Mycoheterotrophic nutrition of selected European orchids in dependence on the light regime

DISSERTATION

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Dr. Heiko Liebel

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Prüfungsausschuss:

Prof. Dr. Gerhard Gebauer (Erstgutachter)
Prof. Dr. Heike Feldhaar (Zweitgutachterin)
PD Dr. Gregor Aas (Vorsitz)
Prof. Dr. Egbert Matzner

Epipogium aphyllum at one of the sampling sites (Photo: H. Liebel)

"The basic ingredient for a love of learning is the same as for romantic love, or love of country, or of God: passion for a particular subject.

Knowledge accompanied by pleasurable emotion stays with us. It jumps to the surface and when summoned, triggers other memory linkages to create metaphor, the cutting edge of creative thought. Rote learning, in contrast fades quickly into jumble of words, facts and anecdotes.

The Holy Grail of liberal education is the formula by which passion can be systematically expanded for both science and the humanities, hence for the best in culture."

(Edward O. Wilson, The creation – an appeal to save life on earth, p. 127, 2006; W.W. Norton & Company Inc., New York, USA)

Content

Zusammenfassung

Motivation und Methode: Diese Dissertation ist das Produkt des langjährigen Forschungsinteresses des Autors für unsere heimischen Orchideen und ist nicht im Rahmen eines definierten Doktorarbeitsprojekts entstanden. Um die Hauptfragestellung dieser Dissertation bearbeiten zu können, wurde die Mykorrhiza bei einer Auswahl europäischer Orchideenarten untersucht, die mit möglichst unterschiedlichen Lichtbedingungen zurechtkommen: Wie leben verschiedene Orchideenarten mit unterschiedlichen Pilzpartnern unter variierenden Lichtbedingungen zusammen? Welche Auswirkungen hat das Lichtklima auf den Nährstoffaustausch zwischen Pilz und Orchidee?

Der Widerbart (*Epipogium aphyllum*) ist die einzige chlorophyllfreie Orchidee der Arbeit. Sie ist in Europa selten und bevorzugt dunkle Waldstandorte. Das Kriechende Netzblatt (*Goodyera repens*) deckt eine weitere Lichtamplitude ab und wächst an seinen dunkelsten Standorten an der Seite des Widerbarts. Gleichzeitig besiedelt es auch komplett offene Habitate oberhalb der Baumgrenze. Aufgrund seines Netzwerks grüner Blattrosetten und der im Labor nachgewiesenen Fähigkeit Nährstoffe zum Pilzpartner zu liefern, wurde die Hypothese aufgestellt, dass die Art auch in der Natur Nährstoffe an den Pilzpartner abgibt, wenn sie voll besonnt wächst. Die entgegengesetzte Richtung eines Nährstoffflusses von der Orchidee zum Pilzpartner wird als der Normalfall angesehen bei chlorophyllfreien (vollständig mykoheterotrophen) und wenigen grünen (partiell mykoheterotrophen) Orchideenarten, die an dunklen Standorten Konkurrenzvorteile haben (z.B. beim Widerbart).

Schließlich wurde auch die Mykorrhiza von vier typischen Offenlandorchideen des Mittelmeerraumes untersucht: Lockerblütiges Knabenkraut (*Anacamptis laxiflora*), Hummel-Ragwurz (*Ophrys fuciflora*), Purpur-Knabenkraut (*Orchis purpurea*) und Pflugschar-Zungenstendel (*Serapias vomeracea*). Bisher wurde angenommen, dass der gesamte Kohlenstoffbedarf dieser Arten durch Photosynthese gedeckt wird (autotrophe Arten).

Stabile Isotopenanalysen und DNA-Analysen der Pilzpartner bilden die Grundlage für die Erforschung der Orchideenmykorrhiza und der Nährstoffflüsse zwischen den untersuchten Orchideenarten und ihren Pilzpartnern. Messungen des Lichtangebotes und des Chlorophyllgehalts in Blättern ergänzen die Studien.

Ergebnisse:

- Ähnlich anderer chlorophyllfreier Orchideenarten (z.B. Vogelnestwurz), lebt der Widerbart mit einer Auswahl weniger Pilzpartner zusammen, die über ihre Ektomykorrhiza die Verbindung zu umliegenden Bäumen herstellen. Somit unterscheidet sich der Widerbart, *Epipogium aphyllum*, von seiner südostasiatischen Schwesterart, *E. roseum*, die zwar ebenfalls blattgrünlos ist, aber ausschließlich mit saprotrophen, holzzersetzenden Pilzarten vergesellschaftet lebt.
- Der Nährstoffffluss zwischen Orchidee und Pilzpartner war beim Kriechenden Netzblatt unabhängig vom Lichtklima und variierte kaum zwischen verschiedenen Untersuchungsgebieten. Die Studie konnte zeigen, dass die untersuchten Individuen abgereichert waren bezogen auf ^{13}C unabhängig davon, ob sie an hellen oder dunklen Standorten wuchsen. Auch das Vorhandensein oder Fehlen von Pilzpartnern mit Ektomykorrhiza-Eigenschaften änderte daran nichts.
- Partielle Mykoheterotrophie (signifikanter Kohlenstofffluss vom Pilz zur Orchidee, neben Assimilation durch Photosynthese) wurde für das Purpur-Knabenkraut festgestellt, auch wenn es in einer komplett besonnten, offenen mediterranen Wiese wuchs. Es war auch diese der vier untersuchten mediterranen Offenland-Orchideenarten die die stärkste Bindung an spezifische Pilzpartner zeigte.

Schlussfolgerungen:

- Die stärkste Bindung an wenige Pilzpartner trat beim blattgrünlosen Widerbart auf. Bei allen anderen untersuchten Orchideenarten der Dissertation variiert das Artenspektrum der Pilzpartner stärker.
- Das Material für die stabilen Isotopenanalysen beim Kriechenden Netzblatt sollten zusätzlich zu ^{13}C und ^{15}N erneut mit Hinblick auf ^{2}H und ^{18}O untersucht werden, um die Orchideenmykorrhiza dieser spannenden Art unter natürlichen Bedingungen besser verstehen zu können.
- Orchideenarten mit hochspezialisierten Anpassungen an enge ökologische Nischen, wie das Zusammenleben mit wenigen spezifischen Pilzpartnern, sind verletzlich bei schnellen Veränderungen in ihren Lebensräumen (z.B. Klimawandel, Umweltverschmutzung, Änderung des Mikroklimas etc.). Deshalb ist ein tiefgründiges Wissen über ihre Nährstoffquellen und Pilzpartner wichtig für ihren nachhaltigen

Schutz. Die Pilzpartner der Orchideen könnten der Flaschenhals für das Überleben an vielen ihrer Lebensräume sein.

Summary

Background: This thesis is a result of continuous interest by the author to study our native orchids rather than a natural outcome of a PhD project. Selected European orchids that thrive at very different light conditions were chosen for this study and the main research question was to investigate their different mycorrhizal associations and fungus-plant matter exchange pathways as adaptations to the different light climate.

Epipogium aphyllum is the only achlorophyllous orchid of this investigation, which is rather rare and only found in some dark forests. *Goodyera repens* occupies habitats with a large range of light climates growing side by side with *E. aphyllum* at a sampling site at Alta (Norway) while other populations thrive at completely open sites above the tree boarder. Due to its photosynthetic activity and proven nutrient transfer to fungal partners *in vitro G. repens* is suspected to be capable of transferring nutrients to fungal partners when growing at open habitats. The opposite direction of nutrient flow (from fungus to orchid) was considered as being the common case among achlorophyllous and some green adult orchids exclusively occurring at dark forest sites. Finally, four Mediterranean orchids of open habitats were studied: *Anacamptis laxiflora, Ophrys fuciflora, Orchis purpurea, Serapias vomeracea.* These orchids were suspected to cover their complete carbon demand via photosynthesis.

Key considerations: Isotope analyses and DNA analyses of fungal partners complemented with light and chlorophyll measurements allowed studying in detail the mycorrhizal modes and sources of carbon gain of the investigated orchid species:

- A high fungal specificity was found for *Epipogium aphyllum* similar to other European achlorophyllous orchids (e.g. *Neottia nidus-avis*). *E. aphyllum* uses a narrow range of ectomycorrhizal fungi as nutrient source, which contrasts the nutritional mode of its sister species from SE Asia that lives associated with a saprotrophic wood-decomposing fungus.
- Nutrient transfer between fungus and orchid in *Goodyera repens* was independent of the light conditions at the investigated sites. The studied individuals were depleted in ^{13}C no matter if they were growing at dark or light sites, or if their fungal partners were believed to have ectomycorrhizal capabilities or not.

- Partial mycoheterotrophy was detected in *Orchis purpurea* even if it was growing at completely open Mediterranean meadows. The latter species showed also the strongest fungal specificity among the four investigated orchids in the Mediterranean region.

Conclusions:

- Fungal specificity is highest in the achlorophyllous orchid *Epipogium aphyllum* and varies in all other orchids of the investigations of this thesis.
- Plant samples especially of *Goodyera repens* should be re-investigated in the future with a new approach that uses ^{2}H and ^{18}O in addition to ^{13}C and ^{15}N to improve the understanding of the mycorrhizal nutrition *in situ*.
- Orchids with precise adaptations to narrow ecological niches, like high fungal specificity, are vulnerable to fast changes in the environment (e.g. human impact, climate change and so on). Therefore, knowing their nutritional mode and their fungal associations is important for their protection. Fungal partners might be the bottleneck for their survival in many habitats.

1. Introduction

Motivation

"Orchids are the most diverse flowering plants on Earth as well as arguably the most aesthetically pleasing" (Wilson 2006, p. 146). Passion and serious interest in orchids and their ecology by the author are the basis for the publication of the presented papers and the doctoral thesis, which was written on personal initiative and with continuous support by Prof. Gebauer predominantly during free-time over several research years.

Basic research on the nutrition of orchids is not only fascinating, but also provides essential prerequisites for the conservation of native orchid species. Studies on mycorrhizal strategies were the basis for a conservation project to save *Epipactis palustris* L. Crantz from extinction in Southern Norway. The last two existing plants at a site were rescued for *ex situ* conservation until the habitat conditions were improved (Røsok et al. 2013). *Ex situ* conservation is only possible if the orchids' mycorrhizal mode and fungal partners are known. In general, orchid conservation is complex as the orchids' ecological interactions are highly specific concerning mycorrhizal fungi, pollinators and host trees (Fay et al. 2015).

Mycorrhizal modes of orchids

All orchids depend from the earliest development stage on mycorrhizal fungi (Leake 1994). The orchids' seeds are tiny and do not contain sufficient nutrients for germination. A large number of fungal taxa facilitating orchid seed germination has been described (see Rasmussen et al. 2015). Fungal partners most often belonging to the ubiquitary saprotrophic or pathogenic genera *Ceratobasidium s.l.*, *Tulasnella* and the Sebacinales (part of the *Rhizoctonia* group; Dearnaley et al. 2012) are known since long time (Bernard 1909) to supply the seeds of many later on chlorophyllous orchids from temperate grasslands or tropical tree crowns with organic and mineral nutrients and facilitate their germination and development.

Based on molecular biological approaches later fungi simultaneously forming ectomycorrhizas with forest trees were discovered as fungal hosts of temperate forest-dwelling orchids (Taylor and Bruns 1997; Bidartondo *et al.* 2000; McKendrick *et al.* 2000). In addition, some orchids from SE Asia were identified to germinate specifically with saprotrophic wood- or litter-decaying fungi, previously considered as non-mycorrhizal (e.g. Yagame et al. 2007). This fully heterotrophic nutritional mode in the seedling (protocorm) stage is called initial mycoheterotrophy and represents an adaptation found in all orchids. Surprisingly, only few genes for pathogen resistance could be discovered in orchid protocorms creating a fungus-

friendly microhabitat (Perotto et al. 2014). In the adult phase most native orchids produce chlorophyll and switch to putatively autotrophic nutrition. Still, mycorrhizal fungi are present in all adult orchids raising the big question of their function. For some orchids this function is rather obvious. These orchids do not perform the step towards autotrophy and remain fully mycoheterotrophic. Thus, initial mycoheterotrophy is considered to be the "evolutionary starting point" of full mycoheterotrophy (Merckx et al. 2013a) as the orchids have the prerequisite of obtaining carbon from fungi already from the initial stage (Waterman et al. 2013).

Out of 25 000 known orchid species (Dressler 2004) more than 235 mycoheterotrophic orchid species are known to lack chlorophyll and the ability to assimilate organic carbon through photosynthesis (Leake 1994, Merckx et al. 2009, Imhof 2010). They depend on fungal partners for all their organic carbon and nitrogen supplies.

It is a matter of ongoing discussion, if nutrients from fungi are transferred without destroying fungal cells as it would be the case in orchids with bidirectional carbon transfer (e.g. in chlorophyllous *Goodyera repens* (L.) R.Br.) or if nutrient transfer occurs after lysis of the fungal pelotons (e.g. in achlorophyllous *Rhizanthella gardneri* R.S. Rogers, Bougoure et al. 2014). There is experimental evidence for both mechanisms (Kuga et al. 2014). Nutrient transfer in a tripartite symbiosis (tripartite symbiosis between tree, fungus and orchid, Bidartondo 2005) could be experimentally shown with the help of ^{13}C-labelled CO_2 applied to the foliage of the host tree and [^{13}C-^{15}N]-glycine fed to the common fungal partner. Both sources traced to the fully mycoheterotrophic *Rhizanthella gardneri* (Bougoure et al. 2010). More than one third of all fully mycoheterotrophic orchids are distributed in Southeastern Asia, which is the global hotspot for mycoheterotrophic orchids (Merckx et al. 2013b) and associate predominantly with saprotrophic and litter and wood decaying fungi (Hynson et al. 2013). Europe, however, is very poor in fully mycoheterotrophic orchid species being restricted to only *Neottia nidus-avis* and *Epipogium aphyllum* Sw. Other species belonging to the genera *Limodorum* and *Corallorhiza* have been misinterpreted in the past to obtain all organic carbon from fungi (Merckx et al. 2013a) but recent research showed that they still obtain carbon to a minor extent from photosynthesis (Girlanda et al. 2006; Zimmer et al. 2008; Cameron et al. 2009). The fungi involved in orchid mycorrhizas in the temperate zone are mostly ectomycorrhizal fungi that transport nutrients from trees to the orchid while most fully mycoheterotrophic orchids in the tropics associate with saprotrophic fungi (Hynson et al. 2013). *Epipogium roseum* (D. Don) Lindl. and representatives of the closely related genus *Gastrodia*

have been shown to associate to saprotrophic fungi, i.e. wood-decomposing fungi, delivering nutrients to the orchid (Yamato et al. 2005; Ogura-Tsujita et al. 2009; Martos et al. 2009; Dearnaley & Bougoure 2010). The dependence on saprotrophic fungi, however, seems to be limited to humid and warm climates in forests with fast rates of litter decomposition where it then seems to be more common than full mycoheterotrophy based on ectomycorrhizal fungi (Lee et al. 2015). Typical representatives of fully mycoheterotrophic orchids appear, for example, in the genera *Cyrtosia*, *Gastrodia*, *Wullschlaegelia* and *Epipogium* (Rasmussen 2002; Yamato et al. 2005; Martos et al. 2009; Ogura-Tsujita et al. 2009; Dearneley & Bougoure 2010). It was a matter of discussion, if the European sister species of *Epipogium roseum*, *E. aphyllum* might be associated with saprotrophic fungi too. It thrives in humid forests as well although the climate at its sites is pronounced colder. This hypothesis was among others addressed in this doctoral thesis and is highly relevant for its protection. *Epipogium roseum* can be successfully cultivated *in vitro* due to its connection to saprotrophic fungi of the family Psathyrellaceae (Yagame et al. 2007). A saprotrophic mycorrhizal strategy in *E. aphyllum* would increase chances to grow also this species *in vitro* and reintroduce it at sites where it has gone extinct.

With the help of carbon (C) and nitrogen (N) stable isotope natural abundance analyzes, a strategy intermediate between autotrophy and full mycoheterotrophy was found to be used by some chlorophyllous orchids: Partially mycoheterotrophic orchids (see Gebauer & Meyer, 2003) thrive mostly in dark forests and their special adaptation to the poor light climate is based on the combination of effective use of fungal organic nutrients combined with photosynthesis that covers the nutrient supply in parts.

Fungal tissue of ectomycorrhizal fungi (ECM) is known to be enriched in the heavy isotopes ^{13}C and ^{15}N compared to surrounding plants (Gebauer & Dietrich 1993; Gleixner et al. 1993). Orchids exploiting fungal organic compounds show isotopically enriched organic matter, which allows estimating the degree of fungally derived C and thus the grade of partial mycoheterotrophy (Gebauer & Meyer 2003). Labelling experiments with ^{13}C confirmed the results from stable isotope analyses in *Corallorhiza trifida* Chatel. showing that the green to greenish-brown orchid receives a large proportion of its C from fungi (Cameron et al. 2009). Molecular DNA analyses revealed that partially mycoheterotrophic orchids show most commonly a connection to ectomycorrhizal fungi (Bidartondo et al. 2004) and only less frequently to mostly saprotrophic fungal partners of the genera *Ceratobasidium* and *Tulasnella* (Liebel et al. 2010, Girlanda et al. 2011). *Epipactis* species for example often live associated

with members of Basidio- and Ascomycota including even truffles (*Tuber spec.;* Selosse et al. 2004).

Latest research on isotope abundances in putatively autotrophic orchids from temperate Europe breaks new ground. With the help of ^{2}H (and ^{18}O) it could be shown that also chlorophyllous orchids associated with fungi of the polyphyletic *Rhizoctonia* group are partially mycoheterotrophic as their isotopic signal in H isotopes differs significantly from autotrophic reference plants while no consistent difference in the isotope abundance of C and N was found. This "hidden partial mycoheterotrophy" was not discovered until 2016 (Gebauer et al. 2016) and might be widespread among orchids that so far have been considered as completely autotrophic (e.g. Sommer et al. 2012).

The isotopic enrichment in the orchids for ^{13}C and ^{15}N depends on the fungal source (saprotrophic fungi, SAP; ectomycorrhizal fungi, ECM) and can be classified as follows (Hynson et al. 2013, mod.):

$$\delta^{15}N \text{ (SAP-MH)} < \delta^{15}N \text{ (autotrophic plants)} < < \delta^{15}N \text{ (ECM-MH)}$$

$$\delta^{13}C \text{ (SAP-MH, wood decaying fungi)} \approx \delta^{13}C \text{ (ECM-MH)} >> \delta^{13}C \text{ (autotrophic plants)}$$

$$\delta^{13}C \text{ (SAP-MH, litter decaying fungi)} \approx \delta^{13}C \text{ (ECM-MH)} > \delta^{13}C \text{ (autotrophic plants)}$$

A clear link exists between the light climate at the site and the amount of fungal organic compounds taken up by some green partially mycoheterotrophic orchids living associated with ECM fungi. More light (higher mean diurnal photon flux density) led to increased C-uptake through photosynthesis and decreased uptake of fungal C (Preiss et al. 2010). A study of albinos and individuals with variegated and green leaves of *Cephalanthera damasonium* (Mill.) Druce showed recently that the amount of fungal compounds taken up by the orchid depends on the leaf chlorophyll content as well (Preiss et al. 2010; Stöckel et al. 2011).

Figure 1: *Calypso bulbosa* at a sampling site at Siljeåsen (Sweden, photo: H. Liebel).

A nutrient transfer from the orchid to the fungus (*Ceratobasidium* symbiont) was suggested only for *Goodyera repens* based on a laboratory experiment with labeled C and N (Cameron et al. 2006; 2008). A direct proof of nutrient transport from the orchid to the fungus *in situ* has not yet been shown. The first documented depletion in ^{13}C in an orchid compared to autotrophic surrounding plants was *Epipactis gigantea* Dougl. ex Hook. Reasons for the depletion in ^{13}C of orchid material compared to autotrophic reference species are not known. Depletion in ^{13}C was recently found e.g. in the boreal forest orchid *Calypso bulbosa* (L.) Oakes fig. 1) from Sweden ($\varepsilon^{13}C$: – 2.3 ‰ compared to autotrophic references; Liebel et al., unpubl. data).

Different mycorrhizal strategies under different light habitat conditions

Mycorrhizal strategies in European orchids depend obviously on different factors. The necessity to exploit fungal partners by using their organic nutrients has been interpreted as an adaptation to dark sites where photosynthesis cannot cover (entirely) their C demand. A variable use of fungal C of partially mycoheterotrophic orchids living with ECM fungi depending on the light conditions was investigated and found especially in representatives of the orchid tribe

Neottiae like members of the genus *Cephalanthera. Cephalanthera damasonium* and *C. rubra* (L.) Rich. for instance have been shown to receive more fungal C when growing in dark habitats than in light habitats where their ^{13}C-signal might not even be distinguished from autotrophic surrounding vegetation (Preiss et al. 2010; Stöckel et al. 2011).

A shading experiment with *Epipactis* species showed as well that less light and photosynthetic activity can be compensated by the exploitation of fungal nutrients (Gonneau et al. 2014). In turn, another field experiment with partially mycoheterotrophic *Limodorum abortivum* (L.) Sw. eliminated all fungal partners by applying fungicides to the soil. The orchid compensated the loss of fungal partners by a stronger pigmentation and increased photosynthetic activity. This underlines the preference to use fungal partners under natural conditions (Bellino et al. 2014). However, it is not known if orchids that supposedly transfer nutrients to fungi (e.g. Rhizoctonias), might deliver more material at light sites than at dark sites. How the mycorrhizal interaction works and why it is useful for an orchid to obtain organic C from fungi at a site with full light conditions are still open questions.

Focus of the thesis

This doctoral thesis aims to investigate further the mode of nutrition of orchids that grow under most different light conditions (see also Table 1).

Main hypotheses addressed in the thesis:

I.) *Epipogium aphyllum* lives in dark forests in association with saprotrophic fungi as described for *Epipogium roseum* (Paper 1).

II.) *Goodyera repens* gets organic compounds from fungi when growing at a dark site. It gives organic compounds to fungi when growing at open sites (Paper 2).

III.) Partial mycoheterotrophy and high fungal specificity does not appear in Mediterranean meadow orchid species growing at completely open, light sites (Paper 3).

Table 1: Investigated orchid species of this thesis, supposed nutritional mode, light and habitat preferences.

Investigated orchid	Supposed nutritional mode	Light preference	Habitat preference
Epipogium aphyllum (Paper 1)	Full mycohetotroph with saprotrophic fungi?	Dark	Boreal and nemoral, humid forests
Goodyera repens (Paper 2)	Mycorrhizal with the ability to deliver and obtain organic compounds to/from fungus?	Dark to light	Boreal and nemoral forests, subalpine grassland
Anacamptis laxiflora, Ophrys fuciflora, Orchis purpurea, Serapias vomeracea (Paper 3)	Partial mycoheterotrophs?	Light	Mediterranean grasslands, shrublands

2. Synopsis

2.1 Paper 1: *Epipogium aphyllum* lives associated with ectomycorrhizal fungi

The ghost orchid *Epipogium aphyllum* (Fig. 2) is known for its unusual behavior in terms of irregular above-ground appearance and the lack of chlorophyll.

Figure 2: *Epipogium aphyllum* at the sampling site at Finsåsskogen (Norway, photo: H. Liebel).

The lack of chlorophyll implicates that this orchid cannot use photosynthesis to assimilate C and actually it occupies its ecological niche in dark forests. *E. aphyllum* may flower in one year and then live below-ground for several years before another shoot is formed. Therefore, *E. aphyllum* is difficult to find and to sample. Only few populations in Europe are large enough so that blooming individuals appear every year. At a field site in Norway an abundant population exists where almost yearly flowering was reported (Hegre 1998): Finsåsskogen (64.21571°N, 12.23274 °E, 60 m asl), county of Northern Trøndelag. This site was chosen to investigate the

nutritional mode of this orchid via stable isotope analyses by taking shoot samples of *E. aphyllum* and leaf samples from adjacent reference plants.

Root samples of the orchid were collected for the identification of fungal associates *via* DNA analyses.

The fungal symbionts of *Epipogium aphyllum* at this site were only two species: *Hebeloma velutipes* and *Inocybe geophylla*. Both species are considered ECM fungi that are lacking host tree specificity (Kuyper 1986; Aanen et al. 2001; Matheny 2005). The findings are in accordance with those of a study performed simultaneously by Roy et al. (2009) who found fungi of the family of Cortinariaceae as mycorrhizal partners of *Epipogium aphyllum*. These results show that *E. aphyllum* is not restricted to a single fungal genus to cover its nutrient supply. The fungal association with ECM fungi is unexpected as *E. aphyllum*'s sister species *E. roseum* and other close-related orchid species like *Cyrtosia javanica* Blume, *Gastrodia confusa, G. falconeri, G. similis* and *G. sesamoides* associate with litter and wood decomposing SAP basidio- and agaricomycetes (Yamato et al. 2005; Martos et al. 2009; Ogura-Tsujita et al. 2009; Dearnaley & Bougoure 2010; Lee et al. 2015).

The stable isotope natural abundance of ^{13}C and ^{15}N in plant tissue of *E. aphyllum* confirms the orchid's fully mycoheterotrophic nutritional strategy (Fig. 3). The orchid is significantly enriched in ^{13}C compared to its autotrophic reference plants. The enrichment factor $\varepsilon\ ^{13}C$ of 8.6 ± 0.7 ‰ is in the typical range of other fully mycoheterotrophic orchids that live associated with ECM fungi. The average enrichment factor for all until then investigated fully mycoheterotrophic plants associated to ECM fungi (orchids and representatives of the Monotropoideae) was $\varepsilon\ ^{13}C$ of 7.2 ± 1.6 ‰ (n: 92; Preiss & Gebauer 2008). Data from fully mycoheterotrophic orchids living associated with litter decomposing saprotrophic fungi might be even more enriched in ^{13}C than mychoheterotrophic orchids living together with ECM fungi ($\varepsilon\ ^{13}C$ of around 10 ‰, Martos et al. 2009; Ogura-Tsujita et al. 2009). Mycoheterotrophic orchids living associated with wood decaying saprotrophic fungi are strongest enriched in ^{13}C ($\varepsilon\ ^{13}C$ up to 12 ‰) as wood is more enriched than litter (Lee et al. 2015).

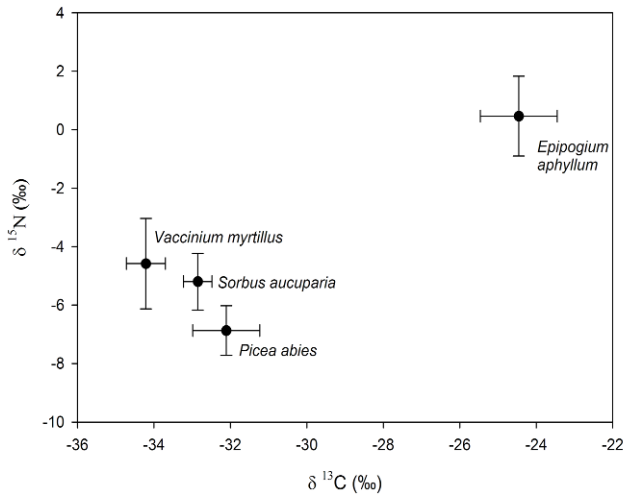

Figure 3: Stable isotope natural abundance of ^{13}C and ^{15}N ± 1 SD in the MHO *Epipogium aphyllum* and its autotrophic reference plants (n=5).

The enrichment factor ε ^{15}N of 6.0 ± 2.1 ‰ found for *E. aphyllum* is a low value in comparison to the arithmetic mean of all investigated mycoheterotrophic plants associated with ECM fungi (ε ^{15}N: 12.0 ± 1.7 ‰, *n* = 92; Preiss & Gebauer 2008). Single ε ^{15}N values in the same range as *E. aphyllum* have been reported from few individuals of *Neottia nidus-avis* (Zimmer et al. 2007; 2008).

The new ε values of this and related studies for ^{13}C and ^{15}N were used to calculate a new overall enrichment factor for mycoheterotrophic plants (ε ^{15}N ± 1 SD of 12.8 ± 3.9 ‰ and ε ^{13}C ± 1 SD of 7.2 ± 1.6 ‰, *n* = 173 based on 13 fully mycoheterotrophic species) which can be used in turn to estimate the ECM fungal contribution to organic nutrient uptake by partial mycoheterotrophic plants where fully mycoheterotrophic plants are lacking. This approach has recently been refined distinguishing between fully mycoheterotrophic orchids and Ericaceae reviewing all available data. The new ε values for fully mycoheterotrophic orchids for ^{13}C and ^{15}N were recalculated: 8.0 ± 0.1 ‰ and 11.5 ± 0.3 ‰, respectively (Hynson et al. 2016).

The N concentration in the *E. aphyllum* samples is significantly ($P < 0.001$) higher than in the leaves of autotrophic reference plants, with 2.8 ± 0.5 mmol g_{DW}^{-1} (DW: dry weight) *versus* 0.9 ± 0.3 mmol g_{DW}^{-1}, respectively. The strikingly high N concentrations in this mycoheterotrophic orchid may be caused by nutrient gain from obligate ECM fungi. Such high N concentrations are in the range usually found for legumes associated with N_2-fixing bacteria (Gebauer *et al.* 1988). Hynson et al. (2016) showed that N concentrations vary significantly between plant families (in this study Orchidaceae *versus* Ericaceae) but not significantly for example between

fully and partially mycoheterotrophic orchids. Even mycoheterotrophic Ericaceae, however, showed lower N concentrations indicating differences in the physiology of involved ECM fungi or their use by the mycoheterotrophic plants. The incorporation of fungal metabolites after lysis of the pelotons inside the root cells of mycoheterotrophic orchids could produce an N surplus.
The study provides new data to improve the understanding of the distribution of an endangered orchid species. The conservation management will need to consider the orchids' fungi, tree hosts and their distribution patterns. *E. aphyllum* is an epiparasitic orchid, e.g. it depends on a living ECM tree and uses ECM networks as carriers of organic nutrients in a tripartite symbiosis.
Uniquely, both saprotrophic and ECM fungal partners are used within the same orchid genus and in closely related phylogenetic clades.

2.2 Paper 2 - *Goodyera repens'* mycorrhizal mode at different light climates

Goodyera repens (Fig. 4) is a very special orchid species, which is especially suited to test the main hypothesis of this thesis. This orchid grows in a large variety of ecological niches, which cover dark forests as well as light completely open habitats. In addition, the species has been investigated *in vitro* before and it was shown for the first time that an orchid can actually transfer nutrients to the fungus (Cameron et al. 2006, 2008). In field experiments examining the stable isotopes of C and N, the orchid differed from other investigated orchids by showing plant tissue depleted in ^{13}C compared to autotrophic reference plants (Zimmer et al. 2007, Hynson et al. 2009, Johansson et al. 2015). In Norway, the species is widespread and could be studied at very different light conditions. This study demanded the biggest organisatory effort for the field work of this thesis. Luckily, trips to the sampling sites in Northern Norway could partly be combined with field work for other professional projects. Leaf samples of *Goodyera repens* and three reference species were collected at four sites in the county of Sør-Trøndelag, Norway (see table 2).

Figure 4: Flowers of *Goodyera repens* (left, photo: H. Liebel) and author during sampling at Alta (Northern Norway, right). Equipment used: Light sensor (upper right), chlorophyll measurement unit, field books and plant samples (photo: A. Liebel).

Table 2: Sampling sites of *Goodyera repens* in the county of Sør-Trøndelag, Norway.

Location	N coordinate [°]	E coordinate [°]	Altitude [m asl]	Number of samples
Olavsspranget	63.44999	10.26819	260	8
Flatholmen	63.43914	10.83037	3	3
Brennberga	63.40156	10.83836	200	4
Jervfjellet	63.33861	10.69483	500	7

The different locations are not more than 40 km apart from each other to ensure similar climatic conditions. All locations are characterized by more or less dark, dense forest of *Picea abies*. The location at Jervfjellet makes the only exception. Here, *Goodyera repens* grows directly at the tree boarder in a completely open subalpine environment. The advantage of this subset of

samples is the large variation in light regimes at the different locations. An unwanted factor which varies largely is the altitudinal difference between the sites.

A second subset of 18 light sensors and orchid samples was taken from a 2 km^2 large area at Lille Raipas in Alta municipality, Finnmark county, Norway (69.93222 °N, 23.37884 °E). Here the altitudinal difference between the sites of the different individuals of *Goodyera repens* was small (80 - 250 m asl).

The leaves of *Goodyera repens* and reference species were collected in a rather open northern boreal birch forest (*Betula pubescens*) in different expositions. Here, the different light climates are basically determined by different expositions and to a minor degree by varying forest canopies.

A large population of *Epipogium aphyllum* was found by the author growing side by side with *Goodyera repens.* Due to its special ecology and rarity, the site coordinates and a comprehensive plant relevé were published in the Journal of the Norwegian Botanical Society (find it attached in the appendix of this thesis; Liebel & Krill 2011).

Light measurements were performed for this project. Close to each of the 40 *Goodyera repens* individuals (max. 20 cm distance), a calibrated light sensor (silicon photodiode BPW 21, Infineon, Germany) was installed at the same height as the basal leaves of the orchids (see also Fig. 7). The light sensors were connected to data loggers (HOBO H8, ONSET, USA) that were buried in the ground. The irradiance was measured every twenty minutes from the day of installation (samples in Southern Trøndelag: 16 May 2011, samples in Finnmark: 15 June 2011) until the day of collection (samples in Southern Trøndelag: 12 August 2011, samples in Finnmark: 26 August 2011). Measured values were converted into photosynthetic active radiation (μmol photons m^{-2} s^{-1}) and averaged as daily means from sunrise to sunset as described by Preiss et al. (2010).

Chlorophyll concentrations in leaves from *Goodyera repens* and surrounding non-orchids were measured *in-situ* with a chlorophyll meter. Five measurements were taken on each leaf and the average value was calculated as described by Stöckel et al. (2011). The output value given by the chlorophyll meter is a Soil Plant Analysis Development (SPAD) value which is correlated to the chlorophyll concentration in the leaf.

Whenever flowering/fruiting individuals were found together with non-flowering rosettes, samples for isotopic abundance of ^{13}C and ^{15}N were collected and the chlorophyll concentrations measured and compared with each other. Interestingly, fruiting/flowering individuals often were paler in colour and contained less chlorophyll than non-

flowering/fruiting plants. The ^{13}C signal however was similar in both pairs indicating that costs for flowering/fruiting were not compensated by a larger nutrient transfer from fungi as it was shown in the genus *Cephalanthera* (Stöckel et al. 2011). The flowering/fruiting individuals were significantly depleted in ^{15}N and showed distinctly lower N concentrations which was interpreted with the nutritional costs due to the formation of fruits and flowers rather than due to changes in nutrition.

The main hypothesis of this study assuming that *Goodyera repens* transfers more nutrients to fungal partners when growing at open sites with strong irradiation has to be rejected. Isotopic signals showed that the orchid is depleted compared to its autotrophic reference plants independently from the light regime or the site. The depletion in ^{13}C depends on the intercellular partial pressure of CO_2 (Farquhar et al. 1989) which is controlled by the opening of the stomata. At shady and humid sites stomata tend to be more open than at strongly irradiated sites and plant tissues are more depleted in ^{13}C due to the higher intercellular CO_2 partial pressure. The same pattern could be observed in the autotrophic reference plants (Fig. 5).

It seems that *Goodyera repens* compensates the lack of irradiance rather with the help of larger clonal networks than by exploiting fungal partners stronger. This hypothesis should be the focus of a future investigation.

The predominant fungal symbionts in *Goodyera repens* were representatives of the genera *Tulasnella* and *Ceratobasidium* which are saprotrophic fungi belonging to the polyphyletic group of Rhizoctonias (Smith & Read 2008) which might be a poor source of fungal C for the orchid (see also the subchapter of the Introduction presenting the "different mycorrhizal strategies under different light habitat conditions"). Few fungal species that have ectomycorrhizal abilities like *Russula, Lactarius* and *Sebacina* were identified as mycorrhizal partners.

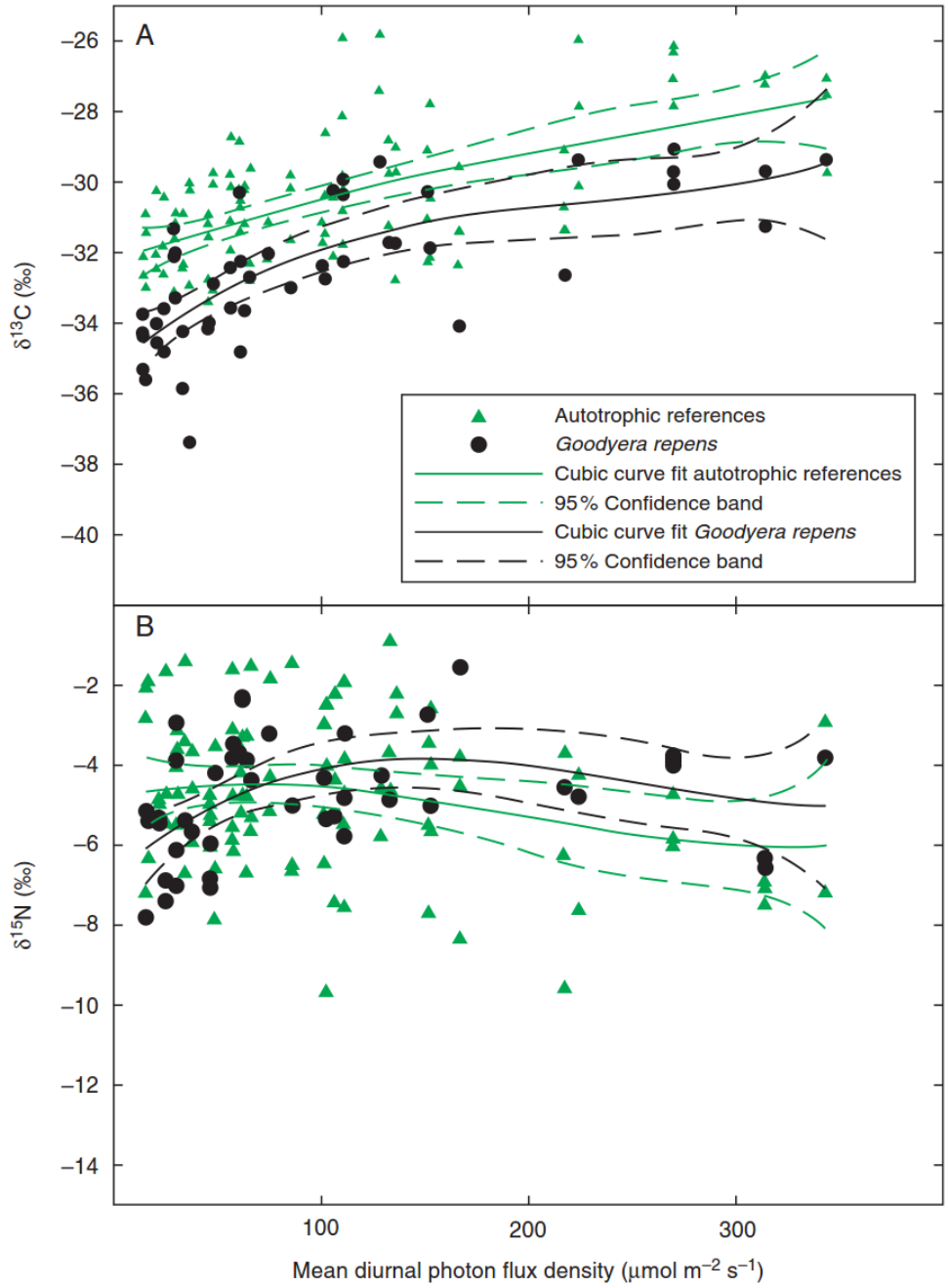

Figure 5: Leaf $\delta^{13}C$ (A) and $\delta^{15}N$ values (B) dependent on light availability for each investigated individual of *Goodyera repens* and for the respective autotrophic reference plants.

Obviously, *Goodyera repens* does not have the potential to use these fungal partners in an efficient manner as other orchids that obtain significant amounts of their nutrition from ECM partners like *Epipactis helleborine* or *Cephalanthera damasonium* (e.g. Bidartondo et al. 2004, Julou et al. 2005, Liebel et al. 2010, Hynson et al. 2013, see Fig. 6) which can compensate a lack of light by increasing the use of ECM fungal nutrients. The association with these fungal

partners might be a pre-requisite for the colonization of more shaded habitats and the change to mycoheterotrophic nutritional strategies.

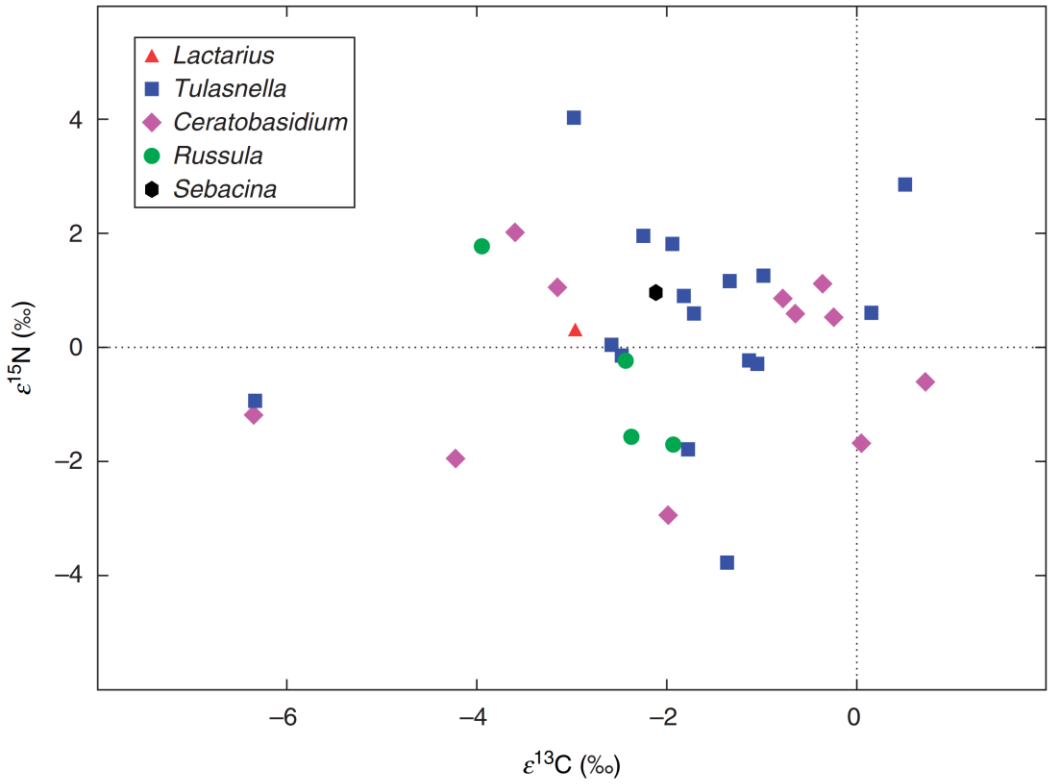

Figure 6: Comparison of the leaf enrichment factors ε ^{13}C and ^{15}N in individuals of *Goodyera repens* associated with different mycorrhizal fungi. For the autotrophic reference plants ε is zero by definition.

Figure 7: Light sensor surrounded by flowering individuals of *Goodyera repens* at the sampling site Olavsspranget close to Trondheim (Norway, photo: H. Liebel).

2.3 Paper 3 - Partial mycoheterotrophy in Mediterranean meadow orchids

Until recently, no orchids from open habitats were known as gaining nutrients from mycorrhizal partners at a significant level (Gebauer *et al.* 2016). New methods combined with each other however facilitated the detailed investigation of orchid mycorrhiza at open habitats. In this study four Mediterranean orchids, *Anacamptis laxiflora*, *Ophrys fuciflora*, *Orchis purpurea*, *Serapias vomeracea* (Fig. 8), were sampled mostly from one site in Liguria (Northern Italy) where the open grassland vegetation might be classified as typical plant societies belonging to the *Festuco-Brometalia* with one wet part of the meadow which might be classified as a *Calthion* (site of *Anacamptis laxiflora*).

Figure 8: Investigated orchid species at the site Cosseria (Italy): *Anacamptis laxiflora* (upper left), *Serapias vomeracea* (upper right), *Ophrys fuciflora* (lower left) and *Orchis purpurea* (lower right corner, photos: H. Liebel).

The mycorrhizal fungi found in intracellular coils of the orchid roots were not only investigated with the help of standard DNA-analysis with the primer pairs ITS1F/ITS4 but also with primers that are especially adapted to trace *Rhizoctonias* (ITS1F/ITS4B, ITS1F/ITS4-Tul). As the presence of several fungal partners in the roots give overlapping DNA signals, cloning was necessary and it was performed with the help of restriction fragment length polymorphism analysis (RFLP). In all four orchid species, *Rhizoctonias* belonging to the genera *Tulasnella, Ceratobasidium* and/or *Sebacina* were found. Fungi belonging to the same genera were also sporadically found in the mycorrhizal network of surrounding grasses *Bromus erectus* and *Carex hirta*. It has been shown that some members of the involved genera might have ectomycorrhizal capabilities (Weiß *et al.* 2004; Selosse *et al.* 2007).

The fungal specificity varied in the different orchids. With the help of a discriminant analysis (DA) it was shown that the spectra of fungal partners of *Orchis purpurea* was clearly different from the three other investigated orchids (Fig. 9), showing for this species a higher fungal specificity.

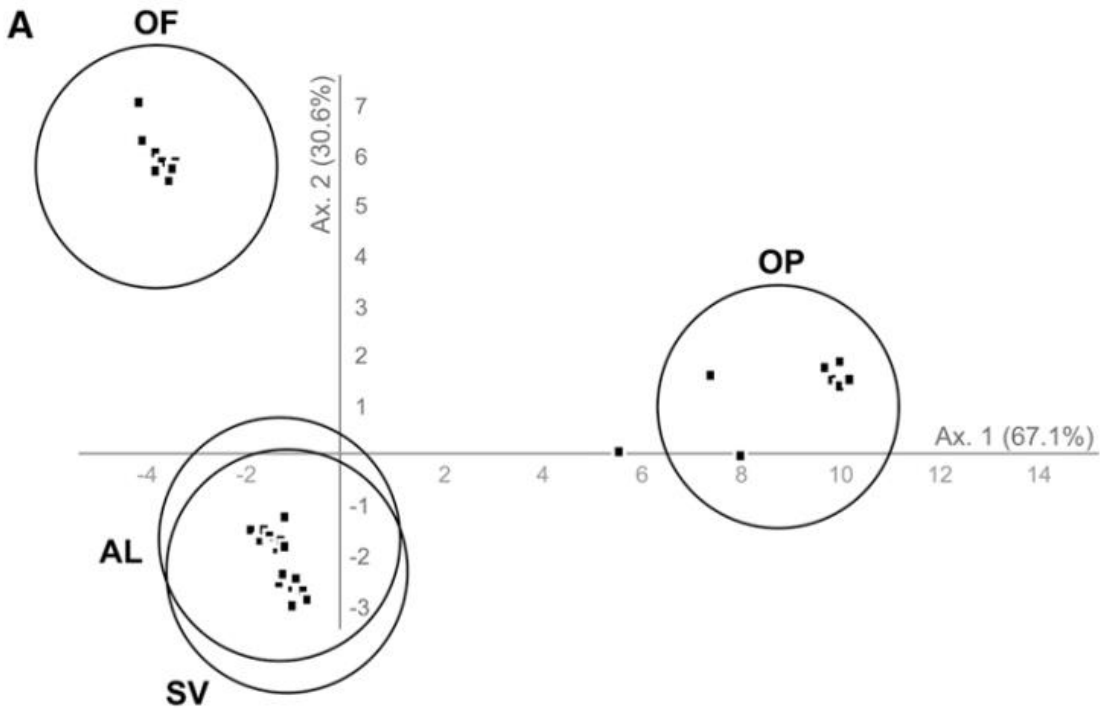

Figure 9: Discriminant analysis (DA) comparing *Rhizoctonia* spectra in *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), *Orchis purpurea* (OP) and *Serapias vomeracea* (SV) roots.

Interestingly, *O. purpurea* is the only orchid that showed both N and C gain from mycorrhizal fungi as it was detected with the help of stable isotope analyses (Fig. 10). This is a direct indication that fungal specificity is not limited to some forest orchids that are dependent on organic C and N delivered from their fungal partners but appears also in meadow orchids.

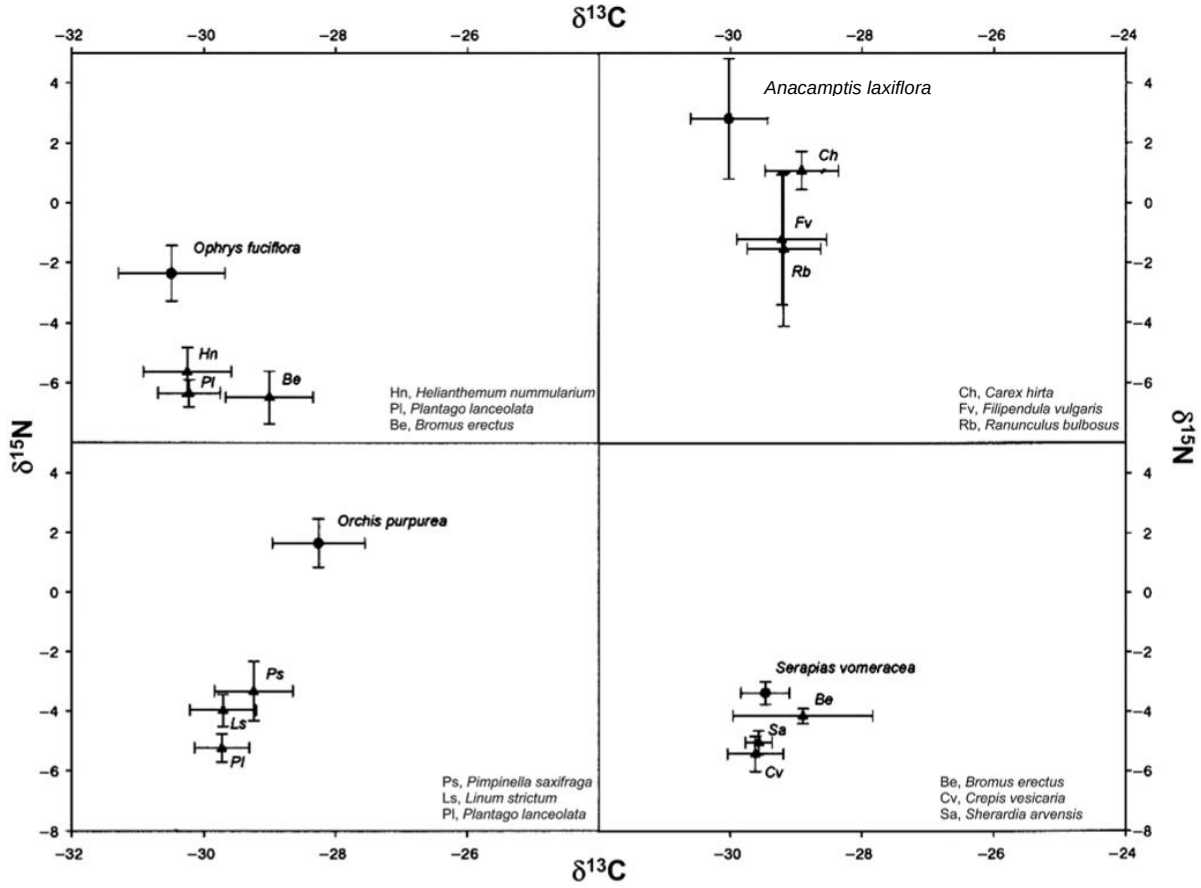

Figure 10: Mean $\delta^{13}C$ and $\delta^{15}N$ values (± 1 SD) in leaves of the four investigated orchids and autotrophic reference plants.

Site effects that might influence the fungal distribution were excluded as *Serapias vomeracea* was sampled at different meadows and showed very similar fungal symbionts. This contrasts the results of Stark et al. (2009) who showed that *Gymnadenia conopsea* (L.) R.Br. is living together with different fungal partners at varying sites. Fungal specificity in *Serapias vomeracea* seems rather similar as in *Pheladenia deformis* (R.Br.) D.L. Jones & M.A. Clem. The latter species is widely distributed all over Australia but uses one single fungal partner belonging to the genus *Sebacina* (Davis et al. 2015). Two mycorrhizal strategies could support a wide distribution:

a) Mycorrhizal specificity with few widespread fungal partners or
b) the use of a wide range of fungal partners, so that one or another is always present at the site to be exploited as orchid symbiont.

In this study isotope signals and fungal partners were identified at one moment during the year – the day of sampling. Later, Ercole et al. (2015) showed that fungal partners in *Anacamptis morio* (L.) R.M. Bateman, A.M. Pridgeon & M.W. Chase changed over the whole year depending on the environmental conditions in the different seasons. Interestingly, isotopic signals did not vary significantly.

Already in our study published in 2011 we claimed that C gain from fungi might be undiscovered in green orchids living associated with *Rhizoctonia* fungi as the C signal might be covered by respiratory effects due to photosynthesis. This finding was recently confirmed with the help of ^{2}H (and ^{18}O) showing that also orchids associated with *Rhizoctonias* obtain nutrients from fungi (Gebauer et al. 2016).

Obviously light is not always the main limiting factor forcing orchids to use mycorrhizal nutrient sources. Other possible factors might be nutrient poor soils that lead orchids to obtain nutrients with the help of fungi. Even a larger tolerance against herbivory might be equilibrated with the help of fungal nutrients (Hynson *et al.* 2013).

3. Discussion and outlook

Discussion of the hypotheses

I.) *Epipogium aphyllum* lives in dark forests in association with saprotrophic fungi as described for *Epipogium roseum* (Paper 1).

Hypothesis I is rejected. There is clear evidence that *Epipogium aphyllum* uses ectomycorrhizal fungal partners to obtain nutrients. This contrasts the strategy of other achlorophyllous orchid species belonging to the same genus, but living associated with saprotrophic fungi. It would be interesting to investigate the reasons for the different adaptations within different representatives of the same genus.

II.) *Goodyera repens* gets organic compounds from fungi when growing at a dark site. It gives organic compounds to fungi when growing at open sites (Paper 2).

Hypothesis II is rejected. Despite extremely varied sampling sites concerning the light climate *Goodyera repens* was depleted in ^{13}C no matter which site it was growing at or which fungi it was associated. It seems that nutrient transfer from the orchid to the fungus might not be the reason for the depletion in ^{13}C. The phenomenon of depleted ^{13}C isotopes is rather widespread in orchids and should be investigated further.

III.) Partial mycoheterotrophy and high fungal specificity does not appear in Mediterranean meadow orchid species growing at completely open, light sites (Paper 3).

Hypothesis III is rejected. Our study clearly shows that efficient partial mycoheterotrophy appears also in orchids growing at completely open, light, Mediterranean sites. Mycorrhizal specificity is not limited to forest orchids only. Partial mycoheterotrophy should be re-investigated in the future especially in orchids of open habitats and nutrient limited sites with the help of new approaches (see Gebauer et al. 2016). The four Mediterranean orchids of this study and a selection of other species should be in the focus of further research (e.g. *Hammarbya paludosa*, *Goodyera repens*, *Calypso bulbosa* and others).

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5. Personal contribution

It was the author's idea to present the work carried out during free-time in the form of a doctoral thesis. The personal contribution to the three published papers is listed below:

PAPER I: LIEBEL H. T., GEBAUER G. (2011) Stable isotope signatures confirm carbon and nitrogen gain through ectomycorrhizas in the ghost orchid *Epipogium aphyllum* Swartz. *Plant Biology*, **13**, 270-275.

Concepts and planning:	90 %	Field and lab work:	50 %
Data analysis:	90 %	Manuscript preparation:	75 %

PAPER II: LIEBEL H. T., BIDARTONDO M. I., GEBAUER G. (2015) Are carbon and nitrogen exchange between fungi and the orchid *Goodyera repens* affected by irradiance? *Annals of botany*, **115**, 251-261.

Concepts and planning:	80 %	Field and lab work:	75 %
Data analysis:	90 %	Manuscript preparation:	75 %

PAPER III: GIRLANDA M., SEGRETO R., CAFASSO D., LIEBEL H. T., RODDA M., ERCOLE E., COZZOLINO S., GEBAUER G., PEROTTO S. (2011) Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. *American Journal of Botany*, **98**, 1148-1163.

Concepts and planning:	25 %	Field and lab work:	40 %
Data analysis:	25 %	Manuscript preparation:	25 %

6. Publications

6.1 Mycoheterotrophy of *Epipogium aphyllum* (Plant Biology)

PAPER I: LIEBEL H. T., GEBAUER G. (2011) Stable isotope signatures confirm carbon and nitrogen gain through ectomycorrhizas in the ghost orchid *Epipogium aphyllum* Swartz. *Plant Biology*, **13**, 270-275.

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RESEARCH PAPER

Stable isotope signatures confirm carbon and nitrogen gain through ectomycorrhizas in the ghost orchid *Epipogium aphyllum* Swartz*

H. T. Liebel[1,2] & G. Gebauer[1]

1 Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, Bayreuth, Germany
2 Present address: Department of Geology and Mineral Resources Engineering, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

Keywords
Fungal DNA analysis; mycoheterotrophy; mycorrhiza; N concentration; Orchidaceae; stable isotopes ^{13}C and ^{15}N.

Correspondence
G. Gebauer, Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany.
E-mail: gerhard.gebauer@uni-bayreuth.de

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*This paper was written in memory of Arne Hegre, who guided us during the plant collection in August 2009, but died shortly after that tour.

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ABSTRACT

***Epipogium aphyllum* is a rare Eurasian achlorophyllous forest orchid known to associate with fungi that form ectomycorrhizas, while closely related orchids of warm humid climates depend on wood- or litter-decomposer fungi. We conducted ^{13}C and ^{15}N stable isotope natural abundance analyses to identify the organic nutrient source of *E. aphyllum* from Central Norway. These data for orchid shoot tissues, in comparison to accompanying autotrophic plants, document C and N flow from ectomycorrhizal fungi to the orchid. DNA data from fungal pelotons in the orchid root cortex confirm the presence of *Inocybe* and *Hebeloma*, which are both fungi that form ectomycorrhizas. The enrichment factors for ^{13}C and ^{15}N of *E. aphyllum* are used to calculate a new overall average enrichment factor for mycoheterotrophic plants living in association with ectomycorrhizal fungi (ε $^{13}C \pm 1$ SD of $7.2 \pm 1.6‰$ and ε $^{15}N \pm 1$ SD of $12.8 \pm 3.9‰$). These can be used to estimate the fungal contribution to organic nutrient uptake by partially mycoheterotrophic plants where fully mycoheterotrophic plants are lacking. N concentrations in orchid tissue were unusually high and significantly higher than in accompanying autotrophic leaf samples. This may be caused by N gain of *E. aphyllum* from obligate ectomycorrhizal fungi. We show that *E. aphyllum* is an epiparasitic mycoheterotrophic orchid that depends on ectomycorrhizal *Inocybe* and *Hebeloma* to obtain C and N through a tripartite system linking mycoheterotrophic plants through fungi with forest trees.**

INTRODUCTION

Orchids typically produce extremely light 'dust' seeds that are easily transported by wind over large distances. The tiny seeds do not contain sufficient endosperm for germination and therefore depend on nutrient supply from a fungal partner. Thus, in this very initial phase of development, all orchids gain nutrients by mycoheterotrophic means (Leake 1994). In some developing orchids (*i.e.* protocorms), Bernard (1909) detected easily cultivable saprotrophic or pathogenic rhizoctonia-forming fungi (in the basidiomycete genera *Tulasnella, Thanatephorus, Ceratobasidium* and biotrophic clade B of *Sebacina*; Weiss *et al.* 2004; Smith & Read 2008). Perhaps, with the exception of a few epiphytic tropical orchids, all orchids investigated so far remain mycorrhizal during their entire life cycle (Smith & Read 2008). Most mature orchids are chlorophyllous and therefore cover their carbon (C) demand by fully autotrophic means, or develop a nutritional mode where C gain through photosynthesis is complemented by organic C [and nitrogen (N)] from fungal partners (partial mycoheterotrophs: Gebauer & Meyer 2003). Among the *ca.* 25,000 orchid species (Dressler 2004) however, are about 200 species that remain achlorophyllous throughout their entire life cycle (Leake 1994). These orchids cover their C and presumably mineral nutrient demand through fully mycoheterotrophic means.

Epipogium aphyllum is a rare Eurasian mycoheterotrophic orchid (Hultén & Fries 1986) that has recently been shown to associate with fungi mainly from the genus *Inocybe* and more rarely other basidiomycetes including *Hebeloma*, *Xerocomus*, *Lactarius* and *Thelephora* species (Roy *et al.* 2009b). These fungi simultaneously form ectomycorrhizas (ECM) with neighbouring trees that are thought to be the final organic nutrient source for the orchid. *E. aphyllum* lives several years underground before it forms inflorescence shoots above ground and is therefore called a 'ghost orchid' (Fay & Chase 2009). Interestingly, the sister species to *E. aphyllum*, *E. roseum*, and other representatives of the phylogenetically closely related genus *Gastrodia*, occur in regions with a warm humid climate, where they are associated with wood- or

litter-decomposing saprotrophic (SAP) fungi (Yamato *et al.* 2005; Martos *et al.* 2009; Ogura-Tsujita *et al.* 2009; Dearnaley & Bougoure 2010). Several recent publications describe *E. aphyllum* as a 'saprophytic' plant (*e.g.* Stace 1997; Delforge 2005; Baumann *et al.* 2006; Lid & Lid 2007; Kretzschmar 2008), *i.e.* a heterotroph that decomposes organic matter. However, non-photosynthetic mycorrhizal plants are actually mycoheterotrophs (Leake 1994), *i.e.* plants that obtain organic compounds from fungi.

Stable isotope natural abundance analysis is a powerful tool to distinguish C and N fluxes between fully mycoheterotrophic orchids (MHOs) and their various types of fungal associates (Gebauer & Meyer 2003; Ogura-Tsujita *et al.* 2009) and to identify partial mycoheterotrophs among green orchids (Gebauer & Meyer 2003; Bidartondo *et al.* 2004; Zimmer *et al.* 2007, 2008; Roy *et al.* 2009a). The method is based on the observation that tissues from fruiting bodies of fungi show an isotope pattern characteristically distinct from neighbouring autotrophic plants. ECM and SAP fungi have a higher abundance of the heavy stable isotopes ^{13}C (Gleixner *et al.* 1993; Högberg *et al.* 1999) and ^{15}N (Gebauer & Dietrich 1993) in comparison to neighbouring autotrophic plants. The stable isotope abundance pattern of these two groups of fungi is slightly different (Gebauer & Taylor 1999; Kohzu *et al.* 1999). MHOs relying on ECM or SAP fungi are therefore also enriched in both ^{13}C and ^{15}N similar to their fungal hosts (Trudell *et al.* 2003). However, they are again characterised by a different stable isotope abundance pattern (Martos *et al.* 2009; Ogura-Tsujita *et al.* 2009; Dearnaley & Bougoure 2010).

This study, for the first time, investigates the type of fungal C and N source of *E. aphyllum* collected from a cold, humid climate of a boreal spruce forest in Norway, close to the northernmost border of its distribution. Using stable isotope natural abundance analysis we test the hypothesis that *E. aphyllum* gains C and N from ectomycorrhizal fungi despite its close phylogenetic relationship to orchid species (*e.g. E. roseum* and *Gastrodia* spp.) living in association with saprotrophic fungi. Furthermore, we analysed fungal identity in the coralloid rhizome of *E. aphyllum* using molecular methods. The results of this study contribute to increase knowledge about nutritional modes within the genus *Epipogium*.

MATERIALS AND METHODS

Study site

The study site of *E. aphyllum* is located in an old north-facing Norway spruce forest in Snåsa municipality in the county of Nord-Trøndelag, Central Norway (64.2° N, 12.2° E, *ca.* 60 m asl). Following the Scandinavian classification of vegetation types, the forest belongs to a weak oceanic, middle boreal, bilberry woodland (Fremstad 1997), with *Picea abies* (L.) H. Karst., *Betula pubescens* Erh. and *Sorbus aucuparia* L. as the dominant tree species. Typical understorey plants are *Vaccinium myrtillus* L., *V. vitis-idaea* L., *Linnaea borealis* L. and *Trientalis europaea* L. Due to the Ordovician Snåsa limestone in the area (Roberts 1997), limestone-indicator species like *Gymnocarpium robertianum* (Hoffm.) Newman and the moss *Ctenidium molluscum* (Hedw.) Mitten are found. Dominant mosses are, among others, *Hylocomium splendens* (Hedw.) Schimp., *Plagiothecium undulatum* (Hedw.) Schimp. and *Sphagnum girgensohnii* Russow. *E. aphyllum* has flowered at the site every year since 1972, with the exception of 1976, with a maximum of about 30 inflorescence shoots in 2009 (Hegre 1998; A. Hegre, personal communication). This exceptionally frequent flowering might be explained either by unusual growing conditions or a large population in the area. The soil consists of brown earth with a thick humus layer and pH of 3.9–4.9 (Hegre 1998).

The mean air temperature is 4.3 °C and average annual precipitation is 1000 mm (closest climate station Snåsa, 100 m asl; 7 km west of the orchid site). The coldest month is January (−5.0 °C) and the warmest is July (13.8 °C). Most precipitation occurs in September and October (120 mm and 115 mm, respectively). The driest month is May, with average precipitation of 47 mm. The climate data are based on the observation period 1961–1990 (Meteorologisk Institutt 2009).

Sampling scheme

In August 2009 we selected five individuals of *E. aphyllum* that were growing a minimum of 2 m apart to avoid sampling orchid clones. To evaluate the orchid stable isotope signatures, each of the orchid plots (1 m^2) had to contain three autotrophic reference plants (*Vaccinium myrtillus* and small saplings of *Picea abies* and *Sorbus aucuparia*). Samples were collected from five plots, yielding five replicates. The flower stalk of the orchid and a couple of leaves or needles from each reference plant species was sampled. Plant material was taken at approximately the same height because it is known that the CO_2 uptake and stomatal regulation at different heights above the soil surface result in different $\delta^{13}C$ values due to different CO_2 sources (soil *versus* atmosphere), light climate and water vapour pressure deficit (Farquhar *et al.* 1989; Gebauer & Schulze 1991; Bauer *et al.* 2000).

Analysis of stable isotope abundance and N concentration

Leaf and orchid stalk samples were oven-dried at 105 °C and ground to a fine powder to achieve a representative sub-sample of plant tissue for further analysis. Relative C and N isotope abundances were measured using a dual element analysis mode with an elemental analyser coupled to a continuous flow isotope ratio mass spectrometer, as described in Bidartondo *et al.* (2004). Measured isotope abundances are denoted as δ values, which are calculated according to the following equation: $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy to light isotopes in the samples and the respective standard. Standard gases were calibrated with respect to international standards using the reference substances ANU sucrose and NBS 19 for C isotopes and N1 and N2 for N isotopes, provided by the International Atomic Energy Agency (Vienna, Austria). Reproducibility and accuracy of isotope abundance measurements were routinely controlled with the test substance acetanilide (Gebauer & Schulze 1991). At least six test substances of varying sample weight were routinely analysed 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%.

Statistics

As described by Preiss & Gebauer (2008), the $\delta^{13}C$ and $\delta^{15}N$ values of the orchids and the non-orchid autotrophic reference plants were used to calculate normalised enrichment factors for each sample as $\varepsilon_S = \delta_S - \delta_{REF}$, with S being the single value of a sample from the fully MHO, and REF being the mean value of all autotrophic reference plants from the respective plot. It has been demonstrated that the ^{13}C and ^{15}N signatures of fully autotrophic C_3 plants in temperate climates do not systematically depend on life form or mycorrhizal status (Gebauer & Dietrich 1993; Gebauer & Meyer 2003; Zimmer *et al.* 2007). However, in boreal climates significant differences in plant $\delta^{15}N$ abundance can be expected due to limited N availability (Schulze *et al.* 1994; Michelsen *et al.* 1998). We kept the spectrum of reference plants as diverse as possible (tree saplings/shrubs, evergreen/deciduous, ectomycorrhizal/ericoid/arbuscular or non-mycorrhizal) to minimise errors when calculating relative enrichment of the orchid, and tested the reference plants' $\delta^{15}N$ abundances for significant differences.

To test for significant differences between the target MHO and the autotrophic reference species, Mann–Whitney *U*-tests for every reference species (Holm 1979) were used.

Statistical analyses were performed with SPSS v.11.5 (SPSS Inc., Chicago, IL, USA). Data are given as means ± 1 SD.

Molecular identification of mycorrhizal fungi

From each of the five individual *E. aphyllum* plants, two root sections colonised by fungi were sampled and placed in lysis buffer (CTAB). These samples were frozen and thawed three times before grinding the softened tissue with a micropestle. Genomic DNA was extracted following methods described elsewhere (Gardes & Bruns 1993) but using the GeneClean® II Kit (Q-BioGene, Carlsbad, CA, USA) for DNA binding and purification. Two root sections per plant were combined into a single DNA sample. Using polymerase chain reaction (PCR), the nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primers ITS1F and ITS4. With this method, the fungus present at the highest concentration can be detected. Investigation of full fungal diversity in the roots would require molecular cloning.

Positive PCR products were purified using ExoSAP-IT (GE Healthcare, Waukesha, WI, USA). DNA sequencing was performed on an ABI3730 Genetic Analyzer using the BIGDYE® v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. Electrophoretograms were checked using SEQUENCHER v.4.5 (Gene Codes Corp., Ann Arbor, MI, USA). All sequences were compared to GenBank using BLAST to ascertain taxonomic affinity.

RESULTS AND DISCUSSION

Stable isotope abundance and total N concentration

E. aphyllum at the study site (Fig. 1) is significantly enriched in both ^{13}C ($P < 0.01$) and ^{15}N ($P < 0.01$; Fig. 2) relative to each of the autotrophic reference plants. As significant differences ($P < 0.05$) were detected in $\delta^{15}N$ abundances between the reference plants *Sorbus aucuparia* and *Picea abies* in this study, the orchid's $\delta^{15}N$ abundance could not be compared to an average value of the autotrophic reference plants.

An average enrichment factor for all investigated fully mycoheterotrophic plants (MHPs) associated with ECM fungi (orchids and representatives of the Monotropoideae) was calculated by Preiss & Gebauer (2008) as $\varepsilon\ ^{13}C = 7.2 \pm 1.6$‰ ($n = 92$). The enrichment factor of *E. aphyllum* in this study, $\varepsilon\ ^{13}C = 8.6 \pm 0.7$‰, is in the typical range for other fully MHOs associated with ECM fungi. The $\varepsilon\ ^{13}C$ values calculated so far for MHO species living in association with wood- or litter-decomposer SAP fungi are in the range of 10‰ (Martos *et al.* 2009; Ogura-Tsujita *et al.* 2009), and thus suggest that this group of MHPs might be even more enriched in ^{13}C in comparison to accompanying autotrophs from the forest understorey than MHPs on ECM fungi.

The enrichment factor $\varepsilon\ ^{15}N = 6.0 \pm 2.1$‰ found for *E. aphyllum* is lower than the arithmetic mean of all investigated MHPs associated with ECM fungi ($\varepsilon\ ^{15}N = 12.0 \pm 1.7$‰, $n = 92$; Preiss & Gebauer 2008). The lowest mean $\varepsilon\ ^{15}N$ value from plants living in association with ECM fungi in the dataset of Preiss & Gebauer (2008) is 9.4 ± 1.4‰, measured in nine individuals of *Pterospora andromedea*

Fig. 1. *Epipogium aphyllum* at the sampling site at Snåsa, Norway.

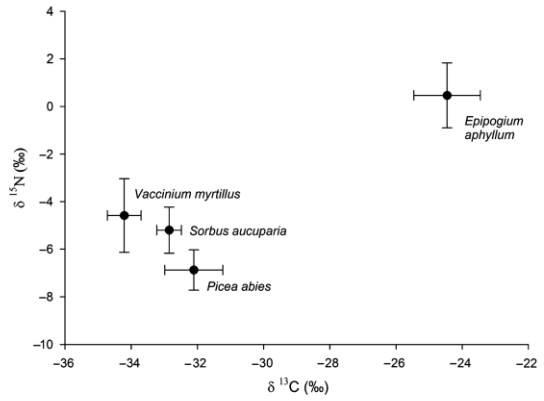

Fig. 2. Stable isotope natural abundance of ^{13}C and $^{15}N \pm 1$ SD in the MHO *Epipogium aphyllum* and its autotrophic reference plants (n = 5).

(Ericaceae), but single ε ^{15}N values in the same range as in *E. aphyllum* or lower have been reported for a few individuals of *Neottia nidus-avis* (lowest ε $^{15}N = 5.7‰$ in a German *Fagus sylvatica* forest; data based on Zimmer *et al.* 2007, 2008). Elucidation of reasons for the higher variability of ε ^{15}N in comparison to ε ^{13}C between MHP species associated with ECM fungi remains a matter for future investigations. *Gastrodia confusa* growing in bamboo forests in Japan has an enrichment factor ε $^{15}N = 2.9 \pm 0.5‰$ (n = 5), which is much lower than that found for all currently investigated MHPs associated with ECM fungi, and is most probably linked to dependence on wood-decay SAP fungi of *Gastrodia confusa* (Ogura-Tsujita *et al.* 2009). In the tropical and subtropical MHOs *Gastrodia similis, G. sesamoides* and *Wullschlaegelia aphylla* that live associated with wood- or litter-decomposer SAP fungi, low ε ^{15}N values have also been reported (Martos *et al.* 2009; Dearnaley & Bougoure 2010).

The ε values of the five individuals of *E. aphyllum* were used to calculate a new overall average enrichment factor for MHPs living in association with ECM fungi. Adding the five data points of this study to the compilation of 92 individual MHPs of Preiss & Gebauer (2008) and ε values calculated from Zimmer *et al.* (2008; n = 17), Hynson *et al.* (2009; n = 37), Ogura-Tsujita *et al.* (2009; n = 5), Roy *et al.* (2009a; n = 12) and Liebel *et al.* (2010; n = 5), new enrichment factors (±1 SD) for ε ^{15}N of $12.8 \pm 3.9‰$ and ε ^{13}C of $7.2 \pm 1.6‰$ (n = 173 based on 13 fully MHP species) were calculated. At present, these values are the most comprehensive basis to estimate the ECM fungal contribution to organic nutrient uptake by partial MHPs where full MHPs are lacking.

The N concentration in *E. aphyllum* samples is significantly ($P < 0.001$) higher than in the leaves of autotrophic reference plants, at 2.8 ± 0.5 mmol·g_{DW}^{-1} (DW: dry weight) *versus* 0.9 ± 0.3 mmol·g_{DW}^{-1}, respectively. The strikingly high N concentrations of this MHO may be caused by nutrient gain from obligate ECM fungi. Such high N concentrations are in the range usually found for legumes associated with N_2-fixing bacteria (Gebauer *et al.* 1988). Fungi have similar C concentrations, but considerably higher N concentrations than higher plants (see *e.g.* Gebauer & Dietrich 1993; Gebauer & Taylor 1999). Thus, the incorporation of fungal metabolites after lysis of pelotons inside root cells of MHOs could produce a surplus of N.

Molecular identification of mycorrhizal fungi

All investigated orchid rhizome cells contained fungal pelotons (40–90% colonisation). Mycorrhizal fungi could be identified from four of the five collected rhizome samples. For the fifth rhizome sample, PCR products were obtained but the sequencing step failed. The fungal DNA sequences obtained from the rhizome of the four *E. aphyllum* individuals closely matched *Hebeloma velutipes* (n = 3, 97% match, AF430254) and *Inocybe geophylla* (n = 1, 99% match, FJ845414), all of which are considered ECM fungi lacking host tree specificity (Kuyper 1986; Aanen *et al.* 2001; Matheny 2005). Both unique DNA sequences from this study have been submitted to GenBank (HM130665, HM130666).

The fungal data obtained in this study are in accordance with the findings of Roy *et al.* (2009b). *E. aphyllum* strongly prefers Cortinariaceae fungi, but is not restricted to a single fungal genus to cover its nutrient supply. It remains to be tested whether narrower specificity towards fungi is manifested during germination, as observed in partially mycoheterotrophic *Cephalanthera* orchids (Bidartondo & Read 2008). Yamato *et al.* (2005) isolated the litter-decomposer SAP fungi *Coprinus* and *Psathyrella* (Coprinaceae) from *E. aphyllum*'s sister species, *E. roseum*. Other close relatives of *Epipogium*, *Gastrodia confusa, G. similis* and *G. sesamoides*, have also been shown to associate with litter- and wood-decomposer SAP basidiomycetes of the genera *Mycena*, *Resinicium* and *Campanella* or *Marasmius*, respectively (Martos *et al.* 2009; Ogura-Tsujita *et al.* 2009; Dearnaley & Bougoure 2010). These saprobic fungi act as an effective nutrient source for these orchids in warm humid climates (Yamato *et al.* 2005; Yagame *et al.* 2007; Ogura-Tsujita *et al.* 2009), but they appear unsuited as fungal hosts for *E. aphyllum* in the boreal climate of Central Norway. *Chamaegastrodia sikokiana* is a species taxonomically closely related to *E. aphyllum*, which associates with fungi of Ceratobasidiaceae that may form ECM with trees (Yagame *et al.* 2008; Bougoure *et al.* 2009). Further stable isotope natural abundance investigations of species belonging to the phylogenetic complex of *Epipogium* and *Gastrodia* will help us understand whether they depend on SAP or ECM fungi and how this is related to their habitat preference. The evolutionary trajectories followed by *Epipogium* and *Gastrodia* are still a matter of debate (Rothacker 2007), but when revealed they will prove useful in resolving the shifts to mycoheterotrophy and among fungal hosts in these orchids.

CONCLUSIONS

The study provides new data to improve understanding of the distribution of an endangered orchid. Conservation of these endangered species will need to consider their fungi and tree hosts. *E. aphyllum* is an epiparasitic orchid, *e.g.* it depends on a living ECM tree and uses ECM networks as carriers of organic nutrients in a tripartite symbiosis. Uniquely, both SAP and ECM fungal partners are used

within the same orchid genus and in closely related phylogenetic clades.

The stable isotope abundances of ^{13}C and ^{15}N strengthen the conclusion that *E. aphyllum* gains organic nutrients from ECM fungi rather than from SAP fungi, as its enrichment factors are similar to those described earlier in MHOs associated with ECM fungi. Measuring the natural abundance of ^{13}C and ^{15}N has the potential to elucidate the final organic nutrient source in other MHOs living together with fungi that might be ectomycorrhizal.

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6.2 Nutrient exchange between fungi and Goodyera repens depending on light regime (Annals of Botany)

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Are carbon and nitrogen exchange between fungi and the orchid *Goodyera repens* affected by irradiance?

Heiko T. Liebel[1,†] Martin I. Bidartondo[2] and Gerhard Gebauer[1,*]

[1]*Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany and* [2]*Imperial College London and Royal Botanic Gardens, Kew TW9 3DS, UK*
* *For correspondence. E-mail gerhard.gebauer@uni-bayreuth.de*
[†]*Present address: Bavarian Environment Agency, Gsteigstr. 43, 82467 Garmisch-Partenkirchen, Germany.*

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• **Background and Aims** The green orchid *Goodyera repens* has been shown to transfer carbon to its mycorrhizal partner, and this flux may therefore be affected by light availability. This study aimed to test whether the C and N exchange between plant and fungus is dependent on light availability, and in addition addressed the question of whether flowering and/or fruiting individuals of *G. repens* compensate for changes in leaf chlorophyll concentration with changes in C and N flows from fungus to plant.
• **Methods** The natural abundances of stable isotopes of plant C and N were used to infer changes in fluxes between orchid and fungus across natural gradients of irradiance at five sites. Mycorrhizal fungi in the roots of *G. repens* were identified by molecular analyses. Chlorophyll concentrations in the leaves of the orchid and of reference plants were measured directly in the field.
• **Key Results** Leaf $\delta^{13}C$ values of *G. repens* responded to changes in light availability in a similar manner to autotrophic reference plants, and different mycorrhizal fungal associations also did not affect the isotope abundance patterns of the orchid. Flowering/fruiting individuals had lower leaf total N and chlorophyll concentrations, which is most probably explained by N investments to form flowers, seeds and shoot.
• **Conclusions** The results indicate that mycorrhizal physiology is relatively fixed in *G. repens*, and changes in the amount and direction of C flow between plant and fungus were not observed to depend on light availability. The orchid may instead react to low-light sites through increased clonal growth. The orchid does not compensate for low leaf total N and chlorophyll concentrations by using a ^{13}C- and ^{15}N-enriched fungal source.

Key words: *Goodyera repens*, orchid, nutrition, mycoheterotrophy, mycorrhiza, light availability, *Tulasnella*, *Ceratobasidium*, stable isotopes, ^{13}C, ^{15}N, chlorophyll concentration.

INTRODUCTION

All orchids have an initial mycoheterotrophic start into their life cycle as the tiny seeds need fungal partners to germinate. Perhaps with the exception of a few epiphytic tropical orchids, all orchids investigated so far remain mycorrhizal during the rest of their life cycle (Smith and Read, 2008). Most mature orchids are chlorophyllous and therefore cover their carbon (C) demands putatively by fully autotrophic means, or they develop a nutritional mode where the C gain through photosynthesis is complemented by organic C (and nitrogen) from fungal partners (partial mycoheterotrophs; Gebauer and Meyer, 2003). Among the approx. 25 000 orchid species (Dressler, 2004) are also about 200 species that remain achlorophyllous throughout their entire life cycle (Leake, 1994). These orchids cover their C and presumably mineral nutrient demands by fully mycoheterotrophic means.

Stable isotope natural abundance analysis is a powerful tool to distinguish C and nitrogen (N) fluxes between fully mycoheterotrophic orchids and their fungal associates (Gebauer and Meyer, 2003; Trudell *et al*., 2003; Ogura-Tsujita *et al*., 2009; Roy *et al*., 2009; Liebel *et al*., 2010; Liebel and Gebauer, 2011) and to identify partial mycoheterotrophs among green orchids that are associated with ectomycorrhizal fungi (Gebauer and Meyer, 2003; Bidartondo *et al*., 2004; Zimmer *et al*., 2007, 2008; Roy *et al*., 2009; Liebel *et al*., 2010). The method is based on the observation that the fruiting bodies of fungi show an isotope pattern characteristically different from that of neighbouring autotrophic plants. Ectomycorrhizal (ECM) and saprotrophic (SAP) fungi have a higher abundance of the heavy stable isotopes ^{13}C (Gleixner *et al*., 1993; Högberg *et al*., 1999) and ^{15}N (Gebauer and Dietrich, 1993) in comparison with neighbouring autotrophic plants (see review by Major *et al*., 2009).

As shown for some green representatives of the ectomycorrhizal fungi-associated orchid genus *Cephalanthera*, partial mycoheterotrophy is not a static nutritional mode but rather a flexible mechanism depending on irradiance (Preiss *et al*., 2010) and chlorophyll concentration in the orchid leaves (Stöckel *et al*., 2011) and other photosynthetic parts of partially mycoheterotrophic orchids (Bellino *et al*., 2014). *Cephalanthera* compensates for photosynthesis-limiting light availabilities in dark forests, or low chlorophyll concentrations in variegated and albino leaves, by increasing leaf ^{13}C abundances due to increasing proportional gains of

TABLE 1. *Sampling sites of* Goodyera repens *and sampled reference species*

Location	N co-ordinate	E co-ordinate	Altitude (m a.s.l.)	No. of *Goodyera* samples	Reference species (sample number)
Forest 1	63·44999°	10·26819°	260	8	*Vm* (8), Vv (8), *Mb* (2), *Cs* (5), *Oa* (1)
Forest 2	63·43914°	10·83037°	3	2	*Oa* (2), *Mb* (2), *Vv* (2)
Forest 3	63·40156°	10·83836°	200	4	*Sa* (2), *Lp* (2), *Vm* (4), *Vv* (4)
Open land 1	63·33861°	10·69483°	500	7	*Cs* (6), *Vu* (1), *Vm* (7), *Vv* (2), *Rc* (5)
Open land 2	69·93222°	23·37884°	80–250	17	*Vv* (17), *Bn* (2), *Lb* (4), *Eh* (4), *Vm* (7), *Vu* (13), *Jc* (1), *Cs* (3)

Vm, *Vaccinium myrtillus*; *Vv*, *Vaccinium vitis-idea*; *Mb*, *Maianthemum bifolium*; *Cs*, *Chamaepericlymenum suecicum*; *Oa*, *Oxalis acetosella*; *Sa*, *Sorbus aucuparia*; *Lp*, *Luzula pilosa*; *Vu*, *Vaccinium uliginosum*; *Rc*, *Rubus chamaemorus*; *Bn*, *Betula nana*; *Lb*, *Linnaea borealis*; *Eh*, *Empetrum hermaphroditum*; *Jc*, *Juniperus communis*.

fungal-derived C. For the partially mycoheterotrophic *Limodorum abortivum*, a field experiment excluding mycorrhizal partners using fungicides showed that orchids to some degree can adapt their capability to capture light, as indicated by increasing pigment concentrations and decreasing relative ^{13}C abundance as autotrophic plants do at shady sites (Bellino *et al*., 2014).

Goodyera repens and *G. oblongifolia* (Zimmer *et al*., 2007; Hynson *et al*., 2009), and a few orchid species within the genera *Aceras*, *Ophrys* and *Orchis* (Liebel *et al*., 2010), all associated with saprotrophic fungi of the polyphyletic rhizoctonia group, were shown to hold significantly depleted leaf $\delta^{13}C$ values compared with autotrophic reference plants. A transfer of nutrients from the orchids to their mycorrhizal associates was hypothesized to be the reason for the depletion (Hynson *et al*., 2009). This explanation was supported by the findings of Cameron *et al*. (2006, 2008) who showed in *ex situ* experiments using labelled C and N compounds that green *G. repens* plants transfer C to the fungus *Ceratobasidium cornigerum*. Carbon transfer from the orchid towards the fungus was thus considered as the fourth possibility for mature orchid nutrition besides (1) full dependence on fungi, or full mycoheterotrophy; (2) partial acquisition of nutrition from fungi, or partial mycoheterotrophy; and (c) complete autotrophic nutrition (100 % photosynthesis). All orchid nutritional pathways include initial mycoheterotrophy by seedlings.

Based on these findings, we hypothesized that the amount of orchid to fungus C flux in the case of *G. repens* is reduced or even reversed with decreasing light availability, while the fungus to orchid transfer of organic N remains unaffected. A decrease in the orchid to fungus C flux should result in a relative increase in ^{13}C abundance, and an unaffected fungus to orchid N flux should not lead to changes in orchid ^{15}N abundances.

Goodyera repens grows in forests and bogs of Europe and North America. We used natural light climate gradients in *G. repens* habitats of Scandinavia, where it is a common orchid, to test this hypothesis. Natural light climate gradients mirror the range of light conditions in which *G. repens* is living. The use of natural light climate gradients avoids any risk of experimental error that can never be excluded with experimental light climate manipulations under field or greenhouse conditions. The risk of experimental error is particularly high when manipulating complex and highly sensitive bipartite systems such as orchid mycorrhizas. In our case the 'experiment' is the *in situ* process in nature *per se*, i.e. a natural light gradient. Stable isotope natural abundance integrates this process and associated matter fluxes over time (see Fry, 2006). Based on this conceptual consideration, we can argue that stable isotope natural abundance observed under field conditions has more power than any kind of manipulation experiment.

Furthermore, we observed in the field that flowering/fruiting individuals of *G. repens* were yellowish in comparison with dark-green non-flowering/non-fruiting individuals. Thus, we also used this experiment to test whether *G. repens* compensates for changes in leaf chlorophyll concentration with C and N flows from fungal partners to the orchid – as was found in albino and variegated individuals of *Cephalanthera damasonium* (Stöckel *et al*., 2011).

MATERIALS AND METHODS

Study site and sampling scheme

As detailed in Table 1, leaf samples of *Goodyera repens* and autotrophic reference species were collected at four sites in the county of Southern Trøndelag, Norway (forest 1–3, open land 1) and at one large site in the county of Finnmark, Norway (open land 2).

The different locations in Trøndelag were not more than 40 km apart from each other to ensure similar climatic conditions. Plant names used in the following were taken from Lid and Lid (2005). The three forest locations were characterized by more or less dark, dense *Picea abies* forest with some *Betula pubescens*, *Sorbus aucuparia* and *Pinus sylvestris* in the canopy layer. The understorey vegetation consisted, among others, of *Vaccinium* species, *Luzula pilosa*, *Trientalis europaea* and *Chamaepericlymenum suecicum*. The open land 1 location was the only exception in Trøndelag. Here, *G. repens* grew directly at the tree edge in an open sub-alpine environment. The vegetation cover was *Calluna vulgaris*, *Empetrum hermaphroditum*, *Vaccinium myrtillus*, *V. uliginosum*, *V. vitis-idaeus*, *Rubus chamaemorus*, *Chamaepericlymenum suecicum*, *Arctous alpinus*, *Avenella flexuosa*, *Picea abies*, *Betula pubescens* and *B. nana*. The advantage of this sub-set of samples is the large variation in light regimes at the different locations.

A second sub-set of orchid samples was taken from a 2 km^2 area in Northern Norway, Finnmark county. Here, the leaves of *G. repens* and reference species were collected in an open northern boreal birch forest (*Betula pubescens*). The different light climates were basically determined by different exposure and to a minor degree by varying forest canopies. The ground vegetation was dominated by Ericaceae species such as

Vaccinium myrtillus, *V. vitis-idaeus*, *V. uliginosum*, *Empetrum hermaphroditum*, *Cassiope tetragona*, *Calluna vulgaris* and *Phyllodoce caerulea*. Other species are, for example, *Sorbus aucuparia*, *Juniperus communis*, *Chamaepericlymenum suecicum*, *Equisetum scirpoides*, *Melica nutans* and *Listera cordata*. A detailed geobotanical description of the site can be found in Liebel and Krill (2011).

Individuals of *G. repens* for the investigation were selected at a wide distance apart from each other to minimize sampling of orchid clones. To evaluate the orchids' stable isotope signatures, each of the orchid plots (1 m^2) had to contain three autotrophic reference plants. Sampling of reference plant material is needed to obtain a measure for the enrichment or depletion in ^{13}C and ^{15}N abundance in target species compared with autotrophic species growing under identical microclimate and soil conditions. One to three leaves of the orchid and a couple of leaves from each reference plant species were sampled. Plant material was collected plotwise in close spatial proximity following the sampling criteria detailed by Gebauer and Meyer (2003). If flowering/fruiting individuals were present in the sampling plots, additional leaf samples were taken for the measurement of the stable isotope abundance of ^{13}C and ^{15}N. The chlorophyll concentration of each sampled *G. repens* leaf (flowering/fruiting and non-flowering/non-fruiting individuals) and of reference plants was measured directly in the field. Samples for stable isotope analyses were collected at the end of the light measurement period (for sampling dates, see later).

Stable isotope abundance and C and N concentration analysis

Leaf samples were oven-dried at 105 °C and ground to a fine powder. Relative C and N isotope abundances were measured using a dual element analysis mode with an elemental analyser coupled to a continuous flow isotope ratio mass spectrometer as described in Bidartondo *et al.* (2004). Measured isotope abundances are denoted as δ values, which were calculated according to the following equation: $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ [‰], 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 C isotopes, and N1 and N2 for N 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. The calculation of C and N concentrations in the leaf samples followed the protocol of Gebauer and Schulze (1991).

Molecular identification of mycorrhizal fungi

Older roots of *G. repens* were densely covered by fungal mycelium, and fungal colonization was also present inside the roots (see Fig. 1A). The roots were cleaned, sectioned and examined for mycorrhizal colonization with a microscope. From each sampled individual of *G. repens*, two root sections with abundant pelotons were sampled, placed in lysis buffer [cetyltrimethyl ammonium bromide (CTAB)] and stored frozen until analysis. Genomic DNA was extracted following methods described elsewhere (Gardes and Bruns, 1993) but using the GeneClean® II Kit (Q-BioGene, Carlsbad, CA, USA) for DNA binding and purification. Using polymerase chain reaction (PCR) with PicoMaxx (Agilent, Santa Clara, CA, USA), the nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primer combination of ITS1F and ITS4. Positive PCR products were purified using ExoSAP-IT (GE Healthcare, Waukesha, WI, USA). DNA sequencing was performed on an ABI3730 Genetic Analyzer using a BigDye® v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and absolute ethanol/EDTA precipitation. Electrophoretograms were checked using Sequencher v.4.5 (Gene Codes Corporation, Ann Arbor, MI, USA). All sequences were compared with GenBank using BLAST to ascertain taxonomic affinity. All unique DNA sequences were submitted to GenBank (KP056301–KP056306).

FIG. 1. (A) Roots of *Goodyera repens*. Notice the abundant root hairs. (B) Low chlorophyll concentration of a flowering shoot of *G. repens* surrounded by dark green non-flowering rosettes.

Light availability and chlorophyll concentrations

The micro-scale light climate was measured close to each sampled individual of *G. repens*. For this purpose, a calibrated light sensor (silicon photodiode BPW 21, Infineon, Germany) connected to a mini data logger (HOBO H8, ONSET, USA) was installed next to the orchid at a similar height to the evergreen rosette plant early in the growth period, a short time after snowmelt. Irradiance was measured every 24 min in the period 17 May–11 August 2011 for the sample's sub-set from Southern Trøndelag and 16 June–25 August 2011 for the sample's sub-set from Finnmark county. The obtained values were averaged as daily means for each orchid plot (from sunrise to sunset or during midnight sun for 24 h). The measured values were converted into photosynthetically active radiation (μmol photons $m^{-2} s^{-1}$) using an intercalibration curve. Mean daily irradiance (minimum plot to maximum plot in μmol photons $m^{-2} s^{-1}$) at the five sampling locations was as follows: forest 1, 15–64; forest 2, 31–62; forest 3, 15–47; open land 1, 111–343; and open land 2, 49–217.

Strong differences in leaf colour were observed between flowering and non-flowering individuals of *G. repens* (see Fig. 1B). For this reason, the chlorophyll concentration of sampled leaves of *G. repens* was measured directly in the field with

a hand-held chlorophyll meter (SPAD-502, Konica Minolta Sensing Inc., Osaka, Japan). The measurement for each sample was repeated five times to obtain a representative mean value as the chlorophyll concentration varied slightly depending on the measurement position on the leaf. The output SPAD (Soil Plant Analysis Development) value was used to calculate the chlorophyll concentration [Chl] (as $mg\,m^{-2}$) using the following equation (Monje and Bugbee, 1992):

$$[\mathrm{Chl}] = 1{\cdot}034 + 0{\cdot}308\mathrm{SPAD} + 0{\cdot}110\mathrm{SPAD}^2$$

Characteristics that were compared for pairs of fruiting and non-fruiting individuals of *G. repens* are the total N and C concentrations, the $\delta^{13}C$ and $\delta^{15}N$ values and the chlorophyll concentrations in the leaves. Pairs were collected at forest 1 (four pairs), forest 2 (two pairs), forest 3 (three pairs) and open land 1 (four pairs).

Statistics

In order to enable site-independent comparisons, the $\delta^{13}C$ and $\delta^{15}N$ values of the orchids and the non-orchid autotrophic reference plants were used to calculate normalized enrichment factors for each sample as $\varepsilon_S = \delta_S - \delta_{REF}$, with S being the single value of a *Goodyera* sample and REF being the mean value of all autotrophic reference plants from the respective plot (Preiss and Gebauer, 2008). It has been shown that the ^{13}C and ^{15}N signature of fully autotrophic C_3 plants that are not living in symbiosis with N_2-fixing bacteria in temperate climates does not systematically depend on their life form or mycorrhizal status (Gebauer and Dietrich, 1993; Gebauer and Meyer, 2003; Zimmer *et al.*, 2007). However, in boreal climates and also in Western Australia, significant differences in plant ^{15}N abundance can be expected due to severely limited N availability (Schulze *et al.*, 1994; Michelsen *et al.*, 1998; Sommer *et al.*, 2012). We kept the spectrum of reference plants as diverse as possible (tree saplings/shrubs, evergreen/deciduous, ectomycorrhizal/ericoid- arbuscular- or non-mycorrhizal). To test for significant differences between $\delta^{13}C$ and $\delta^{15}N$ values in *G. repens* and the autotrophic reference species, Mann–Whitney U-tests were carried out, as the data were not normally distributed. A Kruskal–Wallis H-test was carried out to test for significant differences within the autotrophic references with regard to $\delta^{13}C$ and $\delta^{15}N$ values. Spearman correlations were calculated to test for relationships between $\delta^{13}C$ values and $\delta^{15}N$ values and the light availability for both samples of *G. repens* and of autotrophic reference species. Polynomial (cubic) regression analyses were performed to compare visually the $\delta^{13}C$ and $\delta^{15}N$ values with the light climate for both *G. repens* and autotrophic references. Statistical analyses were performed with SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) and SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA).

RESULTS

Stable isotope abundances and light climate

The overall results of the C and N stable isotope abundance analysis for *Goodyera repens* and autotrophic reference species are shown as δ values in Fig. 2. Significant differences ($P < 0{\cdot}001$; Kruskal–Wallis H-test) were detected in $\delta^{15}N$ between *Rubus chamaemorus* and the other autotrophic references. *Rubus chamaemorus* was significantly enriched in ^{15}N [mean $\delta^{15}N$ $2{\cdot}1 \pm 0{\cdot}8$ (s.d.) ‰ vs. $-4{\cdot}7 \pm 0{\cdot}8$ ‰ in the other autotrophic references] and was therefore not used as a reference plant for further comparisons between autotrophic species and *G. repens*.

A statistical difference in the $\delta^{13}C$ values between *G. repens* and autotrophic reference species was found with the help of a Mann–Whitney U-test ($P < 0{\cdot}001$, $U = 1505$). No significant difference was found between the target orchid and the reference plants for $\delta^{15}N$ values ($P = 0{\cdot}692$, $U = 2512$). *Rubus chamaemorus* was significantly separated from all other species of this study by its high $\delta^{15}N$ values ($P < 0{\cdot}001$, $U = 0$).

The relative enrichment factor ε was calculated for each plot where *G. repens* and its autotrophic reference species were collected. The ε values indicate that *G. repens* individuals independent from any kind of site factors tended to be slightly enriched in ^{15}N and clearly depleted in ^{13}C compared with their autotrophic references. Even if the different sampling locations are characterized by different light climates, no clear pattern of enrichment or depletion was found for especially high-light locations such as open land 1 or especially dark locations such as forest 3 (see Fig. 3).

The $\delta^{13}C$ values in leaves of autotrophic references ranged from $-33{\cdot}4$ to $-25{\cdot}8$ ‰ and showed a significant positive correlation with light availability ($r = 0{\cdot}542$, $P < 0{\cdot}001$; Spearman correlation; see Fig. 4). The $\delta^{13}C$ values in leaves of *G. repens* ranged from $-37{\cdot}4$ to $-29{\cdot}1$ ‰ and also showed a significant positive correlation with light availability ($r = 0{\cdot}751$, $P < 0{\cdot}001$; Spearman correlation) like the autotrophic references. The fitted regression curves for *G. repens* and autotrophic species are close to parallel to each other over the whole irradiance spectrum. The 95 % confidence intervals of the regression curve of *G. repens* do not overlap with those of the autotrophic references, which indicates a significant difference between the two functional groups. The $\delta^{13}C$ values of *G. repens* are more negative than the values of the reference species throughout the range of different light regimes.

No indication was found that higher amounts of nutrients were transferred towards the fungal symbionts in plants exposed to direct sunlight than in plants growing at shady sites.

The $\delta^{15}N$ values in leaves of autotrophic references ranged from $-9{\cdot}7$ to $-0{\cdot}3$ ‰ and showed no significant correlation with light availability ($P = 0{\cdot}241$, $r = -0{\cdot}116$; Spearman correlation). The $\delta^{15}N$ values in leaves of *G. repens* ranged from $-7{\cdot}8$ to $-1{\cdot}6$ ‰ and showed a weak but significant correlation with light availability ($P = 0{\cdot}004$, $r = 0{\cdot}406$, Spearman correlation) in contrast to the autotrophic references. Considering the spread of data in Fig. 4, this correlation has to be interpreted with care.

Molecular identification of mycorrhizal fungi

Tulasnella and *Ceratobasidium* were the most frequently found fungal partners in the roots of *G. repens*. In some cases, as additional partners, fungi belonging to known ectomycorrhizal genera such as *Lactarius*, *Russula* and *Sebacina* were found. The presence of ectomycorrhizal fungi in *G. repens* roots did

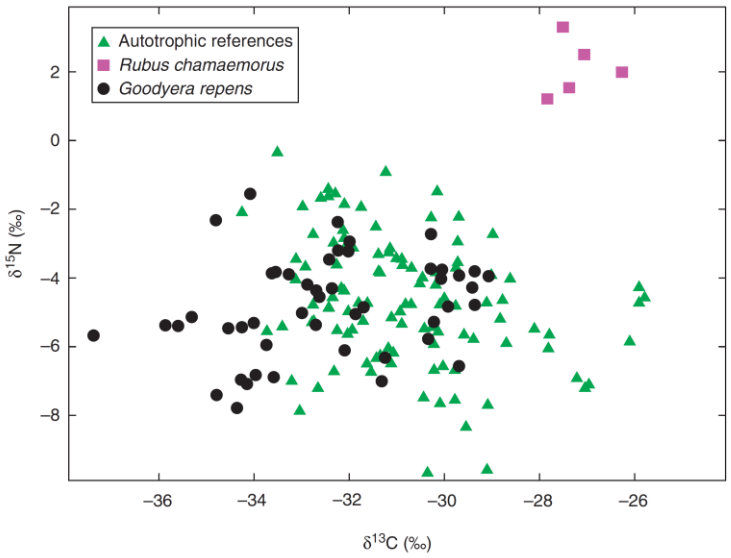

FIG. 2. Overview over all leaf $\delta^{13}C$ and $\delta^{15}N$ values of *Goodyera repens* and of reference plants investigated in this study. Note the unique $\delta^{15}N$ values found for *Rubus chamaemorus*.

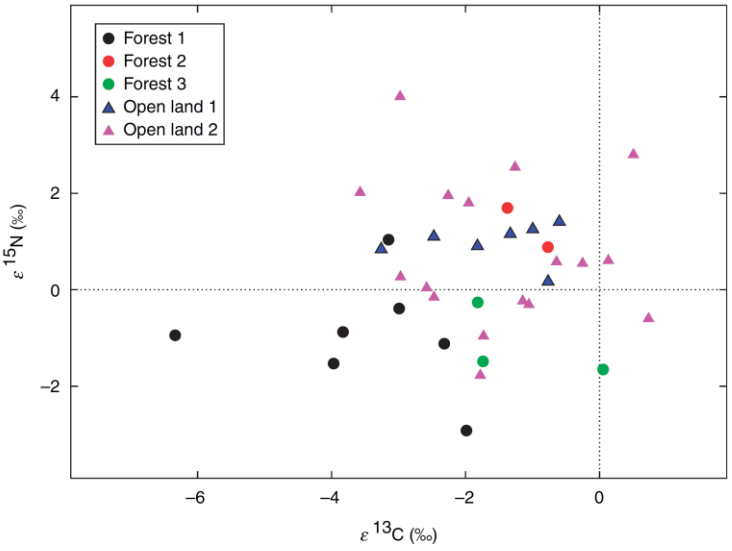

FIG. 3. Comparison of the enrichment factors ε for ^{13}C and ^{15}N in *Goodyera repens* leaves collected at the different sampling sites. For the reference plants, mean ε is zero by definition.

not result in a significant enrichment or depletion in *G. repens* leaf ^{13}C or ^{15}N (Fig. 5).

Comparison of fruiting and non-fruiting individuals

Flowering/fruiting individuals of *G. repens* were compared with non-flowering/non-fruiting individuals at each plot where both stages were present (Fig. 6). The total N concentration was on average 73 % lower in flowering/fruiting individuals compared with non-flowering/non-fruiting individuals. The range was between 45 and 91 %, which means that all investigated flowering/fruiting individuals showed lower leaf total N concentrations. The results of the leaf total N concentrations compared with the results of the leaf chlorophyll concentrations are

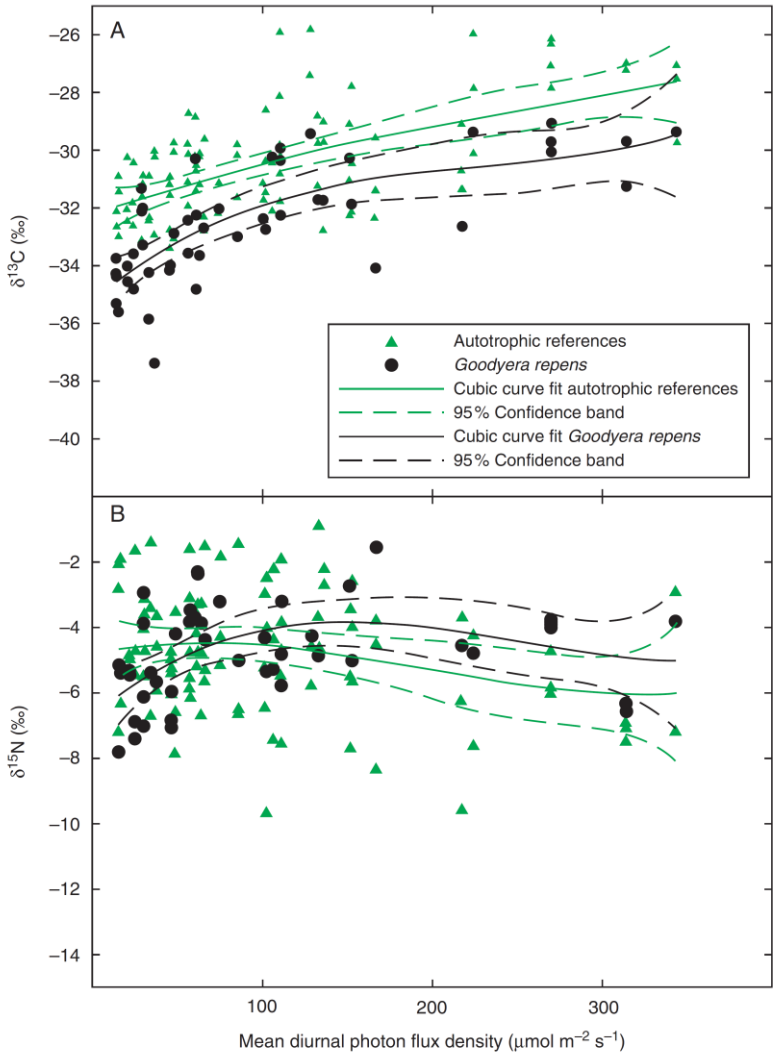

FIG. 4. Leaf $\delta^{13}C$ (A) and $\delta^{15}N$ values (B) dependent on light availability for each investigated *Goodyera repens* individual and for the respective autotrophic reference plants.

in agreement with each other. Lower chlorophyll concentrations were found in leaves of all flowering/fruiting individuals compared with the non-flowering/non-fruiting individuals. The leaf $\delta^{15}N$ values of *G. repens* also showed a similar pattern, with ^{15}N depletion in most cases of flowering/fruiting individuals. In 77 % of the cases, the flowering/fruiting orchid individuals were more depleted in ^{15}N than neighbouring non-flowering/non-fruiting individuals. The leaf total C concentration was in most cases lower in flowering/fruiting than in non-flowering/non-fruiting individuals. The differences, however, are on a much smaller scale than found for the leaf total N concentrations. The lowest leaf total C concentration in a flowering individual was still 97 % of the total C concentration measured in the non-flowering individual from the same sampling site. Interestingly, unlike the pattern found in $\delta^{15}N$, there is no clear change seen in the ^{13}C abundance. Leaf $\delta^{13}C$ values are very similar to each other in both flowering/fruiting and non-flowering/non-fruiting individuals.

DISCUSSION

Our results clearly indicate that leaf $\delta^{13}C$ values of *Goodyera repens* respond to changes in light availability in a similar way to leaf $\delta^{13}C$ values of a wide spectrum of autotrophic reference plants. Leaf $\delta^{13}C$ values of both *G. repens* and reference plants showed a positive correlation with light availability. Increasing

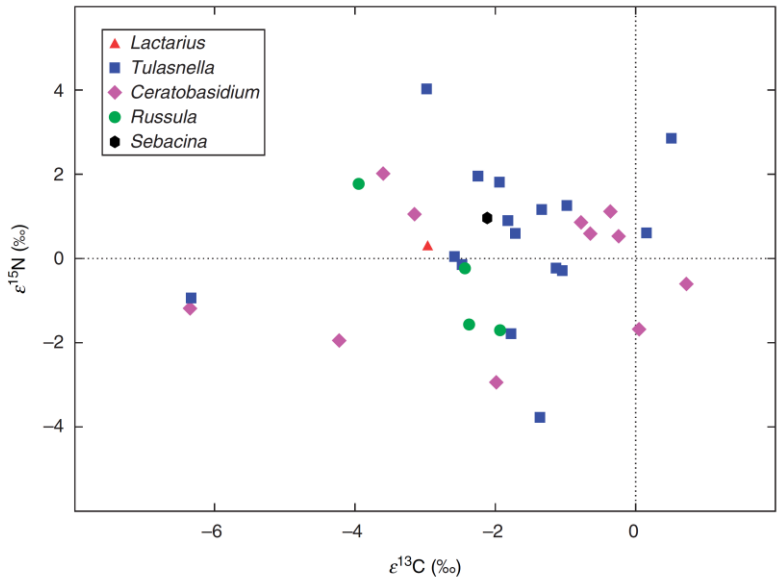

FIG. 5. Comparison of the leaf enrichment factors ε for ^{13}C and ^{15}N in *Goodyera repens* individuals associated with different mycorrhizal fungi (numbers in parentheses indicate the frequency of fungi detected in orchid roots): *Tulasnella* (17), *Ceratobasidium* (11), *Russula* (4), *Lactarius* (1), *Sebacina* (1). In some cases, *Tulasnella* was also found in roots together with *Ceratobasidium*, *Lactarius*, *Russula* and *Sebacina*. For the reference plants, mean ε is zero by definition.

$\delta^{13}C$ values with increasing irradiance are explained by C isotope discrimination during C_3 photosynthesis which depends, among other factors, on the intercellular partial pressure of CO_2 (Farquhar *et al*., 1989). The partial pressure of CO_2 is controlled by the stomata, and their ability to open and close depends on ambient conditions. Under shady and wet conditions, the stomata tend to be more open, leading to a higher intercellular CO_2 partial pressure than under sunny and dry conditions. Thus, our initial hypothesis that the amount and direction of C flow between plant and fungus changes depending on light availability has to be rejected in the case of *G. repens*. *Goodyera repens* responded to changes in light availability similarly to the green orchids *Cypripedium calceolus* (Preiss *et al*., 2010) and *Catasetum viridiflavum* (Zimmerman and Ehleringer, 1990). Both orchids are considered as fully autotrophic species which are adapted to their forest habitats in different ways. While the terrestrial *C. calceolus* has large green leaves to collect light at the forest floor, *C. viridiflavum* lives epiphytically above the shady ground and optimizes photosynthesis further via green flowers. *Goodyera repens* is evergreen with small leaves. *Goodyera* compensates for low-light conditions by growth in large patches of interconnected clones. Areas occupied by clones have been reported to exceed 130 m^2 (Brzosko *et al*., 2013). Large clonal networks are a typical adaptation to colder climates in higher geographical latitude, under nutrient-poor and low-light conditions (Callaghan, 1988; van Groenendael *et al*., 1996; Blinova and Chmielewski, 2008; Wellstein and Kuss, 2011).

Goodyera repens behaved differently in its response to changes in light regime from the orchids *Cephalanthera damasonium* and *C. rubra* (Preiss *et al*., 2010). These *Cephalanthera* species showed no decrease in leaf $\delta^{13}C$ with decreasing irradiance, thus indicating an increase in proportional C gain from the fungal source under low-light conditions (Preiss *et al*., 2010). Similar to our findings, in a full-factorial light manipulation experiment of partially mycoheterotrophic Pyroleae species (*Pyrola picta* and *Chimaphila umbellata*), no proportional dependency on fungal C gains according to a change in irradiation levels could be shown (Hynson *et al*., 2012). A shading experiment with partially mycoheterotrophic *Epipactis helleborine* showed that isotopic C signals in leaves did not differ significantly for a shaded or a sunny situation as the photosynthetic activity changed rather than the C fluxes from fungus to orchid (Gonneau *et al*., 2014). The latter study implies that it might be difficult to detect changes in nutrient fluxes depending on irradiation in the field for some orchid species. Nonetheless, the leaf $\delta^{13}C$ values found for *G. repens* in our investigation confirm previous findings of significant ^{13}C depletion in *Goodyera* leaves in comparison with accompanying reference plants (Hynson *et al*., 2009). This ^{13}C depletion, however, most probably has explanations other than the hypothesized plant to fungus C exchange.

Comparison of flowering/fruiting with non-flowering/non-fruiting individuals of *G. repens* showed that flowering/fruiting individuals had lower leaf total N and chlorophyll concentrations which is most probably explained by the plant using leaf N to form flowers, seeds and the upright orchid shoot, coupled with limited soil N availability to compensate for this investment. Boreal habitats, as in our sites, are considered strongly N limited (Tamm, 1990). The flowering/fruiting costs of *G. repens* are not borne in the previous year as might be the case for non-evergreen orchids such as *Dactylorhiza* species

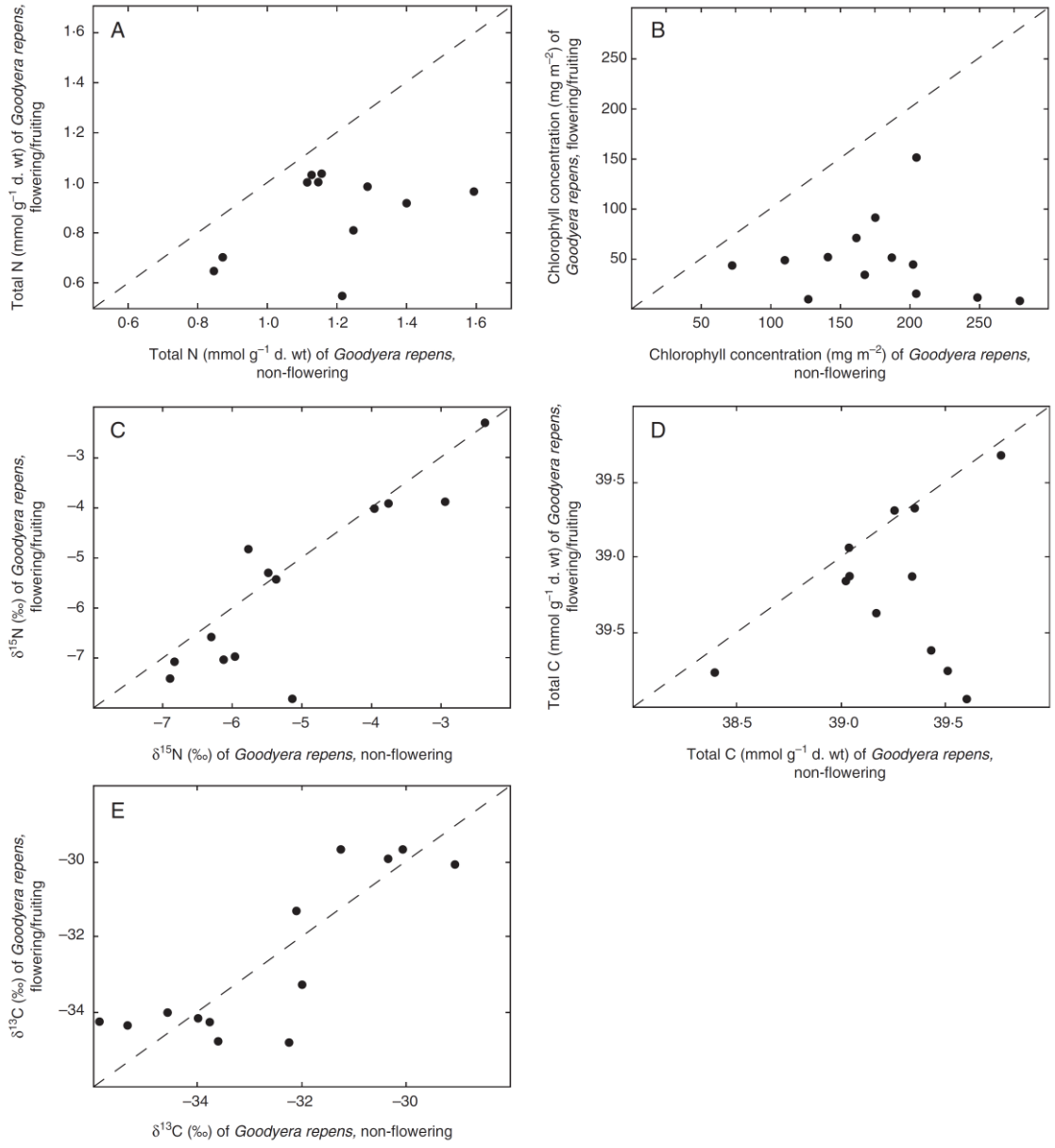

FIG. 6. Comparison of N, C and chlorophyll concentrations and of δ^{15}N and δ^{13}C values in the leaves of flowering/fruiting and non-flowering individuals of *Goodyera repens*: (A) total N concentration; (B) chlorophyll concentration; (C) δ^{15}N; (D) total C concentration; (E) δ^{13}C.

which use the nutrient store of their tubers to form stem, flowers and fruits (Vallius, 2001). In *G. repens*, floral investment causes reduced allocation to other plant functions, as previously shown (e.g. Andersson, 2005). Leaf total C concentrations were also reduced in flowering/fruiting individuals. Leaf ^{13}C abundance did not show any enrichment or depletion in the comparison of flowering/fruiting with non-flowering/non-fruiting individuals, and leaf ^{15}N abundance was depleted in flowering/fruiting individuals. Thus, *G. repens* did not compensate for the lack of chlorophyll and leaf total N with an increased use of a ^{13}C- and ^{15}N-enriched fungal source as was shown for albino and variegated individuals of *C. damasonium* (Stöckel *et al.*, 2011). *Goodyera* individuals die after flowering (Mollison, 1943), and the pale leaf colour is related to senescence. Our

results are in accordance with the conclusions drawn from stable isotope analyses in different compartments of *Epipactis* species. The study of Gonneau *et al.* (2014) indicates that photosynthesis supports mostly fruiting costs, even in partially mycoheterotrophic orchid species. Photosynthetic rates can be adapted to a large extent by orchids, depending on the light regime. Even defoliated individuals of *E. helleborine* maintained the same seed production by increasing stem and fruit photosynthesis without increasing fungus to orchid C fluxes.

The reasons for different responses in leaf C isotope abundance to changes in light availability by *Cephalanthera* species and *G. repens* are difficult to identify. A most likely reason can be traced back to different fungal partners forming mycorrhizas with these orchids. *Cephalanthera* species are known to associate obligately with fungi simultaneously forming ectomycorrhizas with forest trees (Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Liebel *et al.*, 2010; Hynson *et al.*, 2013). As confirmed in this study, *G. repens* preferentially associates with *Tulasnella* and *Ceratobasidium*. Tulasnelloid and ceratobasidioid fungi are considered ubiquitous saprotrophic fungi of the phylogenetically heterogeneous rhizoctonia group (Smith and Read, 2008), though some investigations report ectomycorrhizal abilities for some *Tulasnella* (Bidartondo *et al.*, 2003) and Ceratobasidiaceae lineages (Bougoure *et al.*, 2010; Yagame *et al.*, 2012). Ectomycorrhizal fungi seem to be a prerequisite for the occurrence of irradiance-governed or leaf chlorophyll level-governed exploitation of the fungal partner as they always occur in orchid species of dark habitats and in (partly) albino individuals of mycoheterotrophic orchids (Julou *et al.*, 2005; Abadie *et al.*, 2006; Stöckel *et al.*, 2011). An occasional appearance of typical ectomycorrhizal fungi in orchid roots, such as *Russula* and *Lactarius*, as found in this study for some *G. repens* individuals, seems to be insufficient to turn these adult orchid individuals into efficient fungal exploiters. Saprotrophic fungi of the rhizoctonia group appear to be sufficiently powerful to Fully support seed germination and early protocorm development of the majority of green orchid species. Orchid growth in the early stage of development is extremely slow under natural field conditions. Stöckel *et al.* (2014) reported a maximum orchid protocorm biomass of 0·5 mg of dry weight (d. wt) 1 year after seed burial, which rose linearly to a maximum of 3·8 mg d. wt after 4 years. This biomass production relates to annual C gains from the fungus in the range of only a few micromoles and N gains as low as a few hundred nanomoles. The saprotrophic fungal partners of *G. repens*, however, appear unable to compensate for adult plant C limitations under low-light conditions or for the N demands required for flower development. This limited power of saprotrophic rhizoctonia fungi to support the C and nutrient requirements of adult orchids most probably explains why fully mycoheterotrophic orchids prefer as their hosts fungal lineages other than saprotrophic rhizoctonia fungi. All fully mycoheterotrophic orchids so far investigated exploit either ectomycorrhizal fungi or non-rhizoctonia saprotrophic wood- or litter-decay fungi (Hynson *et al.*, 2013). While ectomycorrhizal fungi and wood- or litter-decay fungi are known to be enriched in heavy C and N isotopes (see Introduction), the isotopic composition of rhizoctonias is still unknown, because these fungi produce no fruiting bodies. Knowledge about the isotopic composition of rhizoctonia fungi would be desirable (Stöckel *et al.*, 2014) in order to understand their potential as targets for minor exploitation by adult green orchids.

Unexpectedly, we observed significantly different leaf $\delta^{15}N$ values in *Rubus chamaemorus* relative to other reference plants. The isotopic positioning of *R. chamaemorus* is similar to plants known to fulfil major parts of their N demand by symbiotic N_2 fixation with bacteria (Shearer and Kohl, 1989; Schulze *et al.*, 1991; Gebauer and Meyer, 2003). The related *Rubus ellipticus* is known to fix N_2 symbiotically with actinomycetes found in root nodules (Becking, 1979). Thus, symbiotic N_2 fixation might be a possible reason for the distinctive leaf $\delta^{15}N$ values in *R. chamaemorus*. Previous searches for nitrogen-fixing nodules in *R. chamaemorus* in Scotland were without success (Nutman, 1976), as were our own searches for nodules on plants from southern Norway (Asker, Akershus county). Thus, the reason for the significant ^{15}N enrichment in *R. chamaemorus* leaves in comparison with accompanying plants merits further investigation.

Goodyera repens is a predominantly rhizoctonia-associated clonal species that may react to a limited light regime by using a large clonal network of photosynthetic rosettes rather than increasing the nutrient flux from fungal partners to balance a lack of irradiance. As a consequence of this and related studies, further light manipulation experiments should be conducted to investigate the orchid-specific mycoheterotrophic changes in nutrient fluxes depending on the light regime. These experiments should elucidate whether there is a relationship between the ability to compensate for light limitation by using a fungal source and/or a different type of orchid mycorrhizal fungus (i.e. ectomycorrhizal vs. saprotrophic). It would also be interesting to correlate the extent of clonality with irradiance at different sites in future studies.

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6.3 Mycorrhizal specificity in Mediterranean meadow orchids (American Journal of Botany)

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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]

[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; and [4]Laboratory of Isotope Biogeochemistry, Center of Ecology and Environmental Research (BayCEER), University of Bayreuth 95440 Bayreuth, Germany

- *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*.

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).

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

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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 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).

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	—

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).

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

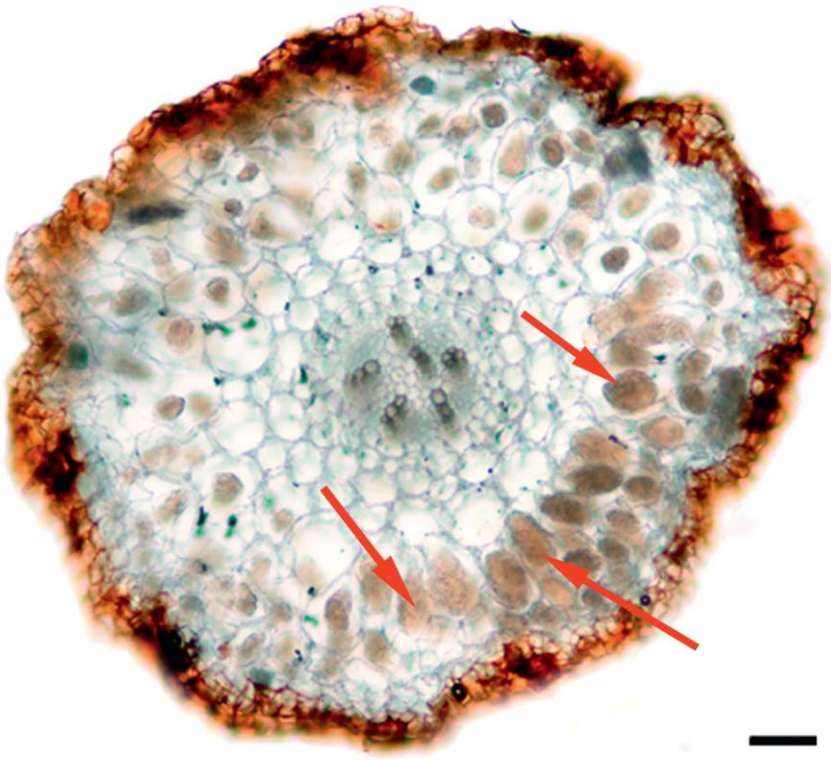

Fig. 1. Light micrograph of unstained, hand section from *Serapias vomeracea*, showing colonization of the cortical cells by fungal hyphae forming pelotons (arrows). Bar = 50 µm.

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.

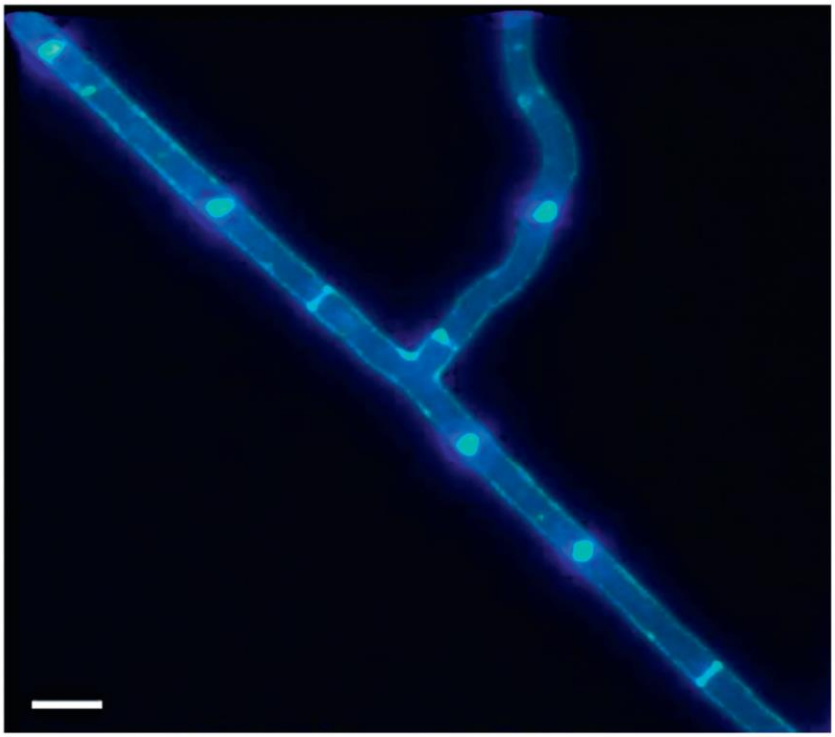

Fig. 2. Fluorescence micrograph of DAPI-stained fungal isolate from mycorrhizal roots of *Serapias vomeracea*, showing typical *Rhizoctonia* features, such as 90° branching of hyphae. Cells are binucleate. Bar =10 µm.

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 10 000 000 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 10 001 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

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

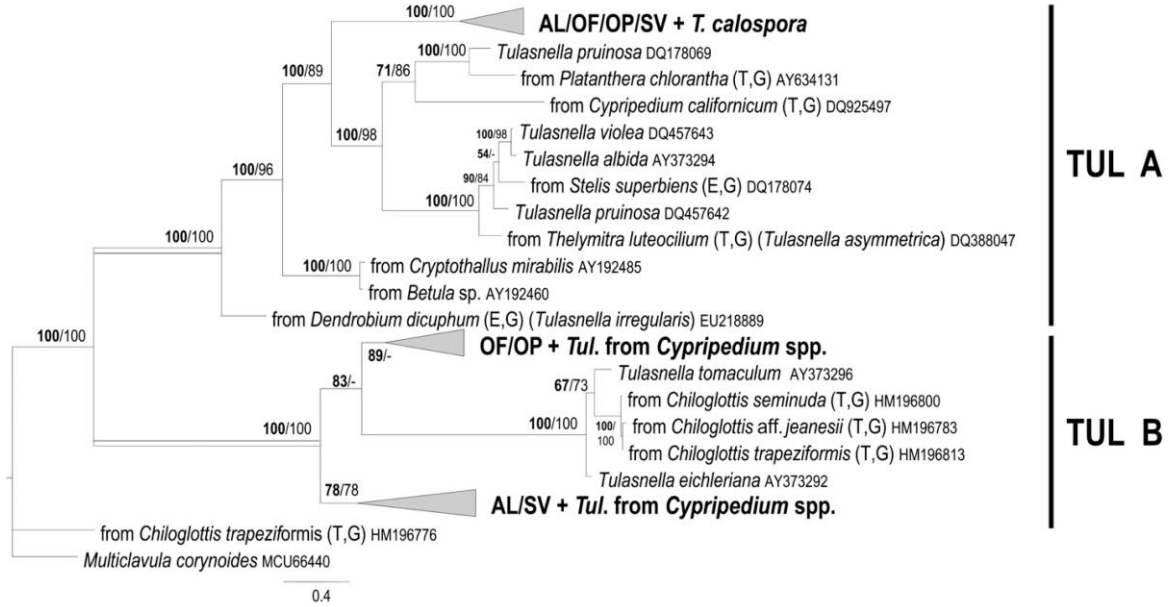

Fig. 3. Bayesian tree obtained from the ITS sequence alignment of tulasnelloid fungi. The GTR+G+I substitution model was used. Sequences from *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), *Orchis purpurea* (OP), *Serapias vomeracea* (SV), and from neighboring non-orchid species were obtained 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.

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).

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,

→

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.


A
from Cymbidium floribundum (T,G) EF393629
from Diplocaulobium anosmum (T,G) AJ313446
from Liparis loeselii (T,G) AM040890
from Anacamptis laxiflora (T,G) AJ549126
from Dactylorhiza incarnata (T,G) AJ549130
from Anacamptis laxiflora (T,G) AJ549133
SV16
AL2
AL1
from Anacamptis laxiflora (T,G) FJ809762
SV6
SV6 isolate
OF7
from Platanthera praeclara (T,G) DQ068773
from Anacamptis laxiflora (T,G) AM697921
AL10 isolate
SV8 isolate
SV8
from Spathoglottis plicata (T,G) AJ313445
from Spathoglottis plicata (T,G) AJ313441
from Vanilla sp. (E,G) DQ834406
SV8
SV5
Tulasnella calospora AY373298
Tulasnella deliquescens AY373291
AL13
AL13 isolate
AL12
AL14
Tulasnella calospora DQ388045
from Acianthus pusillus (T,G) AY643806
from Cymbidium goeringii (T,G) (Tulasnella calospora) EF393631
from Diuris maculata (T,G) (Tulasnella calospora) DQ388042
from Epipactis gigantea (T,G) AY634123
from Acianthus exsertus (T,G) (Tulasnella calospora) DQ388041
from Arundina graminifolia (T,G) AJ313436
SV14
OF9
SV4
AL4
SV18 isolate
SV18
SV17 isolate
AL10 isolate
SV16
from Tipularia discolor (T,G) AY373301
from Platanthera praeclara (T,G) DQ068770
from Microtis parviflora (T,G) (Tulasnella calospora) AY643804
from Goodyera pubescens (T,G) AY373282
from orchid (Tulasnella bifrons) AY373290
from Stelis concinna (E,G) DQ178086
from Stelis halli (E,G) DQ178085
from Dendrobium crumenatum (E,G) AJ313438
from Vanda "Miss Joaquim" (E,G) AJ313443
from Orchis purpurea (T,G) FJ809770
OF9 isolate
SV1 isolate
SV1
SV7
SV10
SV2 isolate
from Ophrys fuciflora (T,G) FJ809771
from Anacamptis morio (T,G) AJ549127
SV13
SV9 isolate
SV12
from Serapias vomeracea (T,G) FJ809763
SV20
SV21
from Liparis lilifolia (T,G) AY373288
from Liparis lilifolia (T,G) AY373287
from Epipactis gigantea (T,G) AY634122
from Platanthera praeclara (T,G) DQ068772
SV15 isolate
from Vanilla sp. (E,G) DQ834405
from Oncidium varimyce (E,G) AJ313449
from Arachis "Maggie Oei" (E,G) AJ313444
from Calypso bulbosa (T,G) (Epulorhiza anaticula) EU218891
Tulasnella danica AY373297
from Platanthera obtusata (T,G) DQ278944
from Stelis superbiens (E,G) DQ178115
SV9
SV11
AL11
Tulasnella violea DQ457643
Tulasnella albida AY373294
from Stelis superbiens (E,G) DQ178074
Tulasnella pruinosa DQ457642
from Thelymitra luteocilium (T,G) (Tulasnella asymmetrica) DQ388047
Tulasnella pruinosa DQ178069
from Platanthera chlorantha (T,G) AY634131
from Cypripedium californicum (T,G) DQ925497
from Cryptothallus mirabilis AY192485
from Betula sp. AY192460
from Dendrobium dicuphum (E,G) (Tulasnella irregularis) EU218889
0.4
TUL A1
39.3% SV sequences (33.3% SV plants);
69.2% AL sequences (50.0% AL plants);
15.4% OF sequences (20.0% OF plants).
TUL A2
TUL A3
42.8% SV sequences (52.4% SV plants);
15.4% OF sequences (20.0% OF plants).
TUL A4

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).

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.*

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.

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*

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.

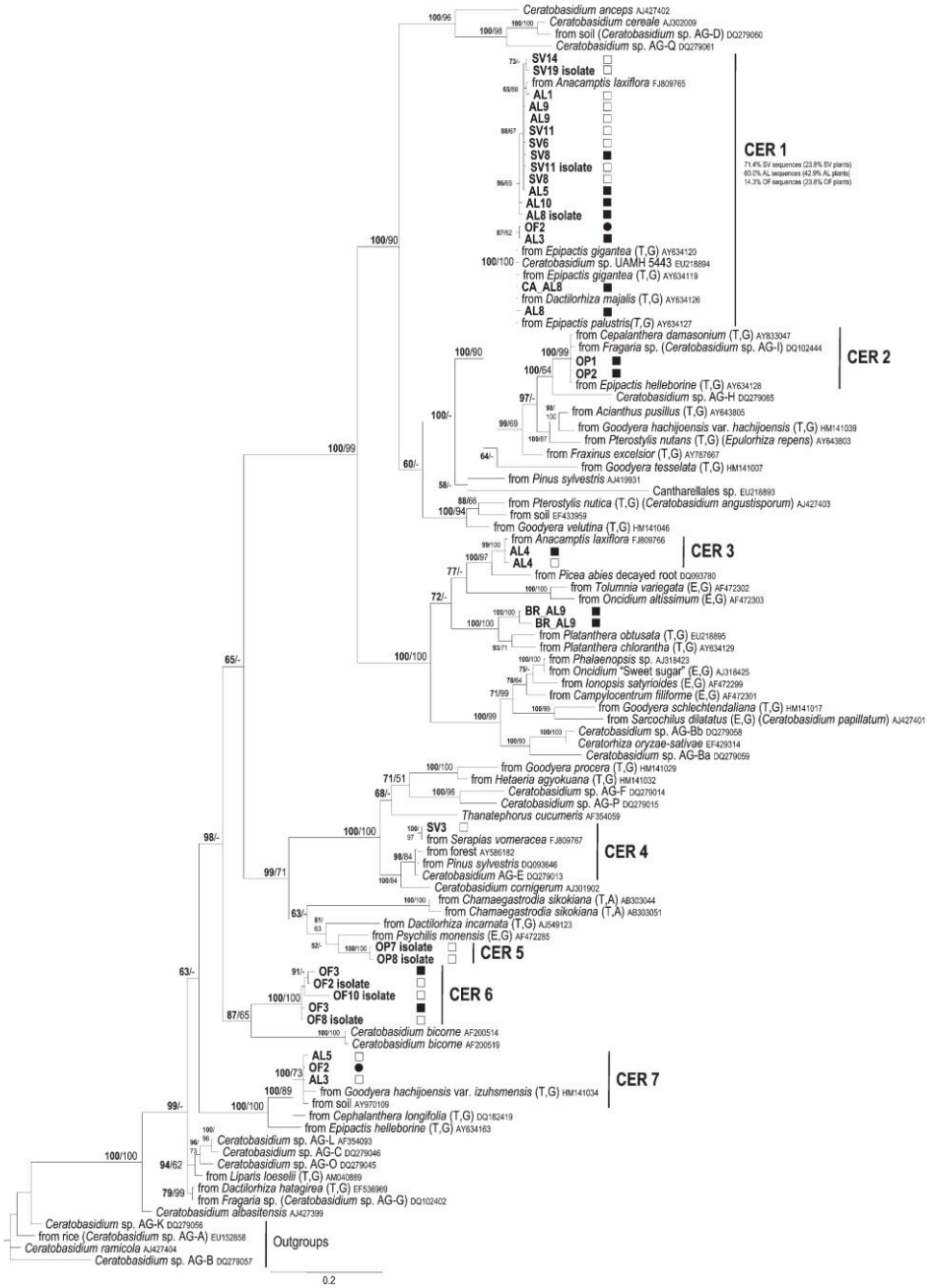

Fig. 6. Bayesian tree obtained from the ITS (ITS1-5.8S-ITS2) sequence alignment of ceratobasidioid fungi. The HKY+G substitution model was used. Sequences from *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), *Orchis purpurea* (OP), and *Serapias vomeracea* (SV) were obtained either with the universal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

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).

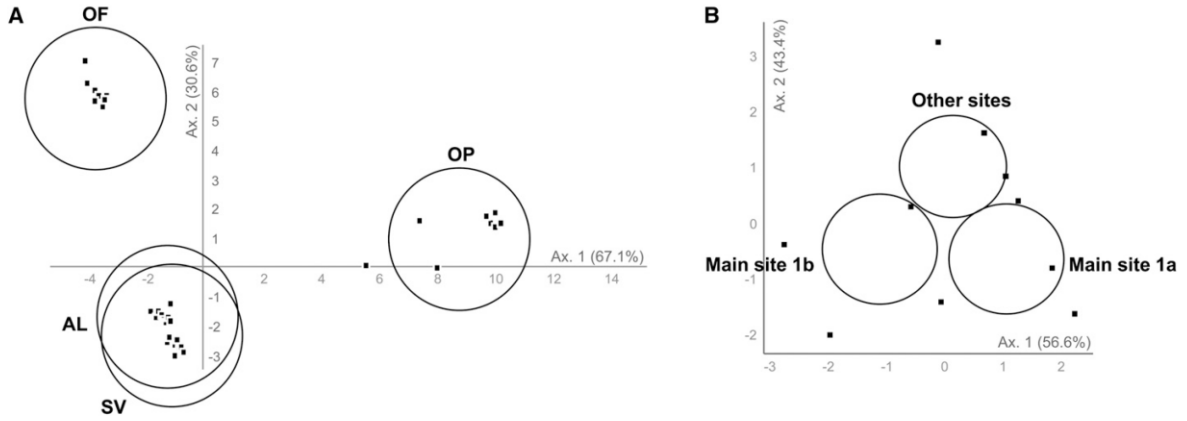

Fig. 8. Discriminant analysis (DA) plots comparing *Rhizoctonia* spectra in (A) *Ophrys fuciflora* (OF), *Anacamptis laxiflora* (AL), *Orchis purpurea* (OP), and *Serapias vomeracea* (SV) roots collected at all sampling sites, and (B) *S. vomeracea* plants collected at the main study site meadows 1a 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.

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.

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).

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.

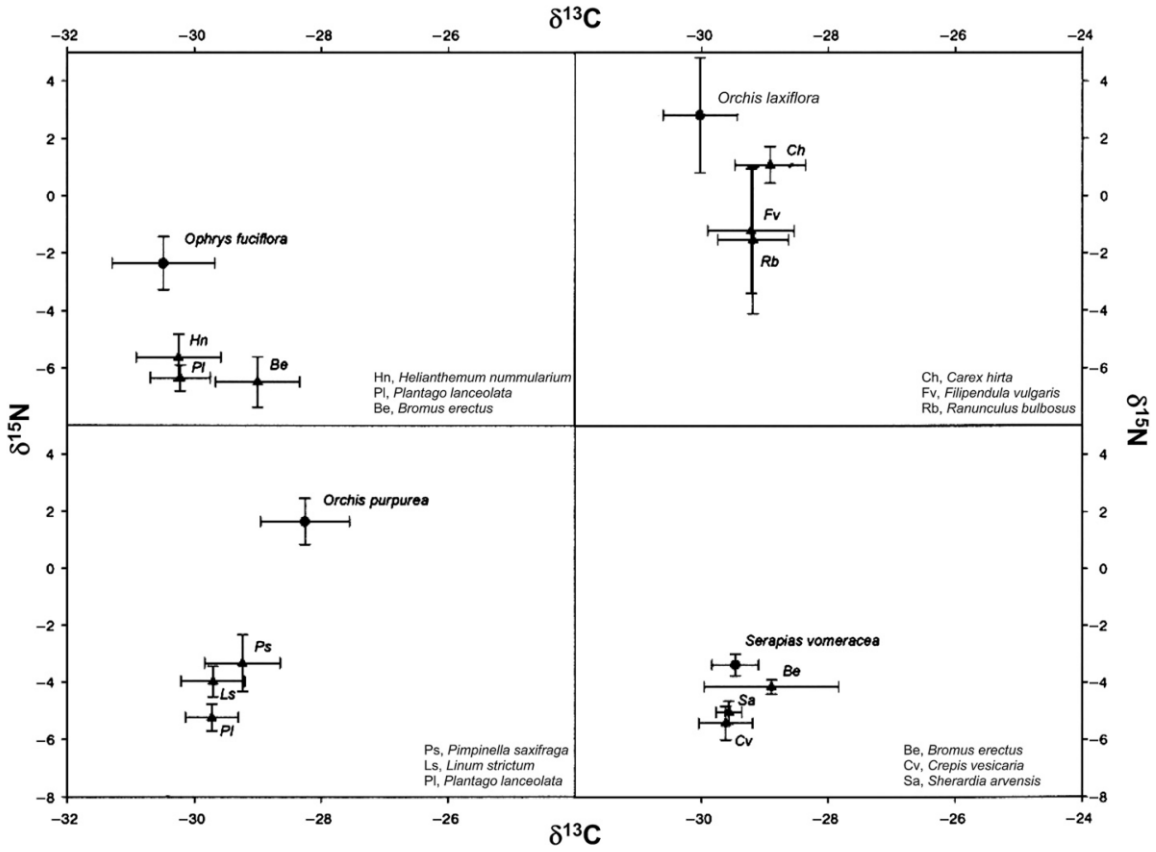

Fig. 9. Mean $\delta^{15}N$ and $\delta^{13}C$ values (±SD) in leaves of the four orchids ($N = 5$ per species) and neighboring autotrophic non-orchid species ($N = 5$; Be, *Bromus erectus*; Ch, *Carex hirta*; Cv, *Crepis vesicaria*; Fv, *Filipendula vulgaris*; Hn, *Helianthemum nummularium*; Ls, *Linum strictum*; Pl, *Plantago lanceolata*; Ps, *Pimpinella saxifraga*; Rb, *Ranunculus bulbosus*; Sa, *Sherardia arvensis*).

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

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 ***	—

Notes: Significance levels for deviations from zero, based on a Mann–Whitney-*U* test with sequential Bonferroni corrections: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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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Appendix A: Related article based on data collected during the diploma thesis in 2007 (American Journal of Botany)

Reference: LIEBEL H. T., BIDARTONDO M. I., PREISS K., SEGRETO R., STÖCKEL M., RODDA M., GEBAUER G. (2010) C and N stable isotope signatures reveal constraints to nutritional modes in orchids from the Mediterranean and Macaronesia. *American Journal of Botany*, **97**, 903-912.

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C and N stable isotope signatures reveal constraints to nutritional modes in orchids from the Mediterranean and Macaronesia[1]

Heiko T. Liebel[2], Martin I. Bidartondo[3], Katja Preiss[2], Rossana Segreto[4], Marcus Stöckel[2], Michele Rodda[4], and Gerhard Gebauer[2,5]

[2]Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, 95440 Bayreuth, Germany; [3]Imperial College London and Royal Botanic Gardens, Kew TW9 3DS, UK; and [4]Dipartimento di Biologia Vegetale, Università di Torino, V.le P.A. Mattioli 25 10125 Torino, Italy

We compared the nutritional modes and habitats of orchids (e.g., autotrophic, partially or fully mycoheterotrophic) of the Mediterranean region and adjacent islands of Macaronesia. We hypothesized that ecological factors (e.g., relative light availability, surrounding vegetation) determine the nutritional modes of orchids and thus impose restrictions upon orchid distribution. Covering habitats from dark forests to open sites, orchid samples of 35 species from 14 genera were collected from 20 locations in the Mediterranean and Macaronesia to test for mycoheterotrophy. Mycorrhizal fungi were identified via molecular analyses, and stable isotope analyses were applied to test whether organic nutrients are gained from the fungal associates. Our results show that orchids with partial or full mycoheterotrophy among the investigated species are found exclusively in Neottieae thriving in light-limited forests. Neottioid orchids are missing in Macaronesia, possibly because mycoheterotrophy is constrained by the lack of suitable ectomycorrhizal fungi. Furthermore, most adult orchids of open habitats in the Mediterranean and Macaronesia show weak or no N gains from fungi and no C gain through mycoheterotrophy. Instead isotope signatures of some of these species indicate net plant-to-fungus C transfer.

Key words: ^{13}C; Macaronesia; Mediterranean; mycoheterotrophy; mycorrhiza; ^{15}N; Neottieae; Orchidaceae; stable isotopes.

Early research by Bernard (1909) first described how orchids live in close mycorrhizal symbiosis with fungi. Since that time, orchid mycorrhizas have attracted much interest from plant ecologists and mycologists. Previous studies investigating orchid nutrition have mainly focused on temperate regions with only marginal consideration of regions with Mediterranean climate (see Gebauer and Meyer, 2003; Selosse et al., 2004; Girlanda et al., 2006), despite the fact that the Mediterranean region shows a much higher orchid diversity—e.g., 56 species in Germany (Rothmaler, 2000) vs. 108 orchid species in Italy alone (Ministero dell'Ambiente e della Tutela del Territorio, 2007). In striking contrast, the adjacent climatically similar Macaronesian region is poor in orchid species—16 orchid species in Macaronesia including only eight orchid species on the Canary Islands (Eriksson et al., 1979; Hohenester and Welß, 1993).

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[5] Author for correspondence (e-mail: gerhard.gebauer@uni-bayreuth.de); phone: +49 (0)921 552060; fax: +49 (0)921 552564

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Orchids typically produce extremely light "dust seeds which are easily transported over large distances by the win The tiny seeds do not contain sufficient endosperm for germina tion and depend on nutrient supply by a fungal partner. In de veloping orchids (i.e., protocorms), Bernard (1909) detecte easily cultivable saprotrophic or pathogenic rhizoctonia-form ing fungi (belonging to the basidiomycete genera *Tulasnell Thanatephorus*, *Ceratobasidium*, and the biotrophic clade B o *Sebacina*; Weiss et al., 2004; Smith and Read, 2008). Perhap with the exception of a few epiphytic, tropical orchids, all o chids investigated so far remain mycorrhizal during their enti life cycle (Smith and Read, 2008).

Some nonphotosynthetic orchids completely depend on the fungal partners throughout life. Altogether, about 200 of thes fully mycoheterotrophic orchids (MHO) have been describe (Leake, 1994). Many MHOs depend on ectomycorrhiz (ECM) fungi that are simultaneously associated with overst rey plants (e.g., Taylor and Bruns, 1997; McKendrick et al 2002; reviewed in Bidartondo, 2005). Furthermore, MHC of the tribe Neottieae often have a pronounced mycorrhiz specificity toward lineages of ECM fungi that are difficult t cultivate (Selosse et al., 2002; Bidartondo et al., 2004). Withi other tribes of the Orchidaceae, ECM fungi have also bee found to form associations with *Hexalectris* and *Corallorhiz* species (McKendrick et al., 2000; Taylor et al., 2003). Thes orchids' dependence on ECM fungi was revealed by molecu lar identification of fungi forming coils (i.e., pelotons) insid orchid roots (Taylor and Bruns, 1997) in combination wit stable isotope natural abundance analysis (Gebauer and Meye 2003). The latter technique is useful to understand pathway for the acquisition of fungi-derived organic C and N based o stable isotope abundances in plant leaf tissue. The method

based on the observation that tissues from fruiting bodies of ectomycorrhizal fungi show a higher abundance of the heavy stable isotopes ^{13}C (Gleixner et al., 1993; Högberg et al., 1999) and ^{15}N (Gebauer and Dietrich, 1993) in comparison to neighboring autotrophic plants. The MHOs relying on ECM fungi are therefore also enriched in both ^{13}C and ^{15}N similarly to ECM fungi themselves (Trudell et al., 2003). Using stable isotope natural abundance analysis, some green orchids previously considered to be fully autotrophic (e.g., *Cephalanthera* and *Epipactis* spp.) were found to also have isotope signatures distinct from those of surrounding plants. Such orchids show ^{13}C and ^{15}N abundances intermediate between autotrophic non-orchid neighboring plants and fully MHOs, and so they are considered to be partially mycoheterotrophic (Gebauer and Meyer, 2003). This physiological phenomenon is not limited to the Orchidaceae; a similar mechanism has recently been discovered in pyroloids (Ericaceae) (Tedersoo et al., 2007; Zimmer et al., 2007; Hynson et al., 2009b). Furthermore, even nonphotosynthetic forms of generally green species may survive due to mycoheterotrophic nutrient supply (Julou et al., 2005; Abadie et al., 2006).

This study investigates whether the occurrence of full and partial C and/or N mycoheterotrophy (i.e., heterotrophy sensu Larcher, 2003 and Lüttge et al., 2005) is coupled to specific types of habitats and how ecological factors constrain the distribution of these nutritional modes. A large range of different Mediterranean and Macaronesian orchid taxa (35 species belonging to 14 genera and three tribes) was tested for their nutritional mode as a function of habitat. Orchids from open habitats, shrubland, forest gaps, and forests were investigated in continental Italy and the islands of Sardinia (Mediterranean) and Tenerife (Macaronesia) to test whether the occurrence of full and partial mycoheterotrophy among orchids is restricted to specific habitat characteristics. We are, however, aware that factors like the geographic distribution of orchid species and their fungal associates cannot be controlled in a large-scale "natural experiment" such as this study.

MATERIALS AND METHODS

Study sites—Orchids of three main regions were investigated: (1) the northern part of continental Italy (N 44.1–45.2°; E 7.1–10.1°), (2) the Mediterranean island of Sardinia (Italy, N 41.2–39.7°; E 9.4–9.8°) and (3) the Macaronesian island of Tenerife (Spain, N 28.2–28.4°; W 16.5–16.8°). The Mediterranean sites are characterized by summer droughts and a maximum of precipitation between October and May (mean annual precipitation at the sites: 800–1150 mm in continental Italy; 450–800 mm on Sardinia). The temperatures rarely drop below 0°C in winter, and they rise in summer to mean temperatures of ca. 25°C in the months July and August. The mean annual precipitation of investigated sites on Tenerife is 400–700 mm (with an additional component from humidity combed out by pine trees from daily orographic fog due to the permanent stream from northeasterly trade winds at a site with *Orchis canariensis*). The rainy period lasts from October to March; the mean annual temperatures vary from 10–18°C according to altitude and exposure (Höllermann, 1982; García Canseco, 2004).

Orchids from 20 sites were investigated. Each of these sites was classified as one of the following habitat types: open habitat, shrubland, forest, and forest gap. Because all orchids investigated in deciduous forests developed leaves after tree canopy development and disappeared before the fall of tree leaves, a further distinction according to orchid phenology between deciduous and evergreen forests was not necessary. The different habitat types are distinguished by accompanying plant species, mycorrhizal associations, and light regime. To clarify the habitat-dependent light regime, we calculated the relative light availability (%) by comparing simultaneously performed measurements of the photosynthetic active radiation (Quantum Sensor, Li-Cor, Lincoln, Nebraska, USA) close to the orchid leaves and above the canopy or outside the forests, respectively. Mean relative light availability was the lowest at forest ($7 \pm 4\%$; $N = 4$) and shrubland sites (7%; $N = 2$) and the highest at open sites ($84 \pm 18\%$; $N = 10$), whereas irradiances at forest gap sites ranged in between ($57 \pm 30\%$; $N = 3$). In continental Italy, the largest distance between any two sites was 270 km, and plants were collected at three open grassland sites, two deciduous broadleaf forest sites, and one forest gap. Sites on Sardinia were distributed across the whole island (maximum distance from each other: 165 km) and orchids were sampled at seven open habitat sites (grassland, degraded steppe, or open places in patchy macchia), two evergreen (*Quercus ilex*) forest sites, and one shrubland site. Plant material on Tenerife was taken from one open grassland site, two gaps of coniferous (*Pinus canariensis*) forest, and one shrubland site with a maximum distance of 40 km. Detailed site descriptions can be found in Appendix S1 (see Supplemental Data at http://www.amjbot.org/cgi/content/full/ajb.0900354/DC1) including vegetation characteristics, light availability data, geographic coordinates, and details on the collected species.

Standardized vegetation surveys per plot to record all plant species surrounding the target orchid within 1 m^2 were set up, and the mycorrhizal type of each species was investigated using the review article on the phylogenetic distribution of mycorrhizas in land plants of Wang and Qiu (2006). Plants that depend on ectomycorrhizal associations (mainly Fagaceae, Pinaceae, and Cistaceae) were found in most plots irrespective of habitat type (see Table 2).

Sampling scheme and investigated species—A total of 35 orchid species were investigated (27 members of the tribe Orchideae, one of the Cranichideae, and seven of the Neottieae). Five of the 35 orchid species were collected in two of the three main regions. In continental Italy, 15 orchid species of all three tribes were sampled, while the 19 orchid species collected on Sardinia belong to the tribes Orchideae and Neottieae and the six species from the Macaronesian region exclusively belong to the Orchideae. All samples were collected in April and May 2007 except for *Barlia metlesicsiana* on Tenerife (collected in January 2008). Orchid species nomenclature follows Baumann et al. (2006) except for the island endemics of Sardinia (Delforge, 2005).

Sites having at least five individuals of an orchid species growing a minimum of 2 m apart from each other (to avoid sampling orchid clones) were located. To evaluate the orchids' stable isotope signatures, each of the orchid plots (i.e., area around the orchid, maximum 1 m apart) additionally had to contain three autotrophic reference plants (listed in Appendix S1). For each orchid species, samples were collected from five plots yielding five replicates to allow statistical validation (except for *Cephalanthera damasonium*, $N = 2$). One to two leaves of the orchid and the reference plants were sampled. Leaf material was taken at approximately the same height because it is known that the CO_2 uptake and stomatal regulation at different heights above the soil surface results in different $\delta^{13}C$ values due to different CO_2 sources (soil vs. atmosphere), light climate, and water vapor pressure deficit (Farquhar et al., 1989; Gebauer and Schulze, 1991; Bauer et al., 2000). As *Neottia nidus-avis* has only a few small bracts, a section of the aboveground inflorescence was collected instead of leaves.

Analysis of stable isotope abundance and N concentration—Leaf and stem samples were oven-dried at 105°C and ground to a 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). Measured isotope abundances are denoted as δ values, which were calculated according to the following equation: $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ [‰], 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 and 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 and Schulze, 1991). Acetanilide has a constant N concentration of 10.36%.

Statistics—ANOVA and post hoc comparisons based on Tukey's honestly significant difference (HSD) test of reference plant $\delta^{13}C$ and $\delta^{15}N$ values

were used to test for differences in their stable isotope abundances at different sites. If no difference is found in the reference species at different sites, the δ values of the orchid samples can be compared with each other directly. Significant differences (for $\delta^{13}C$: $F_{19, 525} = 29.946$, $P < 0.001$ and for $\delta^{15}N$: $F_{19, 525} = 28.630$, $P < 0.001$), however, were found at 50% of the sites. Thus, a normalization of δ values was necessary to compare data among the 20 sites. As described by Preiss and Gebauer (2008), the $\delta^{13}C$ and $\delta^{15}N$ values of the orchids and the nonorchid autotrophic reference plants were used to calculate normalized enrichment factors for each sample as $\varepsilon_S = \delta_S - \delta_{REF}$, with S as a single value of a sample from an autotrophic, partially or fully mycoheterotrophic orchid and REF as mean value of all autotrophic reference plants from the respective plot. Although it has been shown that the ^{13}C and ^{15}N signature of fully autotrophic C_3 plants in temperate climates does not systematically depend on their life form or mycorrhizal status (Gebauer and Dietrich, 1993; Gebauer and Meyer, 2003; Zimmer et al., 2007), we kept the spectrum of reference plants as diverse as possible (monocotyledons/dicotyledons, tree saplings/herbs, evergreen/deciduous, ectomycorrhizal/ericoid-mycorrhizal/arbuscular-mycorrhizal, or nonmycorrhizal) to minimize errors when calculating relative enrichments of the orchids.

To test for significant differences between green orchids and their respective reference plants or between green orchids and fully mycoheterotrophic orchids, the Kruskal–Wallis nonparametric test and Mann–Whitney *U* tests for post hoc comparisons were used. For the calculations of the enrichment factors of *Serapias cordigera* (Sardinia), only two reference species were taken into account. *Centaurium maritimum* was excluded as a reference species because it had surprisingly high $\delta^{13}C$ and $\delta^{15}N$ values. Some members of Gentianaceae are fully mycoheterotrophic (Imhof, 1999; Imhof and Weber, 2000); hence, a partially mycoheterotrophic nutritional mode may be expected in members of this family.

A cluster analysis (Ward linkage, squared Euclidean distance) based on the relative enrichment in ^{13}C and ^{15}N of the different orchid species collected in the three sampling areas in comparison to the respective nonorchid references (ε values) was carried out to identify groups within the dataset.

A correlation analysis evaluates the link between the ε ^{13}C values of the orchids and the relative light availability at the different habitats. Spearman's rank correlation coefficient was calculated, and the correlation was further analyzed with the help of a standard linear regression analysis.

Statistical analyses were performed with SPSS v.11.5 (SPSS, Chicago, Illinois, USA) and PC-ORD v.5.03 (MjM Software, Gleneden Beach, Oregon, USA). Data are given as means ±1 SD.

Molecular identification of mycorrhizal fungi—From each of the five individuals of each orchid species, two root sections colonized by fungi were sampled and placed in lysis buffer (cetyltrimethyl ammonium bromide, CTAB). Roots of four orchid species from continental Italy (*Ophrys fuciflora*, *Orchis purpurea*, *O. laxiflora*, and *Serapias vomeracea*) were analyzed at the Dipartimento di Biologia Vegetale in Torino. From these samples genomic DNA was extracted using a CTAB method (Girlanda et al., 2006), amplified with the primer combinations ITS1F/ITS4, ITS1F/ITS4B, and ITS1/ITS4-tul, and sequenced. If impure electrophoretograms were obtained, then the PCR products were cloned using the pGEM-T kit (Promega, Madison, Wisconsin, USA) as described in Girlanda et al. (2006). All other orchid root samples were analyzed at the Royal Botanic Gardens in Kew. Genomic DNA was extracted following methods described elsewhere (Gardes and Bruns, 1993) but using a GeneClean II Kit (Q-BioGene, Carlsbad, California, USA) for DNA binding and purification. The nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the fungal-specific primers ITS1F/ITS4, ITS1/ITS4-tul and ML5/ML6 using the polymerase chain reaction (PCR) and methods described in Bidartondo and Duckett (2009). Positive PCR products were purified using QIAquickMultiwell PCR Purification Kit (Qiagen, Valencia, California, USA). The PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California, USA) and analyzed as described above. From each clone library at least four colonies were used for reamplification with the corresponding rDNA primers. The DNA sequencing was performed on an ABI3730 Genetic Analyzer using BigDye v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) and absolute ethanol/EDTA precipitation. Electrophoretograms were checked using the program Sequencher v.4.5 (Gene Codes, Ann Arbor, Michigan, USA). All samples with strong PCR amplification of single templates were compared to GenBank using the program BLAST (http://www.ncbi.nlm.nih.gov) to ascertain taxonomic affinity. All unique DNA sequences have been submitted to GenBank (FJ688104–FJ688132 and FJ809762–FJ809770).

RESULTS

Stable isotope abundances—The cluster analysis based on the orchids' isotopic signatures revealed three categories (see online Appendix S2 at http://www.amjbot.org/cgi/content/full/ajb.0900354/DC1 and boxes in Fig. 1): (1) orchids collected in forests, (2) orchids of open habitats and forest gaps, and (3) an intermediate group composed of orchids from all four habitat types (open, forest gap, shrubland, and forest). While orchids from continental Italy and Sardinia cover all three clusters, the group of forest orchids is absent from Tenerife (Fig. 1). Species belonging to this group of typically forest-dwelling orchids are members of the tribe Neottieae characterized by considerable enrichments in ^{13}C and ^{15}N in comparison to nonorchids of the respective habitats (Fig. 1). The highest enrichment in ^{13}C (6.4 ± 1.8‰, $U < 0.01$, $P < 0.001$) and ^{15}N (13.9 ± 1.9‰, $U < 0.01$, $P < 0.001$) was for *Neottia nidus-avis*, the only chlorophyll-lacking orchid of this investigation, which accordingly has enrichment factors characteristic of fully MHOs associated with ECM fungi (Preiss and Gebauer, 2008). The cluster of orchids from open sites and forest gaps is composed of species of the tribes Orchideae and Cranichideae. They are relatively enriched in ^{15}N compared to nonorchid references though their ^{15}N enrichment is considerably lower than that of orchids from forest sites. With regard to the ^{13}C signature, most of these species are statistically indistinct from surrounding photosynthetic reference plants, while some show relative ^{13}C depletion (Table 1). The intermediate orchid group comprises members of all three tribes including two neottioids in continental Italy (*Cephalanthera longifolia* and *Listera ovata*). Plants of this category do not have the typical high enrichment in ^{13}C and ^{15}N as forest orchids do, but they are enriched in ^{13}C compared to nonorchids from their respective sites and to most orchids of open habitats.

Cephalanthera longifolia, one of the five species that were sampled at two different sites, falls into the group of forest orchids (Sardinia) or the intermediate group (continental Italy), depending on the respective habitat type. In continental Italy, *C. longifolia* was collected at a forest gap with relative light availability of 23% and on Sardinia in a densely shaded forest with only 2% of irradiance reaching the understorey plants. *Serapias parviflora* was also collected on two different sites with different relative light availability (62% vs. 90%), and it belongs to the group of orchids of open habitats on Sardinia. Individuals of *S. parviflora* collected at the more exposed grassland terraces on Tenerife are slightly enriched in ^{13}C (not significantly, Table 1) and therefore fall into the intermediate group (Fig. 1).

Regarding the orchids' taxonomy, it becomes apparent that all neottioids are significantly enriched in ^{13}C and ^{15}N compared to autotrophic reference plants (Fig. 1, Table 1), some of them even as strong as obligate mycoheterotrophs (e.g., green *Epipactis helleborine* on Sardinia, ε $^{13}C = 6.7 \pm 1.7$‰, $U = 12.00$, $P = 0.999$) and ε $^{15}N = 13.2 \pm 3.1$‰, $U = 11.00$, $P = 0.841$, $N = 5$; statistically tested against *Neottia nidus-avis* growing at the same site). Most representatives of the Orchideae and Cranichideae show significant relative enrichments in ^{15}N as well, but only a few members of the Orchideae (i.e., *Gennaria diphylla* from Tenerife, *Barlia robertiana*, *Orchis purpurea*, and *Habenaria tridactylites*) are additionally enriched in ^{13}C. For some species of the genera *Ophrys*, *Orchis*, and *Aceras* (all Orchideae), a significant depletion in ^{13}C in relation to their autotrophic reference plants was found (Table 1).

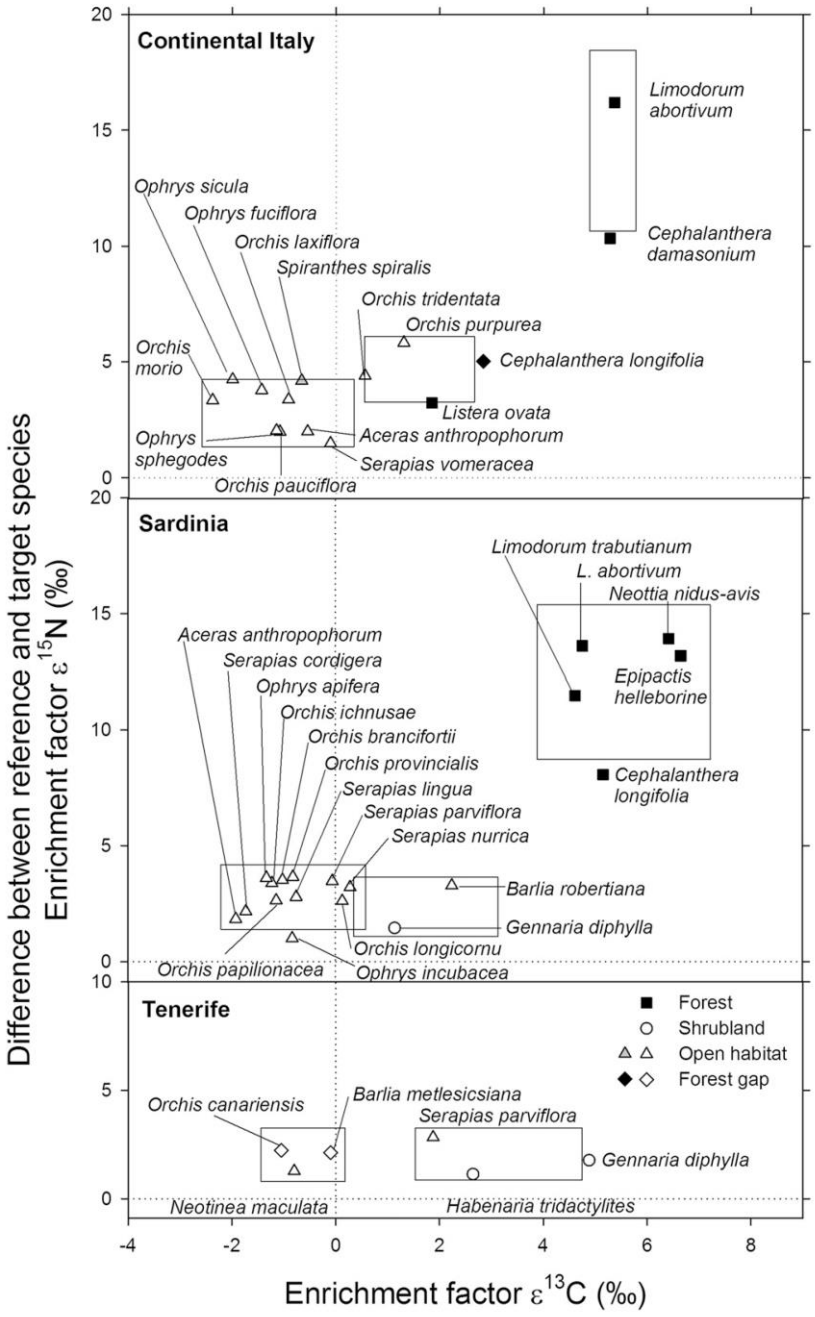

Fig. 1. Enrichment factors (ε) for ^{13}C and ^{15}N of 35 orchid species (including the chlorophyll-lacking *Neottia nidus-avis*) collected at 20 sites in continental Italy, Sardinia, and Tenerife. Orchids of the tribe Neottieae are indicated with black, Orchideae with white, and Cranichideae with gray symbols. The boxes represent one SD of the mean ε values for three groups of orchids as obtained from a cluster analysis: orchids of open habitats (left boxes), typical forest orchids (right boxes, not present on Tenerife), and orchids with intermediate isotope signatures (middle boxes). After normalization, mean ε values of the autotrophic references are equal to zero. All δ values of ^{13}C and ^{15}N of orchids and reference species (Appendix S1) as well as the diagram of the cluster analysis (Appendix S2) are available in the Supplemental Data at http://www.amjbot.org/cgi/content/full/ajb.0900354/DC1.

TABLE 1. Nitrogen concentrations and significances for the relative enrichment or depletion in ^{13}C and ^{15}N of all investigated orchid species compared to their respective autotrophic reference plants.

Region / Orchid species	Relative enrichment (+) or depletion (-) in ^{13}C			Relative enrichment (+) or depletion (-) in ^{15}N			N concentration		
	^{13}C	*U*	*P*	^{15}N	*U*	*P*	$mmol/g_{DM} \pm SD$	*U*	*P*
Continental Italy									
Cephalanthera damasonium	+			+			2.92		
Cephalanthera longifolia	+	5.00	0.002	+	<0.01	<0.001	3.10 ± 0.41	2.00	<0.001
Limodorum abortivum	+	<0.01	<0.01	+	<0.01	<0.001	2.40 ± 0.40	11.00	0.019
Listera ovata	+	1.00	<0.001	+	<0.01	<0.001	3.13 ± 0.37	2.00	0.001
Aceras anthropophorum	−	21.00	0.219	+	13.00	0.044	1.59 ± 0.17	28.00	0.445
Ophrys fuciflora	−	24.00	0.266	+	<0.01	<0.001	2.52 ± 0.26	1.00	<0.001
Ophrys sicula	−	9.00	0.011	+	<0.01	<0.001	1.03 ± 0.17	27.00	0.395
Ophrys sphegodes	−	13.00	0.033	+	10.00	0.015	1.86 ± 0.33	10.00	0.015
Orchis laxiflora	−	9.00	0.011	+	3.00	0.001	1.84 ± 0.13	19.00	0.119
Orchis morio	−	19.00	0.119	+	<0.01	<0.001	1.53 ± 0.26	31.50	0.612
Orchis pauciflora	−	14.00	0.042	+	12.00	0.025	1.11 ± 0.19	1.00	<0.001
Orchis purpurea	+	4.00	0.002	+	<0.01	<0.001	1.66 ± 0.13	27.50	0.395
Orchis tridentata	+	20.00	0.142	+	<0.01	<0.001	1.70 ± 0.25	22.00	0.197
Serapias vomeracea	−	33.00	0.735	+	<0.01	<0.001	1.24 ± 0.20	23.00	0.230
Spiranthes spiralis[a]	−	22.00	0.197	+	<0.01	<0.001	2.43 ± 0.29	<0.01	<0.001
Sardinia									
Cephalanthera longifolia	+	<0.01	<0.001	+	<0.01	<0.001	3.37 ± 0.22	3.00	0.001
Epipactis helleborine	+	<0.01	<0.001	+	<0.01	<0.001	3.68 ± 0.44	1.00	<0.001
Limodorum abortivum	+	<0.01	<0.001	+	<0.01	<0.001	2.16 ± 0.27	<0.01	<0.001
Limodorum trabutianum	+	<0.01	<0.001	+	<0.01	<0.001	2.35 ± 0.20	2.00	0.001
Neottia nidus-avis	+	<0.01	<0.001	+	<0.01	<0.001	2.59 ± 0.12	4.00	0.002
Aceras anthropophorum	−	<0.01	<0.001	+	8.00	0.008	1.48 ± 0.24	36.00	0.933
Barlia robertiana	+	<0.01	<0.001	+	<0.01	<0.001	0.94 ± 0.21	31.00	0.612
Gennaria diphylla	+	21.00	0.168	+	14.00	0.042	1.61 ± 0.43	1.00	<0.001
Ophrys apifera	−	13.00	0.033	+	5.00	0.002	1.68 ± 0.06	<0.01	<0.001
Ophrys incubacea	−	28.00	0.445	+	13.00	0.033	1.50 ± 0.46	15.00	0.053
Orchis brancifortii	−	20.00	0.142	+	<0.01	<0.001	1.39 ± 0.11	34.00	0.800
Orchis ichnusae	−	6.00	0.004	+	<0.01	<0.001	2.01 ± 0.05	25.00	0.306
Orchis longicornu	+	34.00	0.800	+	5.00	0.002	1.73 ± 0.27	9.00	0.011
Orchis papilionacea	−	20.00	0.142	+	<0.01	<0.001	1.73 ± 0.31	5.00	0.002
Orchis provincialis	−	11.00	0.019	+	<0.01	<0.001	1.89 ± 0.20	<0.01	<0.001
Serapias cordigera	−	11.00	0.099	+	18.00	0.440	1.12 ± 0.13	19.00	0.119
Serapias lingua	−	20.00	0.142	+	1.00	<0.001	1.44 ± 0.20	10.00	0.015
Serapias nurrica	+	37.00	1.000	+	10.00	0.015	1.93 ± 0.38	<0.01	<0.001
Serapias parviflora	−	31.00	0.612	+	<0.01	<0.001	1.40 ± 0.33	36.00	0.933
Tenerife									
Barlia metlesicsiana	−	29.00	0.497	+	3.00	0.001	2.03 ± 0.47	14.00	0.042
Gennaria diphylla	+	<0.01	<0.001	+	11.00	0.019	2.09 ± 0.16	19.00	0.119
Habenaria tridactylites	+	6.00	0.004	+	18.00	0.098	1.90 ± 0.30	25.00	0.306
Neotinea maculata	−	23.00	0.230	+	16.00	0.066	2.27 ± 0.27	26.00	0.349
Orchis canariensis	−	12.00	0.025	+	3.00	0.001	1.59 ± 0.13	1.00	<0.001
Serapias parviflora	+	16.00	0.066	+	<0.01	<0.001	1.60 ± 0.19	<0.01	<0.001

Note: Several orchids show significantly higher N concentrations than the reference plants. Species of the tribe Neottieae are given in bold. a, species of the tribe Cranichideae. $N = 5$ for all orchid species, except for *Cephalanthera damasonium* with $N = 2$; $N = 15$ for autotrophic reference species used for comparison per orchid species, except for *Serapias cordigera* with $N = 10$; the full dataset including reference plants is available in Appendix S1 (see Supplemental Data at http://www.amjbot.org/cgi/content/full/ajb.0900354/DC1).

Nitrogen concentrations—The total N concentrations in leaf material of the neottioids (2.85 ± 0.58 $mmol/g_{DM}$, DM: dry mass, $N = 42$) are significantly (Mann–Whitney $U = 294.5$, $P < 0.001$) higher than in leaves of nonneottioid orchids (1.67 ± 0.44 $mmol/g_{DM}$, $N = 150$). However, the group of nonneottioid orchids still has significantly (Mann–Whitney $U = 25364.0$, $P < 0.001$) higher leaf total N concentrations than the group of autotrophic reference species (1.40 ± 0.53 $mmol/g_{DM}$, $N = 513$), though this latter effect was not always significant on a species level based on plot comparisons (Table 1).

Molecular identification of mycorrhizal fungi—All investigated orchid roots contained fungal pelotons inside their root cortex cells, and mycorrhizal fungi could be identified from 50% of these roots partly because some root samples were not well preserved (shipping delays), roots were poorly colonized, and/or pelotons were collapsed. All neottioids from which fungal DNA could be analyzed are associated with ECM fungi (Table 2). The highest specificity to ECM partners is found in *Neottia nidus-avis* and *Limodorum* species. The majority of orchids of open habitats are associated with rhizoctonia-forming basidiomycetes (e.g., *Ceratobasidium* and *Tulasnella*) and ascomycetes (e.g., *Leptodontidium*), while those associated with obligate ECM fungi (*Orchis canariensis* and *Gennaria diphylla*) were found in forest gaps and shrublands.

DISCUSSION

Nutritional modes of orchids in the Mediterranean and Macaronesia—All fungal partners successfully identified in neottioids, solely forest orchids, turned out to be ectomycorrhizal fungi, either exclusively (e.g., *Limodorum abortivum*) or together with root endophytic saprotrophs (e.g., *Epipactis helleborine*) (Table 2). The orchids *Neottia nidus-avis*, *Limodorum abortivum*, and *L. trabutianum* show high mycorrhizal specificity toward only one or two fungal genera. The only fully MHO of this investigation, *Neottia nidus-avis,* is restricted to the fungal genus *Sebacina* in the area that we have examined. This finding is consistent with investigations on this orchid in other parts of Europe showing that *N. nidus-avis* is associated with fungi belonging to the ECM clade of *Sebacina* (McKendrick et al., 2002; Selosse et al., 2002). Sebacinoid fungi have been recognized among the most common ectomycorrhizal 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). Both *Neottia nidus-avis* and *Limodorum* species were regarded as fully mycoheterotrophic orchids at a site in France (Gebauer and Meyer, 2003). The study of Girlanda et al. (2006), however, suggested partial mycoheterotrophy in *L. abortivum* because chlorophyll is formed in the stem and the small leaves of this orchid and photosynthesis was detected. Isotope data in our present work confirm the latter finding. Investigated individuals of *Limodorum abortivum* ($\varepsilon\ ^{13}C = 5.1 \pm 0.5‰$, $U = 29.00$, $P = 0.006$, $N = 10$) and *L. trabutianum* ($\varepsilon\ ^{13}C = 4.6 \pm 1.0‰$, $U = 9.00$, $P = 0.008$, $N = 5$) of this study (Fig. 1) are significantly less enriched in ^{13}C than are fully mycoheterotrophic plants (Preiss and Gebauer, 2008). It thus can be concluded that these *Limodorum* plants are not solely using the organic fungal source but additionally assimilate C through photosynthesis, as recently described for the leafless *Corallorhiza trifida* (Zimmer et al., 2008, but see also Cameron et al., 2009).

Measurement of photosynthetic activity through gas exchange approaches as well as analysis of stable isotope natural abundance are both powerful tools for tracing the carbon gain by partially mycoheterotrophic orchids. However, the information gained from these techniques differs. While gas exchange measurements provide snapshot information about current photosynthetic activity, stable isotope natural abundance data integrate the sources of carbon gain over the entire life history of a plant or plant organ. Therefore, we cannot agree with the conclusion by Leake and Cameron (2010) that the failure to detect photosynthetic CO_2 gain at a single point in the life of a leafless but chlorophyllous orchid (*Corallorhiza trifida*) is sufficient evidence of full mycoheterotrophy, particularly when carbon isotope signatures (Zimmer et al., 2008) and gas exchange data (Montfort and Küsters, 1940) point toward partial mycoheterotrophy. A critical factor for the relative enrichment calculation in heavy isotope abundance of fully or partially mycoheterotrophic plants is the selection of reference plants, which becomes obvious from two recent investigations on mycoheterotrophs living on litter- or wood-decaying saprotrophic fungi. While Ogura-Tsujita et al. (2009; *Gastrodia confusa* with an $\varepsilon\ ^{13}C$ of 10.2‰) used reference plant samples from the understorey vegetation, Martos et al. (2009) used recently fallen tree leaves collected from the ground for *Wullschlaegelia aphylla* ($\varepsilon\ ^{13}C$ of 4.8‰) due to missing understorey reference plants. Leaves from the tree canopy, however, show enrichment in ^{13}C of 4 to 5‰ (Gebauer and Schulze, 1991) or even more (Koch et al., 2004) compared to understorey plants and therefore are not suited for this kind of comparison. A standardized sampling protocol as suggested by Gebauer and Meyer (2003) explicitly requires the collection of autotrophic reference plant

TABLE 2. Orchids and their associated mycorrhizal fungi from continental Italy, Sardinia, and Tenerife and presence (+) or absence (–) of ectomycorrhizal (ECM) plants at the respective sites.

Region	Orchid taxon	Mycorrhizal fungi (*N*)	ECM plants
Continental Italy	Orchideae		
	Ophrys fuciflora	*Ceratobasidium*[a] (2), *Tulasnella*[a, b] (5)	+
	Ophrys sphegodes	*Tulasnella*[a, b] (2)	–
	Orchis laxiflora	*Ceratobasidium*[a] (2), *Tulasnella*[a, b] (5)	–
	Orchis purpurea	*Ceratobasidium*[a](2), *Tulasnella*[a, b] (4)	–
	Serapias vomeracea	*Ceratobasidium*[a] (1), *Sebacina*[a, b] (1), *Tulasnella*[a, b] (5)	+
Sardinia	Neottieae		
	Cephalanthera longifolia	***Hebeloma*** (1), ***Russula*** (2), ***Tomentella*** (1)	+
	Epipactis helleborine	*Leptodontidium* (1), *Pyronemataceae*[b] (3), ***Tuber*** (2)	+
	Limodorum abortivum	***Russula*** (5)	+
	Limodorum trabutianum	***Russula*** (4), *Sebacina*[b] (1)	+
	Neottia nidus-avis	*Sebacina*[b] (5)	+
	Orchideae		
	Barlia robertiana	*Thanatephorus* (3)	–
	Gennaria diphylla	***Cenococcum*** (1), ***Lactarius*** (3), ***Russula*** (1)	+
	Ophrys apifera	*Tulasnella*[b] (4)	+
	Ophrys incubacea	*Thanatephorus* (1), *Tulasnella*[b] (4)	+
	Orchis ichnusae	*Tulasnella*[b] (1)	–
	Orchis longicornu	*Ceratobasidium* (2), *Leptodontidium* (1)	+
	Orchis papilionacea	*Ceratobasidiaceae* (1), *Tulasnella*[a, b] (1)	+
	Orchis provincialis	*Tulasnella*[b] (3)	+
	Serapias lingua	*Ceratobasidium*[a] (1), *Thanatephorus*[a] (1)	–
	Serapias parviflora	*Leptodontidium* (1)	+
Tenerife	Orchideae		
	Gennaria diphylla	*Leptodontidium*[a] (1), *Pezizaceae*[a, b] (3)	?
	Habenaria tridactylites	*Ceratobasidium*[a] (1), *Leptodontidium*[a] (2)	?
	Neotinea maculata	*Leptodontidium*[a] (3), *Ceratobasidiaceae*[a] (3), *Ceratobasidium*[a] (1), *Tulasnella*[a, b] (1)	+
	Orchis canariensis	***Russula***[a] (1), *Tulasnella*[b] (4)	+
	Serapias parviflora	*Leptodontidium*[a] (2)	+

Notes: Obligate ectomycorrhizal fungi are in boldface. *N*: number of orchid individuals in which a fungus was detected; all orchid roots were collected in five replicates; ?: species are present that are not classified for their mycorrhizal condition but that are phylogenetically closely related to ectomycorrhizal plants.

[a]Taxa detected by cloning PCR products

[b]Taxa that contain some ECM lineages

samples from a height above ground similar to where the target species is living to obtain results that are comparable with those from other studies and thus avoid misleading conclusions.

Cephalanthera longifolia and *Epipactis helleborine* collected at a forest site on Sardinia are characterized by strong enrichments in ^{13}C and ^{15}N showing that they are mainly nourished via mycoheterotrophic means. Because enrichment factors of *Epipactis helleborine* are within the range of fully mycoheterotrophic plants, we suggest that this orchid almost completely relies on fungal nutrient supply under extremely dark conditions. In summary, all investigated neottioids of the Mediterranean region turned out to be strongly (or even fully) mycoheterotrophic. There were only four species outside the tribe Neottieae (*Orchis purpurea* in continental Italy, *Barlia robertiana* on Sardinia, *Habenaria tridactylites*, and *Gennaria diphylla* on Tenerife) with apparent organic C and N gain from their fungal partners. Only *G. diphylla* was associated with ECM fungi, but we know from investigations on some fully mycoheterotrophic orchids that saprotrophic fungi can also be an effective nutrient source, at least in warm and humid climates (Yamato et al., 2005; Yagame et al., 2007; Ogura-Tsujita et al., 2009; Martos et al., 2009).

In orchids of open habitats, root endophytes are abundant and diverse. Most of the mycorrhizal associates are part of the saprotrophic, rhizoctonia-forming clades. Some of them (e.g., *Ceratobasidium* spp. and *Thanatephorus* spp.) occur in the roots of several orchid species and may have the potential to link different orchid species through their hyphal network. Orchids from exposed sites were frequently associated with members of the cosmopolitan family Tulasnellaceae (Roberts, 1999), which is in accordance with global investigations of orchid mycorrhizas (Dearnaley, 2007). We have to mention that the ecology of supposedly saprotrophic fungi could be more complex than generally thought. 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 *Pinus sylvestris*-endophytic (Sen et al., 1999), root-growth-promoting (Grönberg et al., 2006), as well as ectomycorrhizal strains (Yagame et al., 2008; Bougoure et al., 2009). Isotope data of some *Aceras*, *Orchis*, and *Ophrys* species (tribe Orchideae) show significant depletion in ^{13}C relative to their autotrophic references (Table 1). This phenomenon occurs in both the Mediterranean and Macaronesian region and has already been found for two *Goodyera* species (Hynson et al., 2009a) and (though statistically not significant) for some other *Orchis* species (Gebauer and Meyer, 2003). Depletion in ^{13}C might be a consequence of a specific flux of organic C compounds from the orchid to the fungus as it has been shown experimentally for the green orchid *Goodyera repens* (Cranichideae) by Cameron et al. (2006, 2008). They demonstrated that in vitro the C flux from *G. repens* to its nonectomycorrhizal fungus (*Ceratobasidium cornigerum*) is over five times higher than the fungus-to-plant C transfer. Depletion in ^{13}C together with enrichment in ^{15}N (as found for some Orchideae of open habitats in this study) could result from two simultaneous processes: (1) organic nutrient gain from fungi leading to enrichment in both ^{13}C and ^{15}N and (2) the plant-to-fungus flux of sugars assimilated through photosynthesis and thus enriched in ^{13}C compared to leaf bulk C (Gleixner et al., 1993). Thus, while the ^{15}N signal from heterotrophic nutrient gain remains within the plant, the ^{13}C enrichment can dissolve—and if more C flows from the plant to the fungus (supposedly under high light availability), it can even turn into a relative ^{13}C depletion.

Nitrogen concentrations—The strikingly high N concentrations of neottioids may be caused by nutrient gain from obligate ECM fungi. Such high N concentrations are in the range usually found for legumes associated with N_2-fixing bacteria (Gebauer et al., 1988). Fungi have similar C concentrations, but considerably higher N concentrations than plants (see e.g., Gebauer and Dietrich, 1993; Gebauer and Taylor, 1999). Thus, the incorporation of fungal metabolites after lysis of the pelotons inside the root cells of mycoheterotrophic orchids could produce a N surplus. Orchid species of open habitats display lower N concentrations although, in many cases, N is still significantly increased compared to that in autotrophic reference plants (Table 1). Previous studies on orchids from Central Europe and Estonia reported similar ranges of leaf N concentrations (Gebauer and Meyer, 2003; Abadie et al., 2006). Because orchids, as well as the majority of reference plants, presumably receive their N through association with mycorrhizal fungi, there must be physiological differences in how this occurs. For instance, the orchids could gain organic N compounds (e.g., amino acids) from their fungi, while other plants under temperate climate conditions may be supplied preferentially with mineral N compounds (Gebauer and Dietrich, 1993; Schulze et al., 1994).

Constraints on orchid nutrition and distribution—Gebauer (2005) suggested that light availability can determine the degree of mycoheterotrophy because the contribution from photosynthesis should be reduced at very dark sites. At a dense *Quercus ilex* forest on Sardinia, *Cephalanthera longifolia* is mainly nourished via mycoheterotrophic means. When growing at more exposed forest gaps, *C. longifolia* is less enriched in the heavy stable isotopes of C and N (Fig. 1) and thus less dependent upon organic nutrient supply from mycorrhizal fungi, fitting Gebauer's hypothesis. At open sites, where orchids were rarely associated with (potential) ECM fungi, we found depletion in ^{13}C for some members of the Orchideae. Our findings indicate that a net plant-to-fungus C flux may occur in these species and that this phenomenon might be coupled to open light-saturated habitats just as strong partial and full mycoheterotrophic nutrition are coupled to light-limited forest understories. A correlation analysis shows a significant negative relation (Spearman $\rho = -0.599$, $P = 0.001$, $N = 39$) between the ε ^{13}C values and the relative light availability at the different habitats, which is also represented in a linear regression analysis (Fig. 2). This statistical analysis underlines that a limited light climate at a site is a driving force for the need of fungal C as described by Preiss et al. (2010).

There are no reports of neottioid orchids in the Macaronesian region (Eriksson et al., 1979; Hohenester and Welß, 1993). Because wind dispersal of the orchids' extremely light dust seeds between the Mediterranean and Macaronesia cannot be excluded, a limited number of ectomycorrhizal plants in the Macaronesian region might be the major reason for this observation. A maximum of 20 ECM plant species is reported to occur on Tenerife, mostly belonging to the family Cistaceae (Hohenester and Welß, 1993). Despite the large number of ECM fungi that are linked to *Cistus* spp. (Comandini et al., 2006), it is questionable whether these shrubs are able to act as efficient host plants in tripartite symbioses between ECM plants, ECM fungi, and orchids in Macaronesia.

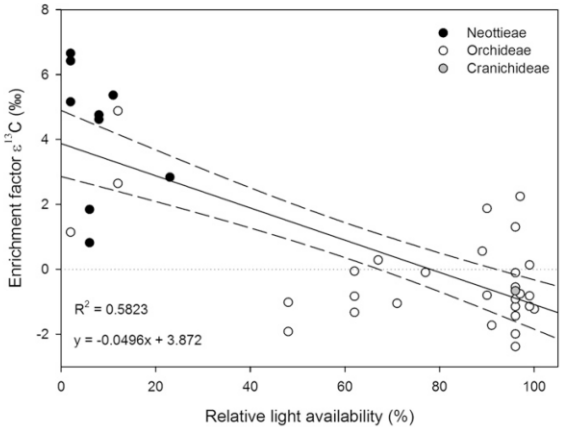

Fig. 2. Enrichment factors (ε) for ^{13}C of all orchid species of this study (excluding *Orchis pauciflora*; no light measurement was done at this site) are shown, separated by sampling sites and depending on the relative light availability at the different sites. A linear regression line is given with 95% confidence intervals (dashed lines).

Conclusions—On the basis of the wide spectrum of species and habitats investigated, we conclude that a potential net orchid-to-fungus C flux is coupled to open, light-saturated habitats, while a high dependence on mycoheterotrophy in orchids seems to be related to only some taxonomic groups (i.e., Neottieae in this study; but note that other tribes among the Orchidaceae occurring in the Mediterranean region are not [Maxillarieae, Malaxideae] or poorly [Cranichideae] represented in this study) and to the light-limited understorey of forest sites. Even though forests are present on the Macaronesian islands, fully mycoheterotrophic orchids are lacking, and the occurrence of partial mycoheterotrophy is rare. We raise the hypothesis that this pattern might be caused by the low diversity of ectomycorrhizal plants and/or suitable ectomycorrhizal fungi. More detailed investigations on the Macaronesian mycorrhizal community and experiments testing whether fully and partially mycoheterotrophic neottioids are able to germinate in the Macaronesian region are needed to confirm this hypothesis.

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Appendix B: Description of a new locality of *Epipogium aphyllum* (Blyttia)

Lille Raipas – a geological-botanical treasure-chest in Alta, Finnmark county

Heiko T. Liebel and Allan Krill

Liebel H.T. & Krill, A. 2011. Lille Raipas – a geological-botanical treasure-chest in Alta, Finnmark county. Blyttia 69: 74-86.

A 3 km^2 area on the hills of Lille Raipas, 7 km southeast of the town of Alta (Finnmark) displays a wealth of geological and botanical treasures. A species list containing more than 200 taxa is presented, including some rare species such as *Epipogium aphyllum*, *Eriophorum x medium* and *Woodsia glabella*. The geology of Lille Raipas is extraordinary. Distinctive rock types, including dolomite, shale, tillite, conglomerate and slate are responsible for different soil conditions and plant communities. Stromatolites, or beds of fossil algae in the dolomites are among the oldest fossils in Europe, dating back more than 1 800 million years. Red breccias are chaotic fragmental rocks that filled cave systems below an ancient flat land surface. A younger land surface, about 650 million years old, consisted of rugged hills of quartzite. It is also well preserved here, as it was covered by glacial moraines of the spectacular Snowball Earth era. All these rocks were buried by hundreds of meters of Alta-slates that were thrust from the northwest during the Caldeonian collision and mountain-building event.
The combination of different geological and botanical cites can be explored on a 3-hour walking trip using the location coordinates and descriptions presented in this article. Lille Raipas has much pedagogical value, as several different links in an intact ecosystem can be easily studied in this small area. It should be monitored and protected from damaging human impact.

Heiko T. Liebel, Allan Krill, Department of Geology and Mineral Resources Engineering, Norwegian University of Science and Technology (NTNU), Trondheim. Heiko.liebel@ntnu.no, allan.krill@ntnu.no

Background

Every year, some dozens of geology students from NTNU (Norwegian University of Science and Technology) travel to Lille Raipas in Alta municipality (Finnmark county) to participate in a field course for geological mapping. The reason for the long trip is the especially varied and distinct geology which is present in an area as small as 3 km^2. The local flora is surprisingly rich as well. Within a time range of one week in June and one week in August 2010, we could carry out an extensive botanical mapping covering most vascular plants in the area. The list of registered plant species for sure is not complete as some grass species were not in bloom yet in June and already impossible to determine in August.

This article aims at documenting plant species and linking them to the regional geology. In addition, we suggest a ca. 3 hour hiking trip on which the most interesting geobotanical sites can be seen.

Historical overview of Alta's geobotanical richness

Alta has been known for its special geology and vegetation since long time. Early geologists that mapped or visited Alta were among others Leopold von Buch (1810), Balthazar Mathias Keilhau 1827-1828 (Dahl 1934) and Olaf Holtedahl (1918). In the old days it was

common for geologists to be scientific generalists. Keilhau for example collected herbarium samples from Finnmark meanwhile he was investigating the geological conditions. Von Buch registered the variation in the vegetation as well. In 1810 he wrote excited about “Alten”: “Zwar sollte wohl die Armut der Vegetation die nördliche Breite verrathen, allein auch das Wenige ist hier von der Natur so reizend geordnet, daß es fast Ueberfluß scheint“ (translation: The poorness of the vegetation should reveal the northern latitude but the little is arranged in such an adorable way that it almost seems to be plentifulness).

Tellef Dahl wrote his thesis about “The Geology of Finnmark” in 1867 and started the interest in new and more detailed studies in the Alta and Raipas area.

Geological information about the area is available through the geological map sheet “Alta” in a scale of 1:50 000 (Zwaan & Gaultier 1980) including a detailed description of rock types, sedimentation and formation conditions. The distribution of ores and rock types was described in a doctoral thesis by Eirik Vik (1985).

Mathias Numsen Blytt was among the well-known botanists that visited Alta and botanised on the Store Raipas in 1841. The data collected got part of his lifetime achievement “Norway’s Flora” (1861). It is not known however if he was familiar with the rich flora at Lille Raipas which is located close-by. Nicolai Lund visited the Alta area at the same time like Blytt and describes his findings in the book, “Trip through Nordlandene and Vestfinnmarken in summer 1841”. He calls Alta “Finnmark’s paradise” (p. 22) and emphasizes this even more: “The whole landscape is adorably beautiful and I have never seen something more neat except the Guuldalen in Trondheim’s diocese” (p. 23). Ove Dahl (1934) stresses that the vegetation in the dolomite areas in Finnmark is particularly interesting and those areas are part of Tellef Dahl’s geological “Raipas system”.

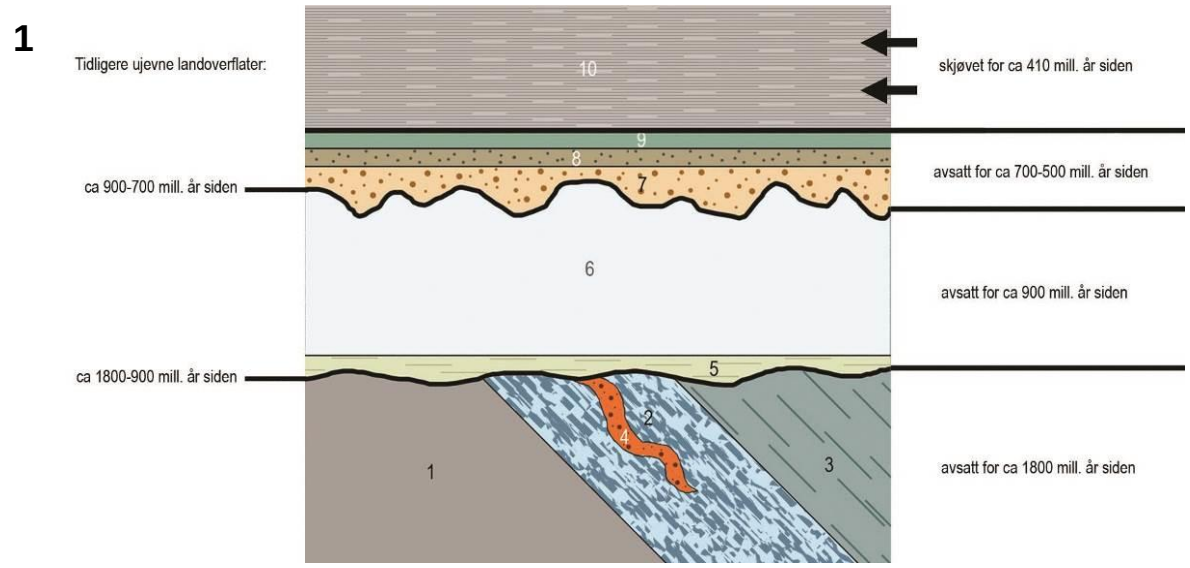

Figure 1. Geology of Lille Raipas. Rock types: 1) Volcanic sandstone and ash, 2) dolomite, 3) mudstone, all tilted and eroded to an old flat land surface. 4) Breccia from collapse of caverns, 5) sand- and siltstone above the erosional unconformity, 6) quartzite, with a rugged landscape above. 7) Tillite, or hardened glacial moraine, 8) sandstone with quartz pebbles, 9) mudstone, 10) Alta slate above a thrust plane.

Geology of Lille Raipas

The geological structure in the area is shown in Figure 1. The oldest geological layers in the area are fine-grained dark sandstones, which were deposited in early Proterozoic time. It is formed of volcanic ash and sand from submarine volcanoes (rock type 1 in Figure 1). The next rock type is dolomite (magnesium-rich limestone, rock type 2 in Figure 1) which was deposited in shallow and warm seawater with a rich growth of photosynthetic blue-green algae. At this moment in Earth's history life was restricted to the oceans. The fossilized blue-green algae, or stromatolites, on the Lille Raipas are more than 1800 million years old and among the oldest fossils found in Norway and Europe. Farther up in the stratigraphy, silt- and mudstones can be found that were deposited from turbid seawater (rock type 3 in Figure 1). The three lowermost layers, volcanic material, limestone and mudstone, are together several hundred meters thick. After being deposited horizontally on an old seafloor, the whole area got tilted and lifted up above sea-level. Erosion started at the land surface and about 1000 million years ago the whole landscape was eroded flat. Large caves formed especially in the dolomites. A new period of sedimentation started including the formation of sand- and mudstones on top of the flat terrain (rock type 5 in Figure 1). The weight of the overlying rocks led to the collapse of the caves. At Lille Raipas there are filled caves, i.e. rock types that formed from material of collapsed cave walls or from material that got washed into the caves in subsurface creeks and then filled the hollow space. These rock types are intensely red colored breccias (rock type 4 in Figure 1). Rock type 5 is represented as well in the filling material of the former caves. In addition, different minerals precipitated from groundwater and those were the basis for the Raipas copper mine during the end of the 18th century.

After the filling of the caves, a thick sediment of pure quartz sand was formed, transported and deposited in fast flowing water in rivers. This quartz sand got cemented to a white, hard quartzite, which can be found over large areas on the Lille Raipas (rock type 6 in Figure 1). The new quartzite landscape was eroded strongly by inland ice belonging to the Precambrian ice age 650 million years ago. This ice age was first described in Eastern Finnmark (Reusch 1891) and got the name "Varanger glaciation". Later on, traces of this ice age were found all over the world on all continents. This was the most comprehensive glaciation on our planet and is now called "Snowball Earth". It seems like all continents and almost all oceans were covered by ice at the same time. Valleys in the hilly quartzite landscape at Lille Raipas got filled with moraines from inland ice (rock type 7 in Figure 1).

When the inland ice melted, sand and gravel were deposited, supposedly from creeks that were flowing on top of the moraines (rock type 8 in Figure 1). The rock types contain quartz-conglomerates with both pebbles of quartz and quartz cement. Afterwards the sea-level rose and at the flooded areas layers of clay and quartz sand were deposited (rock type 9 in Figure 1). About 400 million years ago, approximately at the same time when the first land plants developed, the "Alta slate" was thrust over all the other rocks. This quartz and feldspar rich slate originally was a Precambrian sandstone deposited in rivers. Then it got its flat structure during the thrusting in connection with the Caledonian continental collision and orogeny (rock type 10 in Figure 1). The Alta slate is the youngest rock type on Lille Raipas. Store Raipas, located a little farther south, has even younger thrust nappes.

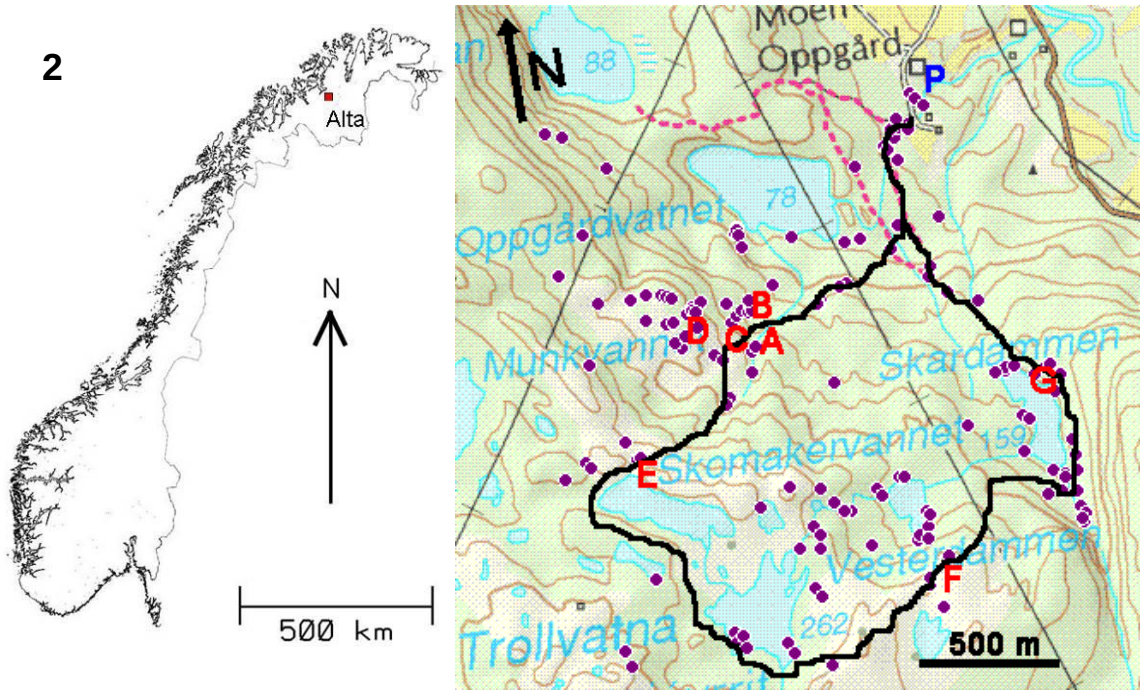

Figure 2. Map over the area with the position of the described localities: A) angular unconformity/alpine butterwort, B) collapsed caves/rich myre, C) modern dolomite cave, D) dolomite/fossils, E) tree trunks, F) Alta slate/*Rhododendron lapponicum*, G) fossil moraine/water plants, P) parking.

Geobotany on Lille Raipas

A whole bunch of botanists visited Lille Raipas over the years, among them J.M. Norman (1879), O. Dahl (1900) and R.E. Fridtz (1900).

Plant registrations are found at http://artskart.artsdatabanken.no to the year 1985. Most botanist reported only single plants (see also table 1) and the most common species and especially grass species are poorly represented.

A comprehensive botanical mapping has never been done on Lille Raipas. Therefore it is impossible to draw conclusions about dynamics within the plant society or the populations of single species.

The area can be classified into lowland middle to northern boreal forest and subalpine birch forest with open wind-exposed ridges in the upper parts.

Quartzites in the east are responsible for the most nutrient-poor soil conditions in the forests while the dolomite areas and areas influenced by groundwater from the dolomites Southwest of Oppgårdvatnet bear the most species-rich vegetation.

On the quartzites the ground vegetation is dominated by *Empetrum nigrum ssp. hermaphroditum, Phyllodoce caerulea* and *Calluna vulgaris.* Among the rarer species, there are *Rhododendron tomentosum* and *Carex adelostoma*.

The vegetation on silt- and sandstones below the unconformity is richer with a large population of *Goodyera repens* and some *Filipendula ulmaria* and *Carex flava*. In the birch forests on the southwesterly side of Oppgårdvatnet the influence of the dolomite can be recognized in the vegetation. *Paris quadrifolia*, *Astragalus frigidus, Cystopteris montana* and *Equisetum scirpoides* can be found in large populations. Even *Epipogium aphyllum* was found flowering (25.08.2010) in a several hundred meters long area in the northeast facing slopes between

Oppgårdvatnet and Gruvevatnet. More than 60 shoots belonging to at least 20 individuals were registered (see Figure 3). *Epipogium* has its northern boundary of distribution in Finnmark county (Hultén & Fries 1986) and the northernmost so far known population grows in Tana municipality (Julelva, found in 1970) at 70.4°N. Lille Raipas is located at 69.9°N. Altogether *Epipogium* was found six times in Finnmark county. The closest localities are 4 km away from Lille Raipas at Elvebakken (Alta municipality, found in 1969) and at valley Tverrelvdalen close to Sønvismoen (Alta municipality, found in 1962). *Epipogium aphyllum* flowers irregularly, it lacks chlorophyll and it gets all organic nutrients from fungal partners like *Hebeloma velutipes* and *Inocybe geophylla* (Roy et al. 2009, Liebel & Gebauer 2011).

Directly on the dolomite rocks in the birch forest, *Woodsia glabella* (see Figure 4) appears in the shady areas while *Asplenium ruta-muraria* can be found on the open rocks. *W. glabella* was reported several times and at different sites on Lille Raipas (1903, 1961, 1978). *A. ruta-muraria*, however, was found only once in 1978.

The vegetation is richer on the tillites, sand- and siltstones that are found above the lower unconformity (locality A) compared to the vegetation on the white quartzites. *Allium schoeno-prasum ssp. sibiricum, Listera cordata* and rarely *Corallorhiza trifida* appear along the small creeks that connect lake Vesterdammen, Middagskarvatnet and Skardammen.

Figure 3. Ghost orchid *Epipogium aphyllum* on dolomite, 25.08.2011.

Figure 4. *Woodsia glabella* on dolomite, 23.08.2011.

Rhododendron lapponicum is a bicentric species in Scandinavia and it was found on a dolomite band within the siltstones in the west of Skomakervannet and on the Alta slate in the east of Vesterdammen.
Aquatic plants appear in all lakes and dams including the largest variation in species at Skardammen (see locality G). *Sparganium hyperboreum* appears in all creeks and lakes.
Table 1 shows a detailed overview over all registered species including information about the rock type they are growing on. All species are documented with coordinates at least once and are registered at www.artsobservasjoner.no (547 observations). Most species appear at more places on Lille Raipas than we could register online. Therefore, the species were classified in three groups: Rare (seen at 1 – 3 locations), spread (seen at 4 – 10 locations) and common (seen at more than 10 locations).

Figure 5. Panorama from Lille Raipas towards Altafjord with white nutrient-poor quartzite in the foreground.

Guide for a geological-botanical excursion

The area at Lille Raipas which is shown in Figures 2 and 5 has a high nature pedagogical value. Some of the most interesting localities can be visited in a 3 hours hike. Special geological and botanical localities are chosen and described in detail. The starting point of the trip is at Oppgård, approximately 7 km Southeast of the centre of Alta (UTM: 34W 0591960, 7760251). The area of the hike does not have any difficulties and the hike takes place in an altitude from 60 to 270 m asl. In addition to the presented localities a lot more can be discovered in the area, both in terms of geology and botany.

Table 1. Taxonomic list of plant species registered on Lille Raipas, and information about their appearance depending upon rock type (S: siltstone, sandstone, tilite, mudstone, conglomerate; D: dolomite, dolomite breccia; K: quartzite; A: Alta slate; U: independant of rock type), frequency and when last found at Raipas before our study (biodiversity made available from: Bergen Museum, Natural History Museum UiO, Tromsø Museum, Science Museum Trondheim, downloaded from Artskart, http://artskart.artsdatabanken.no, 01.09.2010), plant names follow Artsdatabanken's taxonomic database (2011) *: the 1900 record is *ssp. complanatum*.

Scientific name	Appearance	Frequency	Last found
Pteridophyta			
Diphasiastrum complanatum ssp. montellii (Kukkonen) Kukkonen	S	rare	1900 (*ssp. complanatum*)
Huperzia appressa (Bach.Pyl. ex Desv.) Á. & D.Löve	U	spread	-
Huperzia selago (L.) Bernh. ex Schrank & Mart.	U	spread	-
Lycopodium annotinum L.	U	common	1878?
Lycopodium clavatum L.	K	rare	1917
Selaginella selaginoides (L.) P.Beauv.	U	spread	-
Equisetum arvense L.	U	common	-
Equisetum arvense ssp. boreale (Bong.) Á. Löve	U	common	-
Equisetum fluviatile L.	U	common	-
Equisetum palustre L.	U	spread	-
Equisetum pratense Ehrh.	S	rare	1882
Equisetum scirpoides Michx.	S, D	spread	-
Equisetum sylvaticum L.	U	common	-
Equisetum variegatum Schleich. ex Weber & Mohr	S, D	spread	1900
Asplenium ruta-muraria L.	D	rare	1978
Asplenium viride Huds.	S, D, A	spread	1978
Athyrium filix-femina (L.) Roth	S, D	spread	-
Cystopteris fragilis (L.) Bernh.	S, D, A	spread	1978
Cystopteris montana (Lam.) Desv.	D	spread	1900
Gymnocarpium dryopteris (L.) Newman	U	common	1983
Woodsia alpina (Bolton) Gray	S, D	spread	-
Woodsia glabella R.Br. ex Richardson	D	rare	1978
Woodsia ilvensis (L.) R.Br.	S, D	rare	1985
Dryopteris expansa (C.Presl) Fraser-Jenk. & Jermy	S, D, A	common	-
Phegopteris connectilis (Michx.) Watt	U	common	-
Polypodium vulgare L.	S, D	spread	-
Pinopsida			
Pinus sylvestris L.	U	common	-
Juniperus communis L.	U	common	-
Magnoliopsida			
Populus tremula L.	U	spread	-

Salix caprea L.	S	rare	-
Salix glauca L.	U	common	-
Salix herbacea L.	U	common	1841
Salix lanata L.	S	rare	-
Salix myrsinifolia Salisb.	S, K	common	-
Salix myrsinites L.	S, D	common	1961
Salix phylicifolia L.	S	spread	1897
Salix reticulata L.	S, D, A	spread	1961
Alnus incana (L.) Moench	U	common	1913
Betula nana L.	U	common	-
Betula pubescens Erh.	U	common	-
Betula nana ssp. nana x pubescens coll.	U	spread	-
Urtica dioica L.	S	spread	-
Bistorta vivipara (L.) Delarbre	U	common	-
Oxyria digyna (L.) Hill	S, D, A	spread	1878
Rumex acetosa L.	S, D, A	spread	-
Cerastium alpinum ssp. lanatum (Lam.) Ces.	D, A	rare	-
Cerastium fontanum (Baumg.) *ssp. fontanum*	S	rare	-
Minuartia rubella (Wahlenb.) Hiern	D	rare	Ja (uten dato)
Sagina nodosa ssp. borealis G.E.Crow	D	rare	-
Silene dioica (L.) Clairv.	D	rare	-
Caltha palustris L.	S, D	spread	-
Ranunculus acris L.	S	spread	-
Ranunculus repens L.	S	rare	1900
Ranunculus reptans L.	S	rare	1878?
Thalictrum alpinum L.	S, D, A	common	-
Trollius europaeus L.	S, D, A	spread	-
Arabis alpina L.	A	rare	1913
Draba glabella Pursh	D	spread	1961
Drosera longifolia L.	U	spread	-
Drosera rotundifolia L.	U	common	-
Rhodiola rosea L.	K, D, A	spread	-
Parnassia palustris L.	U	common	-
Saxifraga aizoides L.	S, K, D	common	1978
Saxifraga cespitosa L.	D, A	spread	1913
Saxifraga nivalis L.	S, D, A	spread	1878?
Saxifraga oppositifolia L.	S, D, A	spread	-
Saxifraga tenuis (Wahlenb.) Harry Sm. ex Lindm.	A	rare	-
Alchemilla sp.	D	rare	?
Alchemilla glomerulans Buser	A	rare	-
Comarum palustre L.	U	common	-
Dryas octopetala L.	S, D	spread	1978
Filipendula ulmaria (L.) Maxim.	S	spread	-
Geum rivale L.	D, A	spread	-
Potentilla crantzii (Crantz) Beck ex Fritsch	K, D	spread	-
Potentilla erecta L.	S	spread	-
Rubus chamaemorus L.	U	common	-
Rubus saxatilis L.	S, D	spread	1900

Sorbus aucuparia L.	U	common	-
Astragalus frigidus (L.) A.Gray	D, A	rare	1961
Trifolium repens L.	S	rare	-
Geranium sylvaticum L.	U	common	1900
Viola biflora L.	U	common	-
Viola epipsila Ledeb.	U	common	-
Viola palustris L.	S	rare	-
Chamerion angustifolium (L.) Holub	S, D, A	spread	-
Epilobium palustre L.	S	rare	-
Myriophyllum alterniflorum DC.	U	spread	1852
Chamaepericlymenum suecicum (L.) Graebn.	S, K, A	common	-
Hippuris vulgaris L.	U	spread	-
Angelica archangelica L.	S, D, A	spread	-
Anthriscus sylvestris (L.) Hoffm.	S	rare	1900
Moneses uniflora (L.) A.Gray	S, D, A	spread	1961
Orthilia secunda (L.) House	U	common	1961
Pyrola norvegica Knaben	S, D, A	spread	1903
Pyrola minor L.	U	common	1900
Andromeda polifolia L.	U	common	-
Arctous alpinus (L.) Nied.	U	common	-
Calluna vulgaris (L.) Hull	U	common	-
Cassiope tetragona (L.) D.Don	A	rare	1903
Loiseleuria procumbens (L.) Desv.	U	common	-
Oxycoccus microcarpus Turcz. ex Rupr.	U	spread	-
Phyllodoce caerulea (L.) Bab.	U	common	1878
Rhododendron lapponicum (L.) Wahlenb.	D, A	rare	1903
Rhododendron tomentosum (Stokes) Harmaja	U	spread	-
Vaccinium myrtillus L.	U	common	-
Vaccinium uliginosum L.	U	common	1841
Vaccinium vitis-idaea L.	U	common	-
Diapensia lapponica L.	U	common	-
Empetrum nigrum ssp. hermaphroditum (Lange ex Hagerup) Böcher	U	common	-
Trientalis europaea L.	U	common	-
Gentianella aurea (L.) Harry Sm.	D	rare	1897
Menyanthes trifoliata L.	U	common	-
Callitriche palustris L.	S	rare	-
Bartsia alpina L.	S, D, A	common	1864
Euphrasia stricta D.Wolff ex J.F.Lehm. *var. stricta*	S	rare	-
Euphrasia wettsteinii Gussarova	S, D	spread	-
Melampyrum pratense L.	S, K	spread	1903
Melampyrum sylvaticum L.	S, D	spread	1903
Pedicularis lapponica L.	U	common	1897
Rhinanthus minor ssp. groenlandicus (Ostenf.) Neuman	S	rare	-
Veronica serpyllifolia ssp. serpyllifolia L.	S	rare	-
Pinguicula alpina L.	S, D	spread	1961
Pinguicula vulgaris L.	S, D, A	common	1864

Plantago major L.	S	rare	-
Linnaea borealis L.	U	common	1878
Valeriana sambucifolia ssp. procurrens (Wallr.) Á.Löve	U	spread	-
Campanula rotundifolia L.	U	common	-
Antennaria dioica (L.) Gaertn.	U	common	1878
Cirsium heterophyllum (L.) Hill	S, D, A	common	-
Hieracium	U	common	-
Leontodon autumnalis (L.)	S	spread	-
Saussurea alpina (L.) DC.	S, D, A	common	1852
Solidago virgaurea L.	U	common	-
Tussilago farfara L.	U	common	1913
Achillea millefolium (L.)	S	rare	-
Achillea ptarmica (L.)	S	rare	-
Liliopsida			
Triglochin palustris L.	U	common	-
Potamogeton alpinus Balb.	U	common	-
Potamogeton berchtoldii Fieber	S	rare	-
Potamogeton natans L.	U	common	-
Sparganium hyperboreum Laest. ex Beurl.	U	common	-
Paris quadrifolia L.	D	spread	1903
Tofieldia pusilla (Michx.) Pers.	U	common	1900
Allium schoenoprasum ssp. sibiricum (L.) Čelak	S	spread	1985
Coeloglossum viride (L.) Hartm.	S, D, A	spread	-
Corallorhiza trifida Châtel.	S	rare	1903
Dactylorhiza maculata (L.) Soó	U	spread	-
Epipogium aphyllum Sw.	D	rare	1967
Goodyera repens (L.) R.Br.	S, D, A	common	-
Listera cordata (L.) R.Br.	S, D, A	spread	1965
Juncus alpinoarticulatus Chaix	S, A	spread	-
Juncus bufonius L.	S, K	spread	-
Juncus filiformis L.	S, K	spread	-
Juncus trifidus L.	S, K, A	common	-
Juncus triglumis L.	S, D	spread	-
Luzula confusa Lindeb.	A	rare	-
Luzula multiflora ssp. frigida (Buch.) V.I.Krecz.	S	spread	-
Luzula pilosa (L.) Willd.	S	spread	-
Luzula spicata (L.) DC.	A	spread	1864
Eriophorum angustifolium Honck.	U	common	-
Eriophorum ×medium Andersson	A	rare	-
Eriophorum scheuchzeri Hoppe	S, K, A	spread	-
Eriophorum vaginatum L.	U	common	-
Trichophorum alpinum (L.) Pers.	S, D	spread	-
Trichophorum cespitosum (L.) Hartm.	U	common	-
Carex adelostoma V.I.Krecz.	S, D, A	spread	-
Carex aquatilis Wahlenb. *ssp. aquatilis*	S, A	rare	1900
Carex bigelowii Torr. ex Schwein.	S, D, A	common	-

Carex canescens L.	U	common	-
Carex capillaris L.	S, D, A	spread	1900
Carex chordorrhiza Erh. ex L.f.	S, K	spread	-
Carex dioica L.	S, D	common	-
Carex flava L.	S, A	spread	-
Carex lasiocarpa Erh.	S, K, A	spread	-
Carex limosa L.	S	spread	-
Carex nigra (L.) Reichard.	U	common	-
Carex norvegica Retz.	S, A	rare	-
Carex pallescens L.	S	rare	-
Carex panicea L.	K	rare	-
Carex pauciflora Lightf.	S, K	spread	-
Carex paupercula Michx.	U	common	-
Carex rariflora (Wahlenb) Sm.	S	rare	-
Carex rostrata Stokes	U	common	-
Carex rotundata Wahlenb.	S, K, A	common	1900
Carex rupestris All.	S, D, A	spread	1900
Carex vaginata Tausch	U	common	-
Carex serotina L.	S	rare	-
Agrostis mertensii Trin.	S, K	common	-
Alopecurus geniculatus L.	S	rare	-
Anthoxanthum nipponicum Honda	U	spread	-
Avenella flexuosa (L.) Drejer	S, K	common	-
Calamagrostis neglecta (Ehrh.) P.Gaertn. et al.	S, A	spread	1913
Calamagrostis phragmitoides Hartm.	S	rare	-
Deschampsia cespitosa (L.) P.Beauv.	S, D, A	spread	1882
Festuca ovina L.	S, D	common	1961
Festuca rubra L.	U	common	1903
Melica nutans L.	D	spread	-
Nardus stricta L.	S, K, A	common	-
Phleum alpinum L.	S, D	rare	1862
Poa alpina L.	D	spread	1852
Poa annua L.	S, K	spread	-
Poa glauca Vahl	D	spread	-
Poa pratensis ssp. alpigena (Fr. Ex Blytt) Hiitonen	D	spread	-
Trisetum spicatum (L.) K.Richt.	S, D, A	common	-

Locality A: Angular unconformity and Pinguicula alpina

(UTM: 34W 0591625, 7759640)

Description: This locality shows a clear contact between rock type 1 (fine-grained sandstone) and the overlying younger rock type 5 (coarse-grained red sandstone). The contact between the two layers is called angular unconformity: Standing in front of the rock wall, you can see that the lower rock type dips towards right (northwest) while the rock type below dips towards left (southeast, see Figure 6A). At the contact between the upper and the lower rock type, you can put your hand on an ancient land surface which is 1000 million years old. In the rock wall above, *Pinguicula alpina* and *Diapensia lapponica* are common. *Carex*

norvegica and *C. adelostoma* grow on rocks where water flows over them.

Figure 6. A Angular unconformity between rock type 1 and 5; **B** contact between collapsed cave-fill (light) and dark mudstone of earlier cave walls; **C** fossil cyanobacterial stromatolite replaced by white quartz in brown dolomite; **D** tree trunks from a warmer climatic period preserved in Skomakervannet; **E** *Rhododendron lapponicum*; **F** tillite, or hardened glacial moraine, with ice-transported blocks in fine-grained matrix.

Locality B: Collapsed caves and rich peat bog vegetation
(UTM: 34W 0591482, 7759661)
Description: After a few minutes hike towards west, one arrives at a very special but confusing geological locality. A layered fine-grained dark siltstone is in contact with a reddish chaotic sandstone with small layers dipping in all directions (see Figure 6B). This sediment was formed in a collapsed limestone cave. Limestone

and dolomite is eroded easily in running water so that karst caves are formed. If the weight of overlying sediments gets to heavy, caves of this kind may collapse and exactly this may have happened at that place. Even though one has not seen lots of dolomite so far, one may discover that typical rich peat bog species suddenly appear along the creek that passes close to the locality. *Saxifraga aizoides* and *Salix myrsinites* are common.

Locality C: Recent cave in dolomite
(UTM: 34W 0591372, 7759581)
Description: Along the way up to locality C (walk up the slope on the right side (west side) of the creek where the terrain is easier), one walks through species-rich birch forest on dolomite. Dolomite is an easily erodable rock type which is responsible for the richer soil conditions here than in the areas seen so far on the trip. *Cystopteris montana, Pyrola norvegica* and even *Woodsia glabella* is common on the shady rock walls. You might be especially lucky and find *Epipogium aphyllum* and *Gentianella aurea* flowering late in the season. Having arrived at locality C, one stands directly on the unconformity with dolomite and breccia below (west of the creek) and quartzite above (east of the creek). The creek disappears here in a recent karst cave through the dolomite before it gets to the surface again after few meters.

Locality D: Dolomite and Norway's oldest fossils
(UTM: 34W 0591249, 7759589)
Description: The trip takes us farther into the dolomite area at locality D. There, some of Europe's oldest fossils can be seen (ca. 1800 million years old). They appear as round quartz structures in a brownish dolomite (see Figure 6C) and are called stromatolites. Stromatolites are carpets of blue-green algae that appear in shallow tidal zones along the seashore. The area around contains the most nutrient requiring vegetation of the trip with a great many of limestone species like *Minuartia rubella, Draba glabella, Saxifraga nivalis* and *Carex rupestris.*

Locality E: Tree trunks from a warmer time
(UTM: 34W 0591016, 7759223)
Description: Following the valley upwards, one can see the boarder between rock type 3 (dark mudstone) and rock type 5 (red sandstone, UTM: 34W 0591127, 7759233). The special geological feature at this locality is the layer with coarse rounded quartz pebbles. They were deposited as the first sediment after a long period of erosion and the sediment is called "basal conglomerate".
At the westerly end of lake Skomakervannet and in several other small lakes, tree trunks are found in the water that have a diameter that is larger than the ones of trees growing in the area to date (see figure 6D). We interpret those tree trunks as residues of a warmer climatic period which might be the last warm period during the medieval time (Seppä & Birks 2002). It finished in the end of the 14th century, when the climate got colder culminating in the Little Ice Age in the 18th century. At the southwestern end of Skomakervannet, some stromatolites and some breccias can be found that are responsible for soils rich in bases and for limestone vegetation.

***Locality F: Alta slate and* Rhododendron lapponicum**
(UTM: 34W 0591567, 7758592)
Description: On the way to locality F one passes several small lakes and lake Vesterdammen. At the easterly shore, a small population is present of *Eriophorum x medium*. The finding was confirmed by Eli Fremstad (NTNU

science museum) and it was documented as herbarium specimen in the TRH (herbarium of Trondheim).
East of Vesterdammen, there are several benches of grey slates, 2 to 4 meters thick. They are rich in quartz and feldspar and can be called for Alta slate. They were thrust over all underlying rock types during the Caledonian collision and orogeny. On top of those benches, some few centimeters of lose gravel are found from most different rock types, deposited during the last Ice Age. Exactly there, a large mixture of species can be discovered that like either limestone or acidic grounds - among others: *Rhododendron lapponicum* (see figure 6E), *Luzula confusa* and *Salix herbacea*.

Locality G: Fossil moraine and aquatic plants
(UTM: 34W 0592235, 7759383)
Description: We continue the trip from the uppermost layers at Lille Raipas downwards to lake Skardammen. Arriving at locality G, you are standing on a rare rock type. It consists of unrounded pebbles of many different rock types embedded in a red sandstone (figure 6F). The rock type is a fossil Precambrian glacial deposit (moraine).
Skardammen is located quite low in the terrain and several aquatic plants thrive in the shallow parts of the lake. *Potamogeton berchtoldii, Myriophyllum alterniflorum* and *Hippuris vulgaris* are among them. The last part of the round trip leads us down from Skardammen to the parking following the hikinig path and the light-track for Nordic skiing. Along the path *Rhododendron tomentosum* grows on the quartzites and farther down *Moneses uniflora* is common in the forest's ground vegetation. Finally, we reach the cultivated lands around Oppgård where *Alopecurus geniculatus* and *Callitriche palustris* grows along the rural road.

Conservation of the area

The described area on Lille Raipas has large and varied values. More than 200 vascular plant species were registered within two weeks, including three red-listed species (Kålås et al. 2010): *Epipogium aphyllum, Saxifraga tenuis* and *Eriophorum x medium* (all in the category NT, Near Threatened). This is extraordinary at a latitude of almost 70° N, as von Buch recognized earlier.
The stromatolites are among the oldest fossils known in Europe. Filled karst caves are extremely rare to find conserved and there are plenty of them from as old as Precambrian times. Here, well-preserved fossil moraines from the Snowball Earth period cover an ancient hilly landscape.
The area is little influenced by human impact, apart from two electricity lines and some ca. 100 year-old water dams. The area has a large potential for environmental education. It contains a relatively intact ecosystem which shows linkages between lithosphere (different rock types with their life cycles including erosion and sedimentation), pedosphere (different rock types weather in a different way and form different soils), biosphere (plant communities depending on competition, soil, exposition, mycorrhiza), hydrosphere (dams, creeks, peat bogs, well-drained soils) and atmosphere (vegetation depending on altitude, meso- and microclimate, wind exposure).
Therefore, we think that the area should be conserved for further destructive human impact. Meanwhile we want to motivate people interested in nature to take a trip to the most interesting localitites on Lille Raipas equipped with this text and a GPS.

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Remark: The published text was translated from Norwegian.

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Dr. Heiko Liebel