^{13}\mathrm{C} \text{ or } \delta^{15}\mathrm{N} = (R_{\mathrm{sample}}/R_{\mathrm{standard}} - 1) \times 1000[‰] \quad \text{(Eqn 1)}$$

where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard. Standard gases were calibrated with respect to international standards by using the reference substances ANU sucrose and NBS 19 for carbon isotopes and N1 and N2 for nitrogen isotopes, provided by the International Atomic Energy Agency (Vienna, Austria). Reproducibility and accuracy of the isotope abundance measurements were routinely controlled by measures of the test substance acetanilide (Gebauer & Schulze, 1991). At least six test substances with varying sample mass were routinely analyzed within each batch of 50 samples. Maximum variation of $\delta^{13}C$ and $\delta^{15}N$ within as well as between batches was always below 0.2‰. Nitrogen concentrations in the leaf samples were calculated from sample weights and peak areas using a daily six-point calibration curve based on the acetanilide measurements (Gebauer & Schulze, 1991). Acetanilide has a constant N concentration of 10.36%.

$\delta^{13}C$ and $\delta^{15}N$ values of the orchids and the nonorchid autotrophic reference plants were used to calculate normalized enrichment factors for all samples as:

$$\varepsilon_S = \delta_S - \delta_{REF} \quad \text{(Eqn 2)}$$

where *S* is a single value of a sample from an autotrophic, partially or fully mycoheterotrophic orchid, and REF is the mean value of all autotrophic reference plants from the respective plot (Preiss & Gebauer, 2008).

As reference plants, we kept a wide range of species to minimize errors when calculating relative enrichments of the orchids: *Potentilla* sp., *Ranunculus* sp., *Salvia pratensis*, *Bromus* sp., *Sanguisorba* sp. and *Plantago lanceolata*.

Statistical analyses

The composition of mycorrhizal fungal community found in *A. morio* roots in different seasons was compared by means of ordination analysis (canonical variates analysis, CVA). 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). Binary data (occurrence/absence of each OTU in individual orchid plants) were used in the 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.

The effect of season on community composition was also tested for significance by means of permutational multivariate analysis of variance (PERMANOVA; Anderson, 2005), using Jaccard dissimilarity as a distance measure, unrestricted permutation of raw (presence/absence) data and 9999 permutations. Both the *P*-value associated with the permutation test (*P*(perm)) and the Monte Carlo *P*-value (*P*(MC)) were considered. A chi-squared test was applied to evaluate differences in the individual OTU frequency in the different sampling times, and a P-value < 0.05 was considered significant. For stable isotope data, the $\delta^{13}C$ and $\delta^{15}N$ values were tested for differences between *A. morio* and reference plants at each sampling time using the Kruskal–Wallis nonparametric test and Mann–Whitney *U*-tests for *post hoc* comparisons. The same tests were also carried out to assess the significance of differences in *A. morio* $\varepsilon^{15}N$ and $\varepsilon^{13}C$ enrichment values among different sampling times.

Results

Mycorrhizal symbionts of *A. morio*

The cortical parenchyma of *A. morio* roots was found to be colonized by typical fungal pelotons at all sampling times. Although statistical analyses were not performed, at least 50% of the parenchyma cells were usually colonized in root sections. DNA amplification of *A. morio* pelotons yielded 434 ITS sequences between 500 and 700 bp in length, corresponding to a diverse spectrum of fungi, with a dominance of rhizoctonias in the genera *Tulasnella* and *Ceratobasidium* (Supporting Information, Table S1). Other fungi were sporadically detected (< 12% of all ITS sequences), and were assigned to the Basidiomycota (e.g. *Schizophyllum commune*, *Stereum hirsutum*, *Bjerkandera* sp.) and to the Ascomycota (e.g. *Alternaria* sp., *Fusarium* sp.). Among the Ascomycetes, sequences assigned to the Pezizales occurred in 30% of the *A. morio* plants (Table S1).

Phylogenetic analyses for the genera *Tulasnella* and *Ceratobasidium* were performed, and the resulting trees are shown in Figs S1–S4. For both *Tulasnella* (Fig. S1) and *Ceratobasidium* (Fig. S2), the OTUs identified by MOTHUR (> 97% sequence identity) received high support in either the BI or ML phylogenetic reconstructions. To avoid redundancy in the phylogenetic comparison of sequences from *A. morio* and from other orchids (Figs S3, S4), only one representative sequence per OTU was selected. For each genus, the dataset included sequences obtained from other orchid species as well as GenBank reference sequences. ML and BI trees had congruent topologies, so only the BI trees are shown.

In the BI phylogram of *Tulasnella* (Fig. S3), well separated clades could be distinguished. Representative sequences of OTUs *AmTul-1–AmTul-4* were found to be distributed in distant clades (Fig. S3). In particular, OTUs *AmTul-1* and *AmTul-2* fell in a well-supported cluster (PP = 1 and BS = 81) near sequences of *T. calospora*, whereas all other OTUs did not cluster close to identified sequences. OTUs *AmTul-5*, *AmTul-6*, *AmTul-7* and *AmTul-8* clustered in a single well supported clade (PP = 1 and BS = 98), but inter-OTU sequence identity was low (90.3–95.4%). OTU *AmTul-4* clustered independently. All *A. morio* OTUs were close to sequences obtained from other orchids.

The BI tree of *Ceratobasidium* (Fig. S4) shows several well supported clades. Representative sequences of the eight *Ceratobasidium* OTUs identified in *A. morio* were distributed in eight different clades, together with fungal sequences obtained from other orchids.

Owing to their frequent occurrence in *A. morio* roots, a phylogenetic tree was also constructed for the Pezizales (Fig. 1), whereas other fungal endophytes were not analyzed further because of their sporadic occurrence. For the phylogenetic tree of Pezizales, the dataset included GenBank reference sequences and pezizalean ectomycorrhiza sequences from Tedersoo *et al*. (2006) and Hansen *et al*. (2006). All pezizalean sequences from *A. morio* belonged to a single OTU (*AmPez-1*) in the Pezizaceae, which formed a well supported clade (PP = 1 and BS = 99) together with other GenBank sequences exclusively retrieved from orchids.

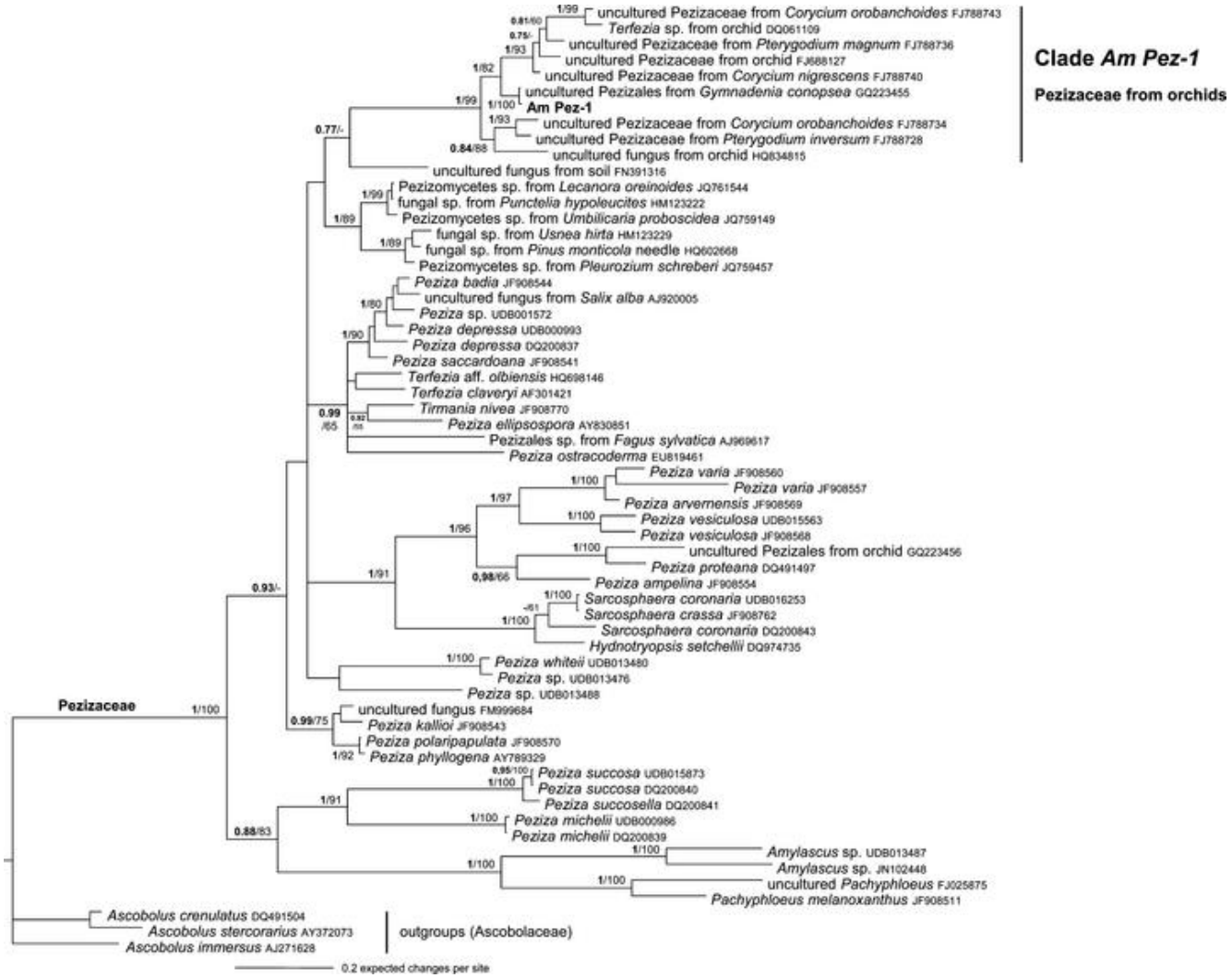

Figure 1.

Bayesian tree obtained from the internal transcribed spacer (ITS) sequence alignment of pezizalean fungi. Fungal sequences from *Anacamptis morio* were aligned with fungal sequences from reference species in the Pezizaceae, as well as sequences identified through Blastn searches. Sequences from Ascobolaceae were used as outgroup taxa, as suggested by Tedersoo *et al*. (2006). Numbers alongside each node represent posterior probabilities (≥ 0.70)/bootstrap support from 1000 maximum likelihood replicates (≥ 50%). –, < 50% bootstrap support.

Temporal variation in mycorrhizal diversity in the wintergreen orchid *A. morio*

Significant variation in the spectrum of mycorrhizal fungi, evaluated in terms of OTU composition and frequency, was found in the roots of *A. morio* collected at five time points during the growing seasons and corresponding to different plant phenological stages (Table 1). The frequency of the different fungal OTUs in these *A. morio* individuals, from sampling T1–T5, is illustrated in Fig. 2, and details of fungal occurrence in the individual plants are reported in Table S1. Some *Tulasnella* OTUs, such as *AmTul-5* and *AmTul-7*, were the most common OTUs in root samples taken at T1 and T2 (i.e. autumn and winter). The chi-squared analysis showed that *AmTul-7*, in particular, was significantly more frequent in these first two time points ($P < 0.05$) than in later sampling, together with *AmTul-2*, whose frequency increased in T2. At T1 and T2, only a small percentage of *Ceratobasidium* and Pezizaceae were found in *A. morio* roots (Fig. 2). In roots collected at T3 (i.e.

early spring), the *AmPez-1* OTU was significantly more frequent than in T1 and T2 ($P < 0.05$) and was detected in most plants. *Ceratobasidium* was scarcely found in the early samplings, but could be frequently detected in roots collected later in the season (T4 and T5, corresponding to flowering and seed setting plants). In particular, *AmCer-5* was significantly more frequent in the T4 and T5 samples ($P < 0.05$), whereas *AmPez-1* decreased in frequency and disappeared in roots sampled at T5 ($P < 0.05$). *AmTul-5*, a frequent *A. morio* symbiont throughout the vegetative season, also showed some variation in its occurrence, with a significantly lower frequency at T3 than at T1 and T5 ($P < 0.05$). Pairwise *a posteriori* PERMANOVA comparisons confirmed the occurrence of significant differences between the T1/T2 and the remaining fungal assemblages, as well as between the T3 and the T5 spectra (P(perm) and P(MC) < 0.05). Temporal variation in fungal OTU profiles in these *A. morio* roots is illustrated in the CVA (Fig. 3). In particular, the first CVA axis (which accounted for 78.8% of total variance) mainly discriminated between the summer and the autumn–winter fungal assemblages, whereas the second axis (14.4% total variance) distinguished the late spring and the late winter/early spring fungal assemblages from the remaining assemblages (Fig. 3). Very similar results were obtained when only rhizoctonias were considered (Fig. S5).

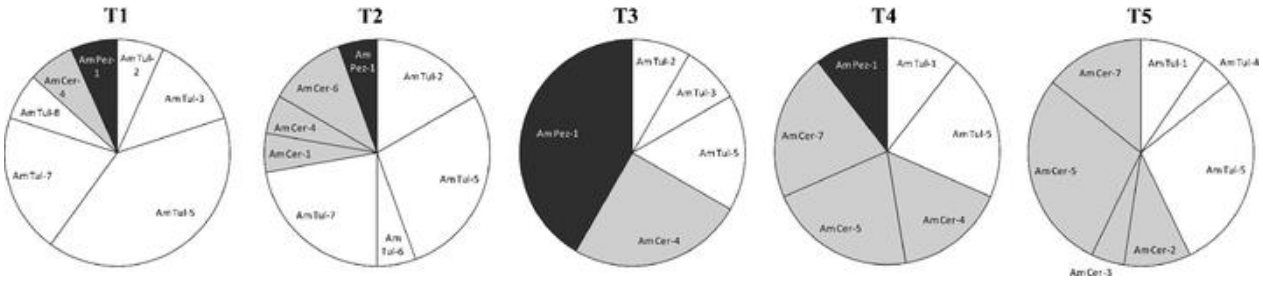

Figure 2.

Pie charts showing the frequency of sequences belonging to the different fungal operational taxonomic units (OTUs). For each sampling time (T1–T5): black, pezizalean fungi; gray, ceratobasidioid fungi; white, tulasnelloid fungi.

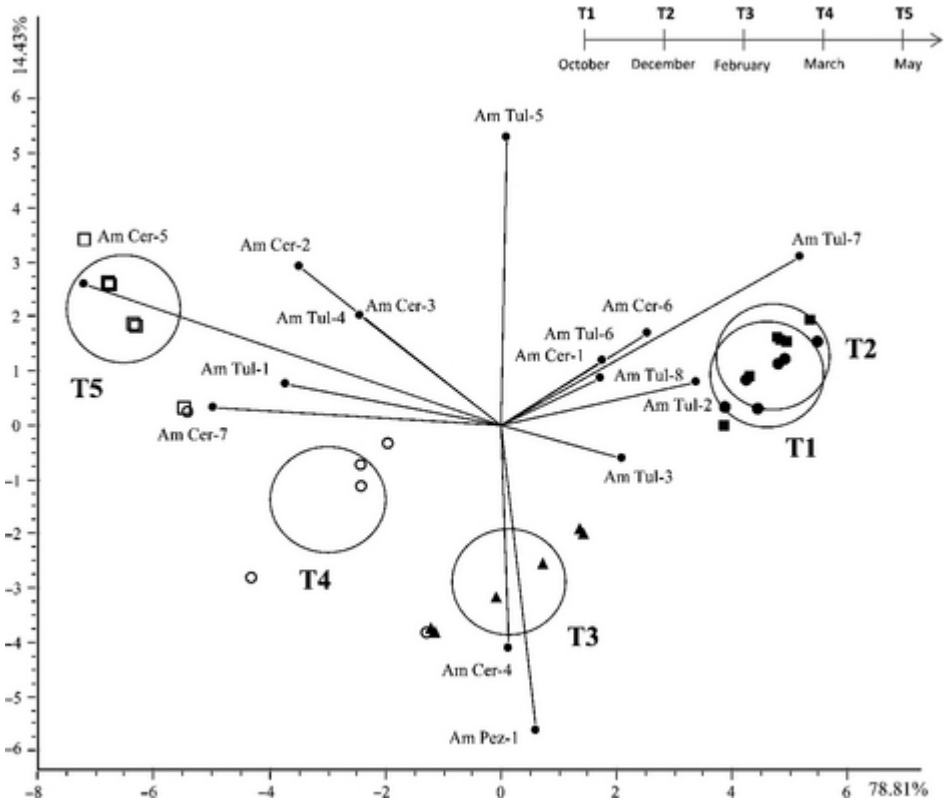

Figure 3.

Canonical variate analysis (CVA) biplots comparing fungal spectra in *Anacamptis morio* roots collected at all sampling times (T1–T5). In the analysis, operational taxonomic units (OTUs) from tulasnelloid, ceratobasidioid and pezizalean fungi were considered. Confidence circles representing each time (circles drawn around group centroids) are shown. Percentage of the total variation accounted for by each CVA axis is reported. Vectors represent correlations of each OTU with the two ordination axes. T1, closed circles; T2, closed squares; T3, closed triangles; T4, open circles; T5, open squares.

The occurrence of these fungal OTUs was investigated 3 yr later in the same *A. morio* population, in a smaller number of orchid individuals collected in autumn and spring. The mycorrhizal spectra in these roots comprised only some of the *Tulasnella* (*AmTul-1*, *AmTul-2* and *AmTul-3*) and *Ceratobasidium* (*AmCer-4*) OTUs previously identified, as well as the Pezizaceae clade (*AmPez-1*). An additional *Ceratobasidium* OTU (*AmCer-8*) was found in a single individual (Table S1). *AmTul-5*, the most commonly detected *Tulasnella* OTU in the 2009–2010 sampling, was not recorded in any of these plants. In this second sampling, the *AmPez-1* OTU was only detected in autumn (Table S1), and the mycorrhizal spectra identified in autumn and winter largely overlapped (PERMANOVA, $F = 0.958$, $P(\text{perm}) = 0.457$, $P(\text{MC}) = 0.413$).

Stable isotope natural abundance in *A. morio* leaves

Stable isotope natural abundance was measured in *A. morio* leaves sampled from T1 to T5 (Table 1). The results of statistical analyses that collectively considered all orchid samples revealed that $\delta^{15}N$ values in *A. morio* were significantly higher than in autotrophic plants, whereas $\delta^{13}C$ values were not significantly different from the autotrophic reference species (Fig. 4). However, when data were analyzed separately for each sampling time (Table S2), it was found that $\delta^{13}C$ values in *A. morio* did not significantly differ from autotrophic reference species (collectively considered) at T1 and T2 (i.e. autumn and winter), whereas they were significantly lower at T3, T4

and T5 (i.e. spring and summer). As some of the reference plant species were not detected throughout the sampling seasons, comparison of the $\delta^{15}N$ and $\delta^{13}C$ values was also made with reference species that were shared among all sampling times, yielding similar results (Table S2).

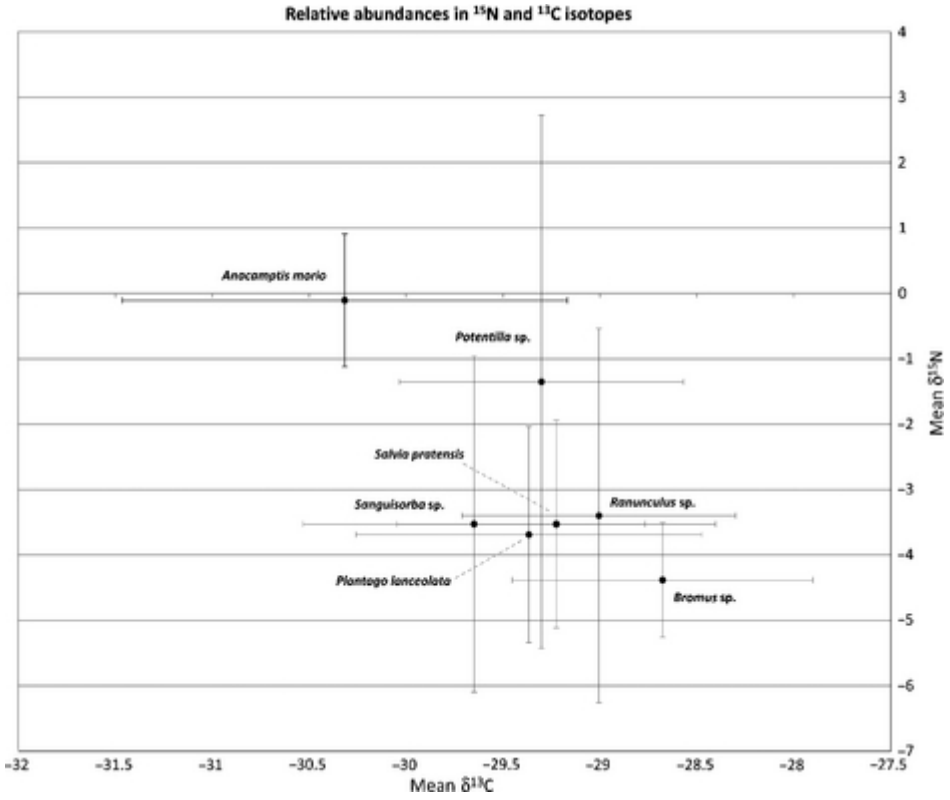

Figure 4.

Mean $\delta^{15}N$ and $\delta^{13}C$ values (mean ± SD) in leaves of *Anacamptis morio* (n = 6 per species) and neighboring autotrophic nonorchid species (n = 5; *Bromus* sp., *Plantago lanceolata*, *Ranunculus* sp., *Salvia pratensis*, *Sanguisorba* sp., *Potentilla* sp.). $\delta^{15}N$ values in *A. morio* were significantly higher than in autotrophic plants, whereas $\delta^{13}C$ values were not significantly different from the autotrophic reference species ($P < 0.05$; Mann–Whitney U-tests for *post hoc* comparisons).

Isotope enrichment factor calculations for *A. morio* leaf tissues (Fig. 5) did not show significant differences in the $\varepsilon^{15}N$ values for samples taken at different times, with the exception of the T4 samples (that had higher values). The $\varepsilon^{13}C$ values confirmed a ^{13}C depletion in all *A. morio* leaf tissues, with a significantly stronger depletion in the T5 samples (Fig. 5).

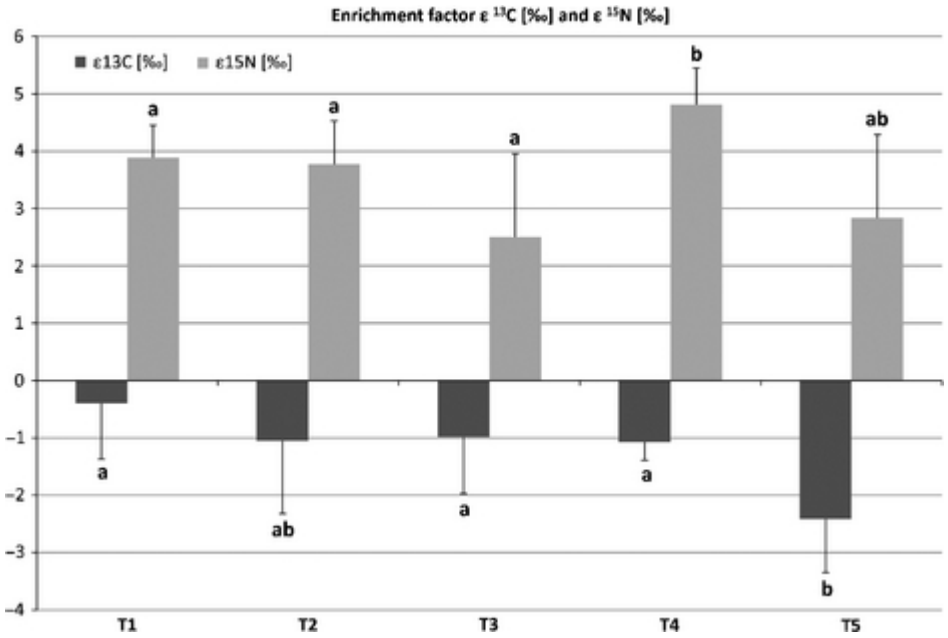

Figure 5.

Enrichment factor (ε) for ^{13}C (black bars) and ^{15}N (gray bars) of *Anacamptis morio* collected at the five different sampling times (T1–T5; mean ± SD of six replicates). After normalization, mean ε values of the autotrophic reference species are equal to zero. All $\delta^{15}N$ and $\delta^{13}C$ of *A. morio* and reference species are listed in Table 1. Different letters indicate, for each isotope, statistically significant differences ($P < 0.05$; Kruskal–Wallis test and Mann–Whitney *U*-tests for *post hoc* comparisons).

Discussion

Anacamptis morio associates with tulasnelloid and ceratobasidioid fungi, but also with a specific Pezizaceae (Ascomycetes)

Most fungal symbionts of photoautotrophic orchids belong to the form-genus *Rhizoctonia* (Taylor *et al*., 2002; Dearnaley *et al*., 2012; Jacquemyn *et al*., 2012). *A. morio* was no exception, as it was found to harbor fungi in the genera *Tulasnella* and *Ceratobasidium*. We did not find fungi with taxonomic affinities to *Sebacina*, also identified in green meadow orchids (Girlanda *et al*., 2011). We cannot exclude the possibility that the absence of *Sebacina* in this study is the result of the small sampling. However, *Sebacina* was not detected in previous investigations on *A. morio* mycorrhiza (Beyrle *et al*., 1995; Bailarote *et al*., 2012; Jacquemyn *et al*., 2014). Thus, as also suggested by Oberwinkler *et al*. (2013), *Sebacina* may be a less common mycorrhizal symbiont of European photoautotrophic orchids, as compared with the more ubiquitous *Tulasnella* and *Ceratobasidium*, with a preference for certain orchid genera.

In our work, sequences from a frequent fungal symbiont in *A. morio* roots matched some uncultured Pezizaceae identified in orchid species from distant geographic regions (e.g. Australia, South Africa and continental Europe). Ascomycetes have often been reported in orchid roots, although their ability to form typical pelotons has been shown, to our knowledge, only by Selosse *et al*. (2004), who discovered that the dominant symbiont of chlorophyllous and achlorophyllous individuals of *Epipactis microphylla* belonged to the ECM genus *Tuber* (Tuberaceae, Pezizales).

Although the family Pezizaceae includes several ECM taxa (Hansen *et al*., 2001; Tedersoo *et al*., 2006), the well supported clade that includes *AmPez-1* OTU from *A. morio* featured fungal sequences exclusively derived from green meadow orchids. Final evidence of the nature of these fungi as true mycorrhizal symbionts will require identification of ultrastructural features typical of Ascomycetes (e.g. simple septa with Woronin bodies) in intracellular pelotons. However, our findings are based on manual isolation of individual fungal pelotons before DNA extraction. This protocol greatly increases the chance to detect true symbionts. This, together with the frequent occurrence of *AmPez-1* in different sampling seasons and years, provides strong support for the hypothesis that fungi in this clade of Pezizaceae form endomycorrhiza in orchid roots. The identification of similar fungi in other orchid species (Stark *et al*., 2009) and the phylogenetic analysis by Waterman *et al*. (2011) also point to the same conclusions.

It would be interesting to understand if fungi in this newly detected clade, like other pezizalean fungi in the genus *Tuber*, can also form ECM, or whether they are exclusively capable of forming orchid mycorrhiza. Although the possibility cannot be excluded, ECM associations have not been reported for fungi in this clade. The *AmPez-1* OTU-containing clade could represent, within the Pezizaceae, a distinct lineage that switched to orchid mycorrhiza from ancestral ECM fungi. Further investigations are needed to better understand the evolution of orchid mycorrhizal symbiosis with members of the Pezizomycetes, a class of fungi with a hitherto underestimated role in nature (Tedersoo *et al*., 2013).

Temporal variation in mycorrhizal fungal diversity in *A. morio*

In this study, we demonstrate that significant temporal variation could occur in the spectrum of mycorrhizal fungi associated with the wintergreen orchid *A. morio*. This finding raises questions regarding both the mechanisms and origin of the dynamic changes in the orchid mycorrhizal interaction. As for the mechanisms, pelotons formed by orchid mycorrhizal fungi, similarly to arbuscules formed by glomalean fungi (Bonfante & Perotto, 1995), are short-lived structures that undergo a rapid turnover within the orchid cells, allowing new colonization events (Rasmussen, 1995; Peterson *et al*., 1996). Thus, the spectrum of symbiotic fungi can, at least potentially, change quite rapidly.

Temporal dynamics of fungal assemblages in mycorrhizal roots have been investigated for some mycorrhizal types, such as arbuscular, ectomycorrhizal and arbutoid mycorrhiza (e.g. Twieg *et al*., 2007; Pickles *et al*., 2010; Dumbrell *et al*., 2011; Matsuda *et al*., 2012; O'Hanlon, 2012; Bennett *et al*., 2013). In some cases, the spectrum of root-associated symbiotic fungi has been found to change over the year. For instance, some authors (Buée *et al*., 2005; Koide *et al*., 2007; Walker *et al*., 2008; Cowden & Peterson, 2013) found variation in the fungal diversity of ECM roots, although Richard *et al*. (2011) described a stable ECM community across seasons. Similarly, temporal changes in plant–AM fungal partnerships were found in arbuscular mycorrhizal roots (Dumbrell *et al*., 2011; Jumpponen, 2011; Bennett *et al*., 2013), although significant seasonal variation was not observed by other authors (see Sánchez-Castro *et al*., 2011).

Studies reporting seasonal variation in mycorrhizal fungal diversity usually consider a single year of sampling (Buée *et al*., 2005; Dumbrell *et al*., 2011; Jumpponen, 2011; Bennett *et al*., 2013; Kohout *et al*., 2013), and it therefore remains unclear whether different fungal assemblages reflect true seasonal variation (i.e. the same fungal assemblages are found repeatedly each year), or whether they simply reflect the dynamism of the soil fungal communities. Most of our data on *A. morio*, a generalist orchid in terms of mycorrhizal interactions (Bailarote *et al*., 2012; Jacquemyn *et al*., 2012, 2014; this study), are derived from 1 yr of sampling, so the question also remains open for this species. However, despite the limited sampling, the absence of the most frequent *Tulasnella*

OTU (*AmTul-5*) in *A. morio* roots collected 3 yr later would suggest a dynamic change of potential mycorrhizal partners in the soil community, rather than selection, from a common pool, of specific fungi in different seasons (and/or phenological stages). In a study by McCormick *et al*. (2006), *Goodyera pubescens* plants could maintain associations with single fungal genotypes over more than 4 yr, but they were found to associate with genetically different fungi after drought. To better understand how changes in plant–fungus combinations can arise in orchids, it would be important to investigate the persistence of potential orchid fungal symbionts in soils. High-throughput sequencing of soil cores (Davison *et al*., 2012) demonstrated that the arbuscular mycorrhizal fungal community is fairly constant during the growing season. By contrast, Jumpponen *et al*. (2010) found indications of seasonal changes of ECM fungi in soil collected close to *Quercus* plants. Profound seasonal changes were also found by 454 pyrosequencing in the composition of the saprotrophic soil fungal community (Voříšková *et al*., 2014).

Despite the highly dynamic fungal community, stable isotopes' natural abundance remained rather constant in leaves of *A. morio* collected from plants at very different stages of their life cycle, from winter dormancy to flowering and fruit setting. Because the C (and N) source at the time of leaf development determines the leaf bulk isotope signature, the small variations in stable isotopes' natural abundance observed in *A. morio* leaves collected from autumn to early spring (T1–T3) could reflect the fact that the basal rosette is mainly formed in autumn, with no new biomass being produced in winter. In these leaves, C and N are probably derived, at least in part, from resources stored in the tuber. However, new leaves formed in late spring were collected at T4 and T5, when the old tuber was drying out and a new tuber was formed. Thus, although we are unable to disentangle direct and indirect fungal contributions in the building of these new leaves, they are probably formed with new C and N resources.

All *A. morio* samples were ^{15}N-enriched, which is in agreement with previous field studies on orchids (see Hynson *et al*., 2013). Although we do not currently know the isotopic N content of rhizoctonias (or the orchid mycorrhizal Pezizaceae), ^{15}N enrichment in photosynthetic orchids has been interpreted as a fungus-to-plant N transfer, as demonstrated in laboratory experiments in mycorrhizal *Goodyera repens* fed with ^{15}N-labeled glycine (Cameron *et al*., 2006). Measurement of C stable isotope natural abundance in *A. morio* indicated instead a significant depletion in the heavier ^{13}C isotope, relative to autotrophic reference species, a peculiar behavior already reported for *A. morio* as well as for other species in the tribes Orchideae and Cranichideae (Hynson *et al*., 2009; Liebel *et al*., 2010). This unusual situation was interpreted by these authors as a consequence of carbon flow from the photosynthetic plant to the mycorrhizal fungus. However, ^{13}C depletion in *A. morio* was also recorded in winter, when low rates of photosynthesis limit the availability of carbon because of cold temperature (T2 plants where under snow cover), thus suggesting that other mechanisms might be responsible for the ^{13}C isotope depletion found in some rhizoctonia-associated photosynthetic orchids.

In conclusion, we showed that the spectrum of orchid mycorrhizal fungi can be highly dynamic in the wintergreen species *A. morio*. However, only slight changes were observed in the same orchid in terms of ^{13}C and ^{15}N natural abundance. These data indicate that *A. morio* plants do not substantially change their isotopic content despite the very different phenological stages (i.e. from basal leaf formation in autumn to flowering and seed setting in summer) and mycorrhizal fungal associates.

We do not currently know what is the contribution, if any, of the associated mycorrhizal fungi (both Basidiomycetes and Ascomycetes) to the orchid C and N metabolism (Hynson *et al*., 2013; Stöckel *et al*., 2014). Therefore, a better understanding of the isotopic signatures and nutrient sources of

rhizoctonias and other mycorrhizal fungi associated with photosynthetic orchids will be necessary to unravel the nutritional mode of these plants.

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