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Ecological and evolutionary consequences of orchid dependence on mycorrhizal fungi

Ph.D. Thesis

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ANNOTATION

Interaction between orchids and fungi belongs to little understood aspects of orchid biology. This thesis investigated an identity of mycorrhizal and other fungal symbionts of several European orchid species and also an influence of these fungal symbionts on ecology, distribution, and evolution of the studied orchid species. Diverse methodological approaches were used including *in situ* seed germination, culture-dependent and -independent techniques for fungal isolation and identification, molecular phylogenetics, stable isotope analyses, and transmission electron microscopy.

DECLARATION **[in Czech]**

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 26. 7. 2014

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LIST OF PAPERS AND AUTHOR'S CONTRIBUTION

The thesis is based on the following papers (listed chronologically):

Jersáková J, **Malinová T**, Jeřábková K, Dötterl S (2011) Biological Flora of the British Isles: *Pseudorchis albida* (L.) Á. & D. Löve. *Journal of Ecology* 99: 1282–1298 (IF=5.43).
Tamara Těšitelová (Malinová) participated in manuscript writing.

Těšitelová T, Tešitel J, Jersáková J, Říhová G, Selosse M-A (2012) Symbiotic germination capability of four *Epipactis* species (Orchidaceae) is broader than expected from adult ecology. *American Journal of Botany* 99: 1020–1032 (IF=2.58).
Tamara Těšitelová was responsible for the field experiment planning and conduction, molecular analyses, data analyses, and manuscript writing.

Kohout P, **Těšitelová T**, Roy M, Vohník M, Jersáková J (2013) A diverse fungal community associated with *Pseudorchis albida* (Orchidaceae) roots. *Fungal Ecology* 6: 50–64 (IF = 2.85).
Tamara Těšitelová participated in planning the study, field sampling, fungal isolation, molecular typing of fungi, data analysis, and manuscript preparation.

Těšitelová T, Jersáková J, Roy M, Kubátová B, Těšitel J, Urfus T, Trávníček P, Suda J (2013) Ploidy-specific symbiotic interactions: divergence of mycorrhizal fungi between cytotypes of the *Gymnadenia conopsea* group (Orchidaceae). *New Phytologist* 199: 1022–1033 (IF=6.74).
Tamara Těšitelová was responsible for planning and conducting of part of the field sampling, for organizing the molecular analyses, for analyzing the data, and writing the manuscript.

Těšitelová T, Kotilínek M, Jersáková J, Joly F-X, Košnar J, Tatarenko I, Selosse M-A: Evolutionary shift from autotrophy to mycoheterotrophy in the orchid genus *Neottia* is associated with a change of *Sebacina* mycobionts. (submitted manuscript)
Tamara Těšitelová participated in planning and conducting of the field sampling and was responsible for organizing the molecular analyses, for analyzing the data, and writing the manuscript.

CONTENTS

CHAPTER I

General introduction

GENERAL INTRODUCTION

Interaction with fungi accompanies land plants since their emergence on the bare soil in Silurian (Simon et al. 1993; Selosse and Le Tacon 1998). This relationship has evolved into the most widespread mutualism on the Earth. More than 80% of land plant species and four derived fungal groups, i.e. Mucoromycotina, Glomeromycota, Ascomycota, and Basidiomycota, form a specialized dual organ – the mycorrhiza (Brundrett 2002; Frank 2005; Smith and Read 2008; Bidartondo et al. 2011). We recognize two most common types of mycorrhiza, arbuscular (endo)mycorrhiza formed by Glomeromycota, and ectomycorrhiza formed by diverse Asco- and Basidiomycota. Despite the phylogenetic diversity of both plant and fungal symbionts, these symbioses represent a finely tuned mutually beneficial relationship (Smith and Read 2008). The plant exchanges a part of its photoassimilates for mineral nutrients and water with the fungus precisely recognizing and rewarding more and less beneficial participants (Kiers et al. 2011). Mutualistic symbiosis with fungi allowed the plants to broaden their ecological niches and could have facilitated their evolutionary radiation and their winning crusade for turning the bare soil into green.

Orchids and mycorrhiza

The orchids (Orchidaceae) with more than 20.000 species and a status of one of the species-richest families (The angiosperm phylogeny group 2009) belong to plants significantly influenced by their mycorrhizal relationship. Orchids evolved *ca* 100 mil years ago during Cretaceous period by splitting at the basis of the monocot order Asparagales (The angiosperm phylogeny group 2009) from arbuscular mycorrhizal predecessors. All orchids are characterized by a new family-specific type of mycorrhiza – orchid mycorrhiza.

Orchid mycorrhiza belongs to endomycorrhizal types: coils of fungal hyphae (pelotons) are formed within cells – between plant plasma membrane and a cell wall (Fig. 1). Pelotons live a limited time and undergo phases of cell colonization and peloton formation followed by peloton lysis. The cells can be then newly re-colonized by hyphae (Smith and Read 2008).

The mechanism of nutrient transfer on the plant-fungus interface is still obscure, and it is under debate if the transfer occurs across intact membranes of fully developed pelotons similarly to arbuscules in arbuscular mycorrhiza or after peloton lysis (earlier called 'digestion'), when the peloton biomass is believed to be absorbed by the plant (Smith and Read 2008). The answer may provide newly developed imaging technologies such as secondary
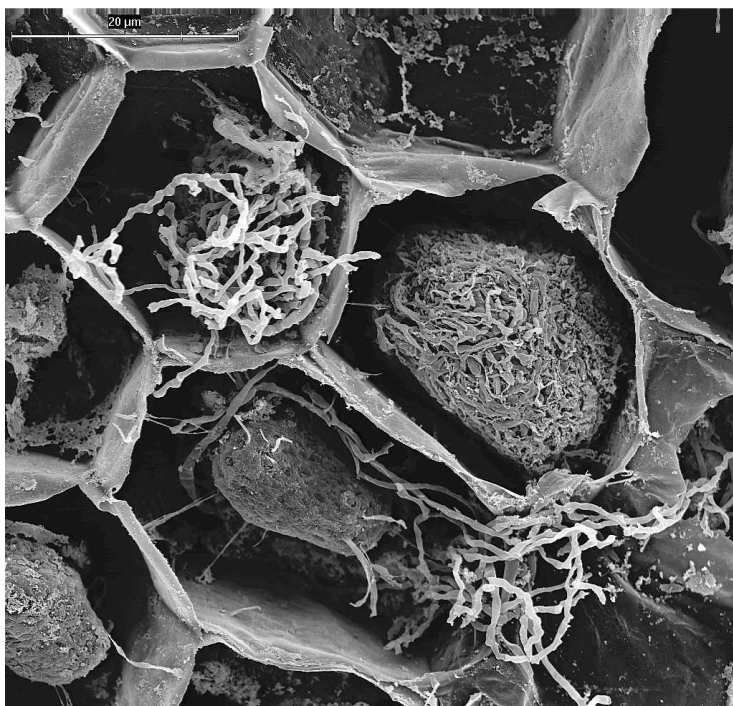
Fig. 1. Pelotons in different growth phases, including fully developed and lysed ones in root cortex cells of *Epipactis helleborine*.

ion mass spectrometry (SIMS). Two recent studies show, however, divergent results: either transfer across both intact and lysed pelotons in a seedling (protocorm) of a *Spiranthes* species green in adulthood (Kuga et al. 2014) or no transfer from intact pelotons in a protocorm of a non-green bellow-ground *Rhizanthella* (Bougoure et al. 2014). It is a question whether this difference may be attributed to the divergence in photosynthetic abilities of the studied orchids or an identity of mycorrhizal fungi, but certainly future research should provide clearer answers.

Fungi forming orchid mycorrhiza

Identification of orchid fungal associates was for a long time dependent on culturing of the fungi on nutrient media which allows determination of fungi based on morphological features of the mycelia. However, most orchid mycorrhizal fungi only rarely fructify in a culture (Rasmussen 1995). The advent of molecular identification significantly improved the taxonomic resolution of taxonomically difficult groups. Direct genotyping of fungi in orchid roots also significantly broadened our knowledge on orchid fungi

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diversity thanks to identification of fungi hard-to-cultivate or even uncultivable *in vitro* (e.g. Taylor and Bruns 1997; Bidartondo et al. 2004; McCormick et al. 2004); however, mycorrhizal fungi of most orchid species and genera are still unknown.

Orchid mycorrhizal fungi do not belong to Glomeromycota as in orchid closest relatives, but mainly to Basidiomycota. The ancestral association is likely formed with members of a polyphyletic fungal group collectively called 'rhizoctonia' involving three main families: Tulasnellaceae and Ceratobasidiaceae from Cantharellales and Sebacinales clade B (Weiß et al. 2004) (both Agaricomycetes *incertae sedis* according to GenBank taxonomy). Rhizoctonias are present as dominant symbionts in all orchid subfamilies, including the basal-most Apostasioideae (Fig. 2; Kristiansen et al. 2004; Yukawa et al. 2009), and are typical associates of green orchids from open habitats (e.g. Rasmussen 1995; *Chapter II*). These fungi are recognized as saprobes, plant pathogens (Smith and Read 2008) or, in case of Sebacinales clade B, mainly endophytes and ericoid mycorrhizal fungi (Weiß et al. 2004, 2011). Moreover, in all these groups, ectomycorrhizal abilities were recently discovered (Bidartondo et al. 2003; Bougoure et al. 2009; Garnica *et al*. 2013; Tedersoo and Smith 2013; Veldre et al. 2013).

Within 43 genera in Vanilloideae, Orchidoideae, and Epidendroideae *ca* 235 non-green heterotrophic orchid species evolved, most of which can be found in the species-richest subfamily Epidendroideae. The non-green species shifted from symbiosis with saprotrophic or endophytic rhizoctonia to other fungal taxa (reviewed in Merckx et al. 2013). In the few investigated non-green species from Vanilloideae, an association with parasitic *Armillaria*, saprotrophic Tricholomataceae, and ectomycorrhizal Russulaceae was found, while the two investigated genera, *Chamaegastrodia* and *Rhizanthella*, from Orchidoideae associated with ectomycorrhizal Ceratobasidiaceae. The highest phylogenetic diversity of mycorrhizal fungi is then found in the derived Epidendroideae (Fig. 2). Species from this subfamily associate with many common ectomycorrhizal Basidiomycota of temperate forests, such as Thelephoraceae, Russulaceae, or Hymenogastraceae, ectomycorrhizal Ascomycota, such as Pyronemataceae and Tuberaceae, and diverse saprotrophic and parasitic fungi including *Armillaria*, *Mycena*, *Resinicium*, or Coprinaceae (Merckx et al. 2013).

Similarly to other plant species, orchid roots may host a broad variety of endophytic fungi, which seemingly do not cause any symptoms of their presence in the roots (*Chapter IV*). Moreover, fungi which form a functional mycorrhiza with some plants, such as orchid-mycorrhizal Tulasnellaceae and

	Botryobasidiaceae	Ceratobasidiaceae	Tulasnellaceae	Sebacinales clade B	other saprotrophic Basidiomycota	other ectomycorrhizal Basidiomycota	ectomycorrhizal Ascomycota
Apostasioideae	X	X	X				
Vanilloideae		X	X		X	X	
Cypripedioideae			X				
Orchidoideae		X	X	X			
Epidendroideae		X	X	X	X	X	X
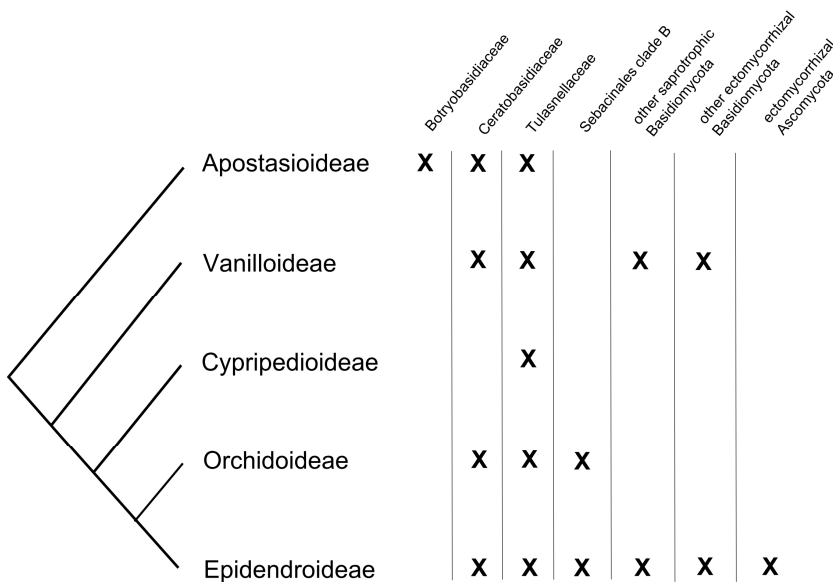
Fig. 2. Phylogenetic tree of subfamilies within Orchidaceae as available at Angiosperm Phylogeny Website (http://www.mobot.org) with dominant mycorrhizal associations. Note that only a limited number of orchid species has been investigated so far and the list may not be complete.

orchid- and ericoid- mycorrhizal Sebacinales clade B (e.g. Selosse et al. 2007; *Chapter VI*), may be endophytic in a variety of other plants (Weiß et al. 2004, 2011; Girlanda et al. 2011; Garnica et al. 2013). This opens a potential for formation of hyphal connections, a common mycelial network, among phylogenetically unrelated co-occurring plants, which may have profound but so far little investigated effect on plant communities (Selosse et al. 2006). The tripartite symbiosis among an orchid, an ectomycorrhizal fungus, and an ectomycorrhizal tree may serve as a clear example of the existence of such networks.

Culture-independent molecular techniques usually reveal wider and often different diversity of root-associated fungi compared to culture-dependent ones, including many endophytes, pathogens, but also soil contaminants (Allen et al. 2003; Kohout et al. 2012; *Chapter IV*). A useful tool to evaluate mycorrhizal ability of such fungi are transmission electron microscopic observations allowing detailed inspection of fungal pores in peloton-forming hyphae (Selosse et al. 2004). This laborious and time-consuming method, however, allows inspection of limited root area, and function of many fungi detected in the roots thus remains little understood. Endophytic fungi or diverse

less abundant associates may still form mycorrhiza and have influence on orchid germination (Zimmerman and Peterson 2007), and combination of both culture-dependent and -independent techniques followed by symbiotic cultivations is likely to shed more light on their function.

Functioning of orchid mycorrhiza

A characteristic feature common to all orchids is the production of vast amounts of minute, dust-like seeds which obligately depend for germination on carbon and nutrient supply by mycorrhizal fungi (Rasmussen 1995; Smith and Read 2008). In the vast majority of species, the initial germination starts with enlarging seed volume and growth of rhizoids without mycorrhizal fungus colonization (but see McKendrick et al. 2000). However, advanced growth under natural conditions only continues after exogenous supply of carbohydrates. A germinating seedling differentiates specialized tissues – a fungus-free plant meristem and mycorrhizal tissue (most of the body) – forming a special growth stage called protocorm (Fig. 3). Protocorms of terrestrial orchids are subterranean, non-green, and fully mycoheterotrophic (i.e. obtaining all carbon from a fungus) even in species photosynthetic in

Fig. 3. Germinating seedlings of *Epipactis atrorubens* cultivated two years in soil. Un. – ungerminated seed, NMr – germinating but non-mycorrhizal seedling. All other seedlings with differentiated tissues are mycorrhizal protocorms.

adulthood.

Mycorrhizal fungi are retained in orchid roots also in adulthood, when most orchids produce green leaves and are fully photosynthetic. The function of mycorrhizal fungi in such plants is little investigated, but microcosm experiments on *Goodyera repens* associated with saprotrophic *Ceratobasidium* showed importance of the fungus for nitrogen, phosphorus, and even some carbon uptake (Cameron et al. 2006, 2007, 2008). Cameron et al. (2006, 2008) also demonstrated the flow of photoassimilated carbon from the plant to the fungus showing mutually beneficial relationship between orchid and fungus, a topic which has provoked controversy for decades (reviewed in Smith and Read 2008; Látalová and Baláž 2009; Rasmussen and Rasmussen 2009).

Non-green species obtain all (or vast majority of) nutrients from the fungus without any obvious reward to the fungus. Despite their phylogenetic spread within Orchidaceae, they have many common features such as reduced leaves and stomata, reduced root system that is filled with a mycorrhizal fungus, appearance above-ground only for flowering, or long periods of dormancy (Leake 1994). Beside these two extremes (green vs. non-green species), intermediate species that are green or partly green and photosynthesize but simultaneously obtain significant amount of carbon from their mycorrhizal fungi were recently discovered in several unrelated orchid genera. These so called partially mycoheterotrophic (a kind of mixotrophy) species were found by analyzing natural abundances of stable isotopes ^{13}C and ^{15}N in orchid tissues (e.g. Gebauer and Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005; Selosse and Roy 2009; Motomura et al. 2010; Yagame et al. 2012). Different ecosystem components have different isotopic compositions due to distinct ways of nutrient acquisition. Thus, autotrophic plants differ in stable isotope content from fungi, and also saprotrophic and ectomycorrhizal fungi differ from each other (e.g. Dawson et al. 2002; Taylor et al. 2003). Similarly, orchids which associate with diverse groups of these fungi and vary in amount of autotrophic carbon in their tissue, feature specific isotopic abundances (e.g. Bidartondo et al. 2004; Martos et al. 2009; Selosse and Roy 2009; Hynson et al. 2013; Stöckel et al. 2014; *Chapter VI*) allowing evaluation of the mycoheterotrophy level. Recently, it has been, however, shown that this method may be little sensitive in detecting carbon flow from saprotrophic rhizoctonia to orchids and partial mycoheterotrophy may be more widespread than expected (Stöckel et al. 2014).

The close relatedness of partially mycoheterotrophic species to fully mycoheterotrophic, non-green species provoked an interest in steps leading to the loss of chlorophyll (Selosse and Roy 2009). Although some level of

mycoheterotrophic carbon uptake in adulthood may be widespread among all orchids (Cameron et al. 2006, 2008; Stöckel et al. 2014), non-green orchids and their closely related green(ish) partially mycoheterotrophic counterparts never associate with saprotrophic rhizoctonia but with diverse ectomycorrhizal Basidiomycota or Ascomycota (e.g. Bidartondo et al. 2004; Selosse et al. 2004; Julou et al. 2005; Ogura-Tsujita et al. 2012; Yagame et al. 2012; *Chapter III*) or saprotrophic Basidiomycota in the tropics (Martos et al. 2009; Ogura-Tsujita et al. 2009). The change in mycorrhizal symbionts thus may be one of the necessary steps in evolution to achlorophylly (Selosse and Roy 2009; Motomura et al. 2010; Ogura-Tsujita et al. 2012; Yagame et al. 2012; *Chapter VI*). Reasons for such shift remain questionable, but, for instance, ectomycorrhizal fungi may represent a more stabile and stronger source of nutrients.

Do mycorrhizal fungi influence orchid ecology and evolution?

Obligate dependence on mycorrhizal fungi poses an important question, how much is such dependence limiting orchids in colonisation of new habitats. In contrast to distribution patterns and ecology of orchids we have only limited knowledge of the distribution of many macromycetes and rather absent data in case of most micromycetes, including rhizoctonia. Distribution of saprotrophic *Tulasnella calospora* could serve as an example – this species, commonly associated with many meadow orchids, has got only three known localities in the Czech Republic (Holec and Beran 2006). In case of orchids associated with ectomycorrhizal fungi, the necessity of presence of a tree or shrub host for the fungus is obvious – such as in a case of a rare Australian bellow-ground orchid *Rhizanthella* which specifically associates with ectomycorrhizal *Ceratobasidium* and grows exclusively under *Melaleuca* shrubs (Bougoure et al. 2009). But such clear examples are extremely rare.

Fungal identification from adult orchids' roots reliably indicates mycorrhizal fungi presence, but other approaches may bring more detailed overview of fungal distribution in soil. One of the approaches are the baiting methods – either using an organic material which is introduced in soil, and saprotrophic rhizoctonia colonizing it may be identified (Masuhara and Katsuya 1994) or seed baiting techniques (Rasmussen and Whigham 1993). Orchid germination is notoriously difficult to follow in nature owing to minute size of the seeds and very slow development, but methods of inserting seeds into a fine nylon mesh invented *ca* 20 years ago have significantly simplified the manipulation with seeds (Rasmussen and Whigham 1993; Masuhara and

Katsuya 1994; van der Kinderen 1995; Brundrett et al. 2003). The mesh size about 40μm allows entrance of soil fungal hyphae and other soil biota and cultivation under nearly natural conditions. The packets can be retrieved after variable time, even years, and obtained seedlings can be then used for identification of mycorrhizal fungi promoting germination and mapping the fungus distribution in soil.

The optimal conditions for symbiosis establishment *in situ* may be, however, difficult to fulfil for both the seed and the fungus, and the ability of the fungus to support germination may alter under different conditions. Orchid germination was shown to be influenced by various soil properties, including soil moisture or pH (reviewed in Jersáková and Malinová 2007). On the fungus side, McCormick et al. (2012) showed that not only the fungus presence but also abundance may influence germination success, and obviously, only a minor part of seeds placed in a packet establishes symbiotic growth (e.g. *Chapter III*). DNA amplification using fungal-specific primers directly from soil may thus bring more independent results (McCormick et al. 2009, 2012), and new high-throughput sequencing methods will certainly bring new views.

Up to now, most studies indicate that orchid germination is very patchy within sites, and that mycorrhizal fungi can be more widespread than orchid populations: introduction of seeds to habitats typical for the orchid species, but unoccupied, usually reveals at least some germination (e.g. McKendrick et al. 2000; Phillips et al. 2011; De hert et al. 2013; *Chapter III*; reviewed in McCormick and Jacquemyn 2014). Little is known about fungal distribution in diverse habitats; however, the fungi may be little habitat specific: *Orchis mascula*, an orchid species growing in a wide variety of habitats from meadows to light forests, is surprisingly specific to a single *Tulasnella* taxon both in West and Central Europe (Jacquemyn et al. 2010; Těšitel et al. unpublished). Similarly, germination of four ecologically divergent *Epipactis* species was not limited by distribution of ectomycorrhizal fungi even in diverse forest types, where adult orchids do not occur (*Chapter III*). Consequently, orchid mycorrhizal fungi do not seem to be limited in their distribution. Despite the general assumptions, orchids may be significantly dispersal limited, and significant propagule pressure may be needed for the establishment due to high patchiness and ephemerality of fungal distribution in soil (Jersáková and Malinová 2007; McCormick and Jacquemyn 2014). Also other factors, including the difficulties with reaching the soil seed bank or transition to above-ground stage, may represent an important bottleneck for orchid population establishment. However, more studies in unoccupied and unsuitable habitats for orchids are needed to fully evaluate the influence of

mycorrhizal fungi distribution for orchid distribution and ecological preferences.

Mycorrhizal fungi may, however, play a role in orchid co-existence. Co-occurring species using the same source compete for resources and theoretically should not co-exist in long term (Tilman 1990), but mycorrhizal fungi segregation is one of the strategies facilitating niche divergence and partition of resources. Indeed, co-existing orchid (Waterman et al. 2011; Jacquemyn et al. 2012ab, 2014) and also other arbuscular mycorrhizal species (van der Heijden et al. 2003; Vandenkoornhuyse et al. 2003) tend to differ in mycorrhizal associations. Jacquemyn et al. (2012ab, 2014) also showed that orchid species diverging in mycorrhizal fungi germinate significantly better in clusters with con-specific adults, what likely influences plant distribution above-ground. The mechanisms of different cytotype co-existence are even more enigmatic since rarer cytotypes may represent newly (even repeatedly) emerging lineages. These often easily hybridize with the dominant cytotype and as a consequence may suffer with reproductive disadvantage being finally outcompeted from a population (Levin 1975) in case of absence of reproductive barriers. Mycorrhizal fungi differentiation between cytotypes and its potential for facilitation of cytotype co-existence has not been investigated so far with exception of *Gymnadenia conopsea* agg., in which a differentiation between diploid and tetraploid plants was found (*Chapter V*).

Obviously, influence of mycorrhizal fungi on orchid evolution is not as straightforward as in case of interactions with pollinators (e.g. Waterman et al. 2011; van der Niet and Johnson 2012). Some species did not change mycorrhizal symbionts during their speciation (Swarts et al. 2010; Phillips et al. 2011; Waterman et al. 2011), while in other cases shifts in mycorrhizal fungi among related species led to spatial segregation (Jacquemyn et al. 2012ab, 2014; *Chapter V*). Although spatial segregation does not directly prevent hybridization, it may alter the frequency of mating interactions if pollinators are shared, reduce occurrence of hybrid plants, and stabilize the species or even cytotype existence. Finally, shift to ecologically different mycorrhizal symbiont may lead to change in habitat preferences and subsequently to allopatric speciation.

Objectives and content of the thesis

Despite the researchers' interest in orchids since Darwin's time, many aspects of orchid biology stay little understood, especially orchid interactions with microorganisms, which only recently with advancement of new molecular techniques became more accessible to numerous researchers. The complexity

of interactions both below- and above-ground and their potential interplay (Waterman and Bidartondo 2008) makes the orchids exciting evolutionary models (Bronstein et al. 2014). Last but not least aspect provoking the interest in orchid research is the conservation aspect. Many species belong to critically endangered species threatened mainly by loss of habitats and fragile links with interacting organisms (Swarts and Dixon 2009). Detailed knowledge of orchid biology could help to understanding of species' requirements and improve restoration efforts.

In this thesis, I tried to shed more light on diverse below-ground interactions of several European orchid species and potential consequences of mycorrhizal fungi identity for orchid ecology, distribution, and evolution. More specifically, I asked which fungi are associated with the studied orchids? Is the mycorrhizal fungi distribution limiting in diverse habitats? Does the identity of mycorrhizal fungi change with changed ploidy level or during evolution to mycoheterotrophy? and others. I used diverse methods applied in orchid fungi research, such as *in situ* seed cultivation, both culture-dependent and -independent approaches for fungal isolation, fungal molecular barcoding, molecular phylogenetics, stable isotope analyses, or transmission electron microscopy.

Chapter II reviews different aspects of biology of an endangered and declining mountain species, *Pseudorchis albida*.

Chapter III examines germination abilities of four ecologically divergent *Epipactis* species in occupied and unoccupied habitats and investigates how abiotic factors and mycorrhizal fungi distribution can influence their germination.

Chapter IV describes diversity of mycorrhizal and endophytic fungi in roots of *Pseudorchis albida* in two sampling seasons and compares efficiency of different isolation approaches.

Chapter V investigates relationship between identity of mycorrhizal associates and ploidy level in *Gymnadenia conopsea* group.

Chapter VI investigates mycorrhizal associations and nutritional mode in two green *Neottia* species and tries to shed more light on the evolution of mycoheterotrophy in the *Neottia* genus.

Chapter VII summarizes the main results of this thesis.

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CHAPTER II

Biological Flora of the British Isles:
Pseudorchis albida **(L.) Á. & D. Löve**

Biological Flora of the British Isles: *Pseudorchis albida* (L.) Á. & D. Löve

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Abstract

• This account presents information on all aspects of the biology of *Pseudorchis albida* (Small White Orchid) that are relevant to understanding its ecological characteristics and behaviour. The main topics are presented within the standard framework of the *Biological Flora of the British Isles*: distribution, habitat, communities, responses to abiotic and biotic factors, structure and physiology, phenology, floral and seed characters, herbivores and disease, history, and conservation.

• *Pseudorchis albida* is a native British herb growing in several plant communities of semi-natural habitats, typically in recently burned heather moorlands, upland well-drained pastures and meadows, and cliff ledges. Although the species is able to tolerate a wide range of ecological conditions from mildly acid to base-rich soils, it is limited mainly to open, short and nutrient-poor stands. Typical British plant communities include *Calluna vulgaris* heathlands, *Anthoxanthum odoratum - Geranium sylvaticum* hay meadows, and *Nardus* grasslands. The species often grows in transitions from heath to grass-dominated communities. The ecology of *P. albida* in the northern acidic environments is poorly understood.

• *Pseudorchis albida* is a perennial geophyte whose populations are maintained predominantly by sexual reproduction, as vegetative spread is limited. The main perennating organ is a highly divided root tuber, which is completely replaced every year. Dormancy – the failure of above-ground parts to appear in a growing season, followed by reappearance of full-sized photosynthetic plants in subsequent seasons – has been observed and typically lasts for one, sometimes two years. The adventitious roots and distal parts of the root tuber are colonized by basidiomycetes from the family Tulasnellaceae.

• The species is pollinated by crepuscular pyralid and pterophorid moths, most likely attracted by the sweet scent. During the day, pollinia can be also transferred by dance flies (Empididae). The breeding system of *P. albida*

is poorly known but high fruit set, often over 90%, raises the possibility of spontaneous autogamy, particularly in arctic-alpine areas. The probability of flowering in consecutive years in *P. albida* is strongly influenced by cost of flowering and fruiting.

- *Pseudorchis albida* has suffered a significant decline in distribution in Britain and other European countries. In Britain, it has been lost from more than 65% of its historical sites, particularly in lowland areas. The main causal factors are the cessation of traditional agricultural practices, habitat fragmentation and disturbance associated with housing and road construction, and agricultural improvement, including reclamation, fertilization and overgrazing. Conservation of remnant populations primarily depends on the maintenance of a short turf by extensive grazing, controlled burning and shrub cutting to promote flowering of adult plants and seedling recruitment.

Key words

communities, conservation, ecophysiology, geographical and altitudinal distribution, germination, herbivory, mycorrhiza, parasites and diseases, reproductive biology, soils

Small White Orchid. Orchidaceae, subfamily Orchidoideae, tribe Orchideae, subtribe Orchidinae. *Pseudorchis albida* is a polycarpic perennial herb with a root tuber divided into 2-4 tapering extensions, 3-5 mm in diameter. Adventitious roots (1-3) thin, 3-7 x 0.1-0.15 cm. Stem 10-40 cm long, erect, with 2-3 brownish, scaly leaf sheaths at the base. Leaves shiny green, 3-5 oblong-lanceolate basal leaves, 2.5-11 x 0.9-3 cm, acute or obtuse at apex; 1-2 narrow pointed bract-like leaves on stem. Inflorescence a narrow, dense, cylindrical, somewhat one-sided spike, 2-8(-15) x 0.6-1.9 cm, with 15-60(-80) flowers. Bracts lanceolate, 5-8 x 2.5 mm, with numerous symmetrical marginal teeth, three-veined, equal or slightly exceeding the ovary. Flowers small, c. 2-3 mm in diameter, whitish to yellowish green, with complete or partial resupination. Outer perianth segments 2-4 x 1-2 mm, obtuse, three-veined, forming the galea; inner perianth segments shorter, faintly three-lobed, two-veined. Labellum 2-4 x 2-3 mm, three-lobed; mid lobe 1 mm long, most often distinctly longer than the side lobes, which are 0-1.3 x 0.5 mm. Spur short, slightly saccate, down-curved, 1.2-3 mm long, 0.8-1 mm in diameter, with concealed nectar. Column short, erect; anther broad, greenish-white; two sectile yellowish pollinia, small three-lobed rostellum, bursicle absent. Ovary sessile, twisted, glabrous, 4-6 x 2-2.5 mm; capsule erect, 4-8 x 2-4 mm. Seeds numerous and tiny (0.45 ±0.05 x 0.19 ±0.03 mm).

The genus *Pseudorchis* was assigned for a long time to *Gymnadenia* (e.g. Ascherson and Graebner 1905-1907; Summerhayes 1951; Luer 1975; Moore 1980; Delforge 2001), but the generic separation is now supported on both molecular and morphological grounds. According to the sequence data of the internal transcribed spacer (ITS) region of ribosomal DNA, *Pseudorchis* is a basally divergent genus within *Platanthera* clade (Bateman et al. 2003, 2006). This distinction is also supported by the chromosome number $2n = 42$ (cf. Pridgeon et al. 2001), as the genus *Gymnadenia* has $2n = 40$ (Marhold et al. 2005).

Pseudorchis albida s.l. shows variation throughout its distribution range, which has resulted in the description of three taxa (Kreutz 2004), although their status is still under debate. Only ssp. *albida* occurs in the British Isles (Harrap and Harrap 2005). Table 1 shows differences in morphological characters and distribution of the three subspecies (Reinhammar 1998; Klein 2000). Phylogenetic data (Bateman et al. 2003) showed negligible ITS divergence between *P. albida* ssp. *albida* and ssp. *straminea* giving little support to recent morphometric (Reinhammar 1995) and allozyme (Reinhammar and Hedrén 1998) studies indicating that the latter should be treated as a full species, segregated from *P. albida* s.l., although there is evidence of ecological segregation (Reinhammar et al. 2002). The morphological study of Reinhammar (1998) revealed an overlap between characteristics of these taxa with no clear separation in Central Europe but slightly better separation in Fennoscandia. This study also confirmed the view of Mossberg and Nilsson (1982) that ssp. *straminea* occurs in Central Europe. Later, Klein (2000) suggested that *P. straminea* should be subdivided into two taxa, *P. albida* ssp. *straminea* and *P. albida* ssp. *tricuspis*. However, the latter taxon is not well defined, its geographical range is entirely within the range of ssp. *albida* and it is probably more appropriately treated as *P. albida* var. *tricuspis* (Beck) Kreutz.

P. albida is a small, native orchid of mesotrophic to acidic grasslands, or heathlands. It is now largely confined to the northern and western parts of the British Isles; like other grassland orchids, it has declined drastically in the southern parts of its range, consequently being classified as vulnerable in Great Britain and endangered in Ireland.

1. Geographical and altitudinal distribution

In the period 1987-1999, *P. albida* was recorded from 165 10-km squares in the British Isles (about 4.3% of the total; Preston et al. 2002; Fig. 1). In the British Isles, *P. albida* is commonest and most widespread in northern and western

Table 1. Summary of differences in morphological characters and distribution among three subspecies of *Pseudorchis albida*, based on information presented in Reinhammar (1998) and Klein (2000)

Character	ssp. *albida*	ssp. *straminea* (Fern.) Á. & D. Löve	ssp. *tricuspis* (Beck) Klein
Plant height (cm)	15-40	10-20	6-17
Lower leaves	erect	spreading	at right angle to stem
Leaf colour	green	yellowish green	yellowish green
Widest place of leaf	near the apex	near the middle	just above the middle
Number of leaves	5-7	4-7	3-4
Flowers in spike	evenly distributed	somewhat one-sided	n.r.
Spike length (cm)	3-7.5	2-5	1.5-3.5
Number of flowers	15-60	10-40	n.r.
Flower colour	greenish-white to yellowish-white	yellowish-white	cream white to yellowish-white
Bract length (mm)	6-8	5.5-7.5	5.5-7.6
Bract margin (at the tip)	numerous symmetrical teeth	sparse, asymmetrical teeth	smooth or crenulate
Sepal length x width (mm)	2-3 x 1.5	3.5-4 x 1.5	3-3.9 x n.r.
Shape of lip	Mid lobe distinctly longer than lateral lobes	Mid lobe slightly longer than lateral lobes	lip lobes of equal length
Lip length x width (mm)	2-2.5 x 1.5-2.5	2.5-3.5 x 2-3	2.8-3.4 x n.r.
Mid lobe length x width (mm)	1	1-1.8 x 0.7-1.2	1.5 x 1.4
Lateral lobe length x width (mm)	0-1.3 x 0.5	1-1.7 x 0.5-0.8	1.5 x 1.4
Spur length (mm)	1.2-2.2	2-2.5	1.8-2.3
Spur shape	slightly saccate	cylindrical	cylindrical
Spur colour	whitish	yellowish	yellowish
Distribution	boreal-montane (from UK across Scandinavia to the northern Urals in the European part of Russia; mountain ranges from Spain across the Alps to the Eastern Carpathians)	west arctic-north atlantic (Iceland, Faroes, Greenland, northernmost part of Finland, widespread in the mountains of Norway and Sweden, Quebec in Canada)	alpine -boreal (Swiss, Italian and Austrian Alps, Tatra mountains, Eastern Carpathian)

n.r., no records.

Scotland, including the Inner Hebrides and Orkney (Lang 2004; Foley and Clarke 2005; Harrap and Harrap 2005). It is almost absent from the central lowlands and southern uplands of Scotland, remaining on few isolated sites in Dumfriesshire, Ayrshire and Roxburghshire. It is absent from the Outer Hebrides. Similarly, the species has decreased in northern England and is now found rather rarely in Cumbria, Northumberland, Durham and mid-west (Hickson 1981) and north-west Yorkshire (Medd 1983). There are historic records for heathy areas in the Weald, and especially near the border of Sussex
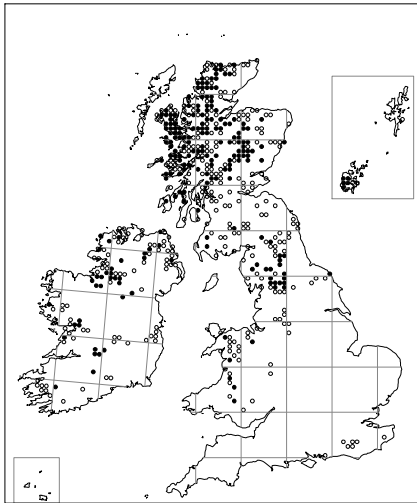
Fig. 1. The distribution of *Pseudorchis albida* in the British Isles. Each dot represents at least one record in a 10-km square of the National Grid. Native: (●) 1970 onwards; (○) before 1970; introduced: (X) 1970 onwards; (+) before 1970. Mapped by Colin Harrower, using Dr A. Morton's DMAP software, Biological Records Centre, Centre for Ecology & Hydrology, Monks Wood, mainly from data collected by members of the Botanical Society of the British Isles.

and Kent, but it is now extinct in southern England. In Wales it is found at a few scattered sites in Breconshire, Merionethshire, Denbighshire and Caernarvonshire. In Ireland it is found mainly in northern counties (Cavan, Sligo, Leitrim; Duffy et al. 2009) and in Galway, with odd sites in counties Tipperary, Limerick, Clare (Breen 1970) and Donegal (Cotton et al. 1994). In Northern Ireland the main concentration lies in the northwest (Londonderry, Tyrone, Fermanagh and Antrim; Nodder 1972). In Tipperary it reappeared in 1991, having last been recorded there in 1898 (Nash 1991), and a similar reappearance was recorded in west Yorkshire after an interval of more than 50 years.

Pseudorchis albida s.l. is one of the few orchid taxa growing in polar latitudes. It has a very wide distribution in boreal-montane Europe extending via Iceland and Greenland to Newfoundland (Fig. 2). It is very abundant in Iceland, and is found in the Faeroe Islands, Denmark, Scandinavia and northern Russia, just creeping over Urals into northwest Siberia; depiction of the genus *Pseudorchis* as ranging across Central Russia to Kamtchatka by Pridgeon et al. (2001) is incorrect. In the south it is found in the Pyrenees, Massif Central, Corsica, Alps, Apennines, Balkans, Carpathians and east to Ukraine (for detailed references see Appendix S1).

II. Habitat

(A) CLIMATIC AND TOPOGRAPHICAL LIMITATIONS

Preston and Hill (1997) include *P. albida* as a member of the European Boreal-montane element of the British flora (i.e. mainly occurring in Europe with extension to more continental parts of Europe, but not Siberia, and associated with coniferous forest zone). The mean January and mean July temperatures,

(a)

(b)
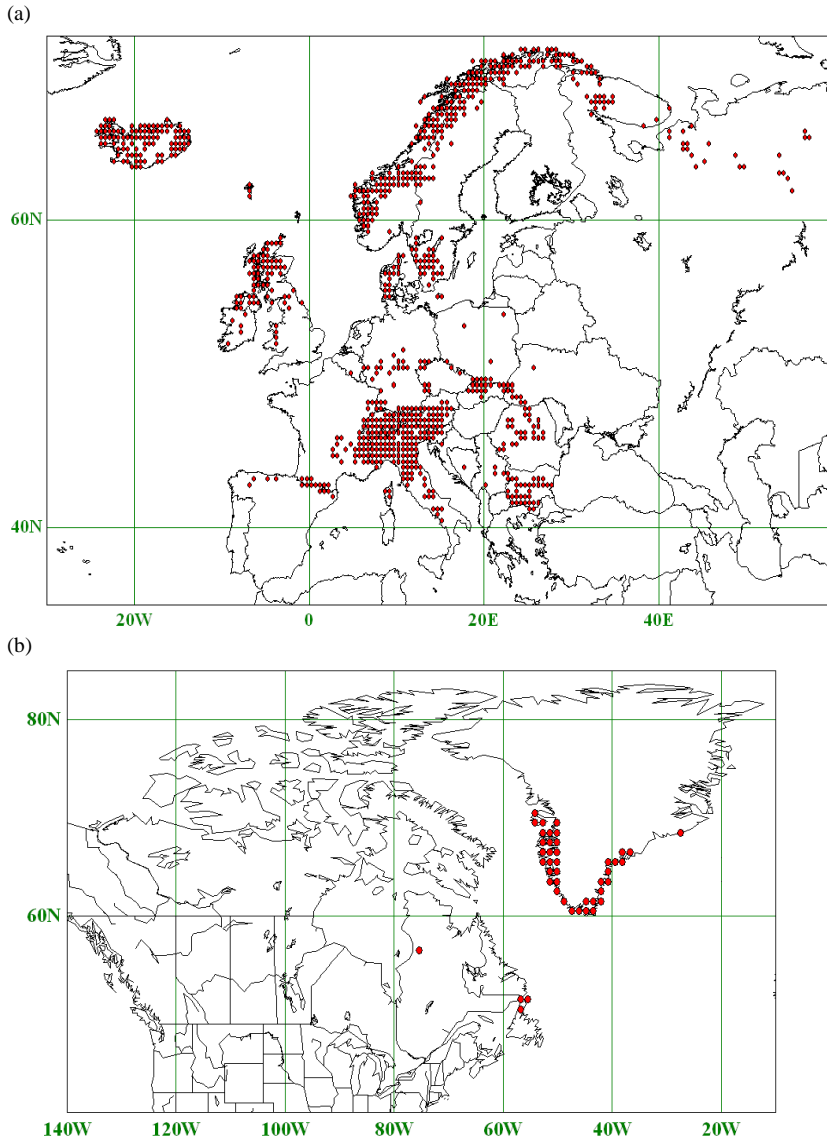
Fig. 2. World distribution of *Pseudorchis albida sensu lato* (including ssp. *albida*, ssp. *straminea*, ssp. *tricuspis*). (a) Europe; (b) N. America. Distribution is based on the references in Appendix S1. Mapped by Jana Jersáková, using Dr. A. Morton's DMAP software.

and mean annual precipitation in the 10-km squares occupied by *P. albida* in Britain are 2.6 °C, 13.0 °C and 1534 mm, respectively (Hill et al. 2004).

The altitudinal range of *P. albida* in the British Isles is from sea level on the west coast of Scotland (Allan et al. 1993) to 500 m at Alston Moor in Cumbria and 550 m at Beinn a' Chaisteil in the Scottish Highlands (Harrap and Harrap 2005). The lower limit of distribution in the Alps is located in Bavaria

about 600 m, in the Inn valley at about 900 m, in Carinthia at 700 m and the Ticino at 750 m. In the Central Alps the lower limit is considerably higher, for example, in the Grisons at 1100 m. The highest frequency of the plant occurs at about 1500 m. The upper limit of its occurrence is recorded as 2550 m at the Stelvio Pass, 2500 m in the Grisons, 2550 m in Wallis and 2370 m in the Tyrol (Ziegenspeck 1936; Presser 2000). In the Italian Abruzzo, the species ascends to 2700 m in Apennine Mountains (Ziegenspeck 1936). In the Czech Republic, the species grows from submontane to subalpine level (minimum, Beskydy, c. 500 m; maximum, Sněžka in Krkonoše Mountains, 1580 m; Štěpánková 2010). In Scandinavia, the altitudinal limits range from 40 m in Våxtorp on southwest coast of Sweden to 1100 m in mountains of Central Sweden (Reinhammar et al. 2002). Klein (2000) reported altitudinal ranges for *P. albida* ssp. *tricuspis* in the Alps as 1010 – 1400 m and in Scandinavia as 700 – 1100 m.

(B) SUBSTRATUM

Throughout its distribution *P. albida* grows in a wide range of soil conditions from the acidic, moist substrata of *Sphagnum* bogs to alkaline, well drained soils on carboniferous limestone (Summerhayes 1951). Ellenberg's indicator values for edaphic characteristics at sites where *P. albida* is found are 6 for pH (the species favours sites with moderately acidic to moderately basic reaction), 5 for moisture (fresh soils of average dampness), 2 for nitrogen (poor sites) and 0 for salinity (*P. albida* does not occur in saline habitats) (Hill et al. 2004). Analyses of physical and chemical properties of soil samples taken across Europe indicate that *P. albida* can occur over a wide edaphic gradient (Table 2).

It has been suggested that differences in demands for substratum can be used as a taxonomic tool for distinguishing *P. albida* subspecies. In Scandinavia where two subspecies occur, ssp. *albida* is indifferent to lime

Table 2. Mean values, standard deviations (SD) and range of selected environmental variables for sites of *Pseudorchis albida*. Analyses calculated on a dry mass basis

Source	Reinhammar *et al.* (2002)		Jeřábková (2006)		Sundermann
	Mean ± SD	Range	Mean ± SD	Range	Range
pH	5.1 ± 0.4	4.5 - 7.2	4.4 ± 0.1	4.1 - 4.6	4.7 - 5.4
Soluble phosphorus (mg 100g^{-1})	4.2 ± 3.8	0.4 - 20.6			
Total phosphorus (mg 100g^{-1})			86.2 ± 23.6	62.2 - 136.4	
Total nitrogen (%)			0.6 ± 0.18	0.4 - 1.1	
Total carbon (%)			9.6 ± 3.1	7.2 - 16.3	
C:N ratio			15.2 ± 1.2	13.9 - 17.8	
Organic content (%)	26.2 ± 21.1	4.3 - 89.4			
Potassium (mg 100g^{-1})	25.0 ± 17.1	4.0 - 112.0			

(Nilsson 1992), while ssp. *straminea* prefers lime-rich habitats or at least base-rich habitats (e.g. on serpentine; Ericsson and Rune 1991). Similarly, ssp. *straminea* growing in Newfoundland and Quebec prefers limestone barrens (Argus and Pryer 1990). In continental Europe, ssp. *albida* prefers acidophilous soils, while ssp. *tricuspis* is calcicolous (Klein 2000). This differentiation is probably not so straightforward, as plants having attributes of ssp. *tricuspis* grow on acidic soils in the Czech Republic (Jeřábková 2006).

III. Communities

Pseudorchis albida ssp. *albida* is described as a species of well-drained poor hill pastures, stream sides, mountain grasslands, cliff ledges, and heather moorlands (Preston et al. 2002), but from a phytosociological point of view, the communities are difficult to classify, as the species often grows in vegetation mosaics or transitions from heath to grasslands. In Scotland, where the species is most abundant, regional floras (McCallum Webster 1978; Duncan 1980; Evans et al. 2002) report its occurrence in acidic hill pastures and short moorland among the heather, often on the edge of sheep paths, but also on rocky outcrops high in the hills, without giving any further vegetation information. A survey of the vegetation of Glen Coe in the Highlands of Scotland reports *P. albida* from two British plant communities: *Calluna vulgaris - Erica cinerea* heath (H10) and *Trichophorum cespitosum - Erica tetralix* heath (M15) (Averis and Averis 2006). A more detailed description is given by Holland et al. (2008) from Tyndrum Community Woodland in west Perthshire, where *P. albida* grows in a patch of H10 heath vegetation on the south facing slope of a moraine hummock within an area of a former pasture (a mosaic of dry grassland, marshy grassland, heath and mire) accompanied by *Calluna vulgaris, Erica cinerea, Festuca rubra, Potentilla erecta, Gymnadenia conopsea, Carex panicea, Linum catharticum, Antennaria dioica, Hypericum pulchrum, Bellis perennis, Hypochoeris radicata, Pilosella officinarum, Gentianella campestris*. On the Isle of Skye in the Inner Hebrides and on the island of Rum, *P. albida* grows with lichens and mosses in the dwarf mountain heath *Calluna vulgaris - Racomitrium lanuginosum* community (H14) (Preston C.D., pers. comm.).

Further south *P. albida* seems to be more common in less acidic, and hence grass-dominated communities. Rodwell (1992) mentions *P. albida* as a component of mesotrophic grasslands, associated with the *Anthoxanthum odoratum - Geranium sylvaticum* (MG3) community typical for well-drained permanent pastures and meadows confined to northern England. It is also a minor component of *Geranium sylvaticum* grassland sub-community (a

member of *Luzula sylvatica-Geum rivale* tall-herb community U17) and herb rich birch-woodlands on limestone in Yorkshire and Durham (Tennant 2008). In the Vicarage Meadows Nature Reserve in Powys, Wales, *P. albida* grows in lowland acid grassland referable to the *Lathyrus montanus – Stachys betonica* sub-community (U14c) of *Festuca ovina – Agrostis capillaris – Galium saxatile* community (Preston C.D., pers. comm.).

In Ireland, the species is an indicator of species-rich *Nardus* grasslands, predominantly found on acidic soils on sloping ground in upland areas (White and Doyle 1982). Typical accompanying species include *Agrostis capillaris, Anthoxanthum odoratum, Calluna vulgaris, Festuca ovina, Nardus stricta, Pedicularis sylvatica, Polygala serpyllifolia, P. vulgaris, Potentilla erecta, Vaccinium myrtillus,* and *V. riviniana*, as well as other orchids such as *Platanthera chlorantha*, *Gymnadenia conopsea* and *Dactylorhiza maculata* (Blackwood 1976). Cotton et al. (1994) found *P. albida* in Sligo, Leitrim and Galway counties in unimproved, unfertilized cattle pastures in hilly areas, often with low cliffs or rocky outcrops nearby. The underlying rock was almost invariably limestone, but the mixture of calcicole and calcifuge plants indicated the partial leaching of base-rich soil, causing sufficiently acid conditions to develop a limestone heath. The common accompanying species included *Calluna vulgaris, Potentilla erecta, Pedicularis sylvatica*, and *Polygala serpyllifolia*.

Elsewhere in Europe, *P. albida* grows predominantly in *Nardus* grasslands on siliceous substrates at various altitudes (Nilsson 1986; Reinhammar et al. 2002, European Commission 2007): alpine grasslands of Nardion and Nardo-Caricion rigidae communities; subalpine grasslands of Nardion strictae community (Lycopodio alpini-Nardetum, Thesio alpini-Nardetum, Solidagini-Nardetum subcommunities); mountain grasslands with presence of alpine species of Nardo-Agrostion tenuis community (Sileno vulgaris-Nardetum strictae subcommunity); and submontane and montane grasslands of Violion caninae community. Typical accompanying species are *Arnica montana, Agrostis capillaris, Deschampsia flexuosa, Polygonum bistorta, Festuca rubra, Galium saxatile, Hypochoeris maculata, Meum athamanticum, Nardus stricta, Pedicularis sylvatica, Platanthera bifolia, Polygala vulgaris, Potentilla erecta* and *Viola canina*. In Central Europe, the *Nardus* grasslands with *P. albida* sometimes grade into wet *Sphagnum* meadows of Caricion fuscae community. The species can be found also in mountain *Trisetum* meadows (Polygono-Trisetion community) and secondary alpine and mountain heathlands of Genistion and Juncion trifidi communities. In the Pyrenees, *P. albida* occurs in subalpine and lower alpine closed

mesophile *Festuca eskia* grasslands of north-facing slopes and depressions with *Arnica montana*, *Ranunculus pyrenaeus*, *Selinum pyrenaeum*, *Trifolium alpinum*, *Campanula barbata*, *Gentiana punctata*, and *Phyteuma betonicifolium* (European Commission 2007). In the West Carpathians at subalpine and alpine levels, *P. albida* grows in Dryado octopetalae-Caricetum firmae community accompanied by *Carex firma*, *Dryas octopetala*, *Bartsia alpina*, *Soldanella carpatica*, *Tofieldia calyculata*, *Pinguicula alpina* and *Vaccinium vitis-idaea* (Šibík et al. 2004). In its eastern Russian distribution range, *P. albida* grows in the extreme north (Murmansk Province) in swampy Sphagno-Tofieldietum pusillae tundras and *Sphagnum* bogs. Towards the south in Arkhangelsk Province, the species occurs in coniferous forests with nemoral herbs, as well as on wet meadows (Vakhrameeva et al. 2008).

     Pseudorchis albida ssp. *straminea* occurs in the low alpine zone, predominately in lime-rich *Dryas* heaths. This subspecies is also reported from serpentine (Ericsson and Rune 1991). Common companion species are *Dryas octopetala*, *Silene acaulis*, *Salix reticulata*, *Thalictrum alpinum*, all favouring lime (Nilsson 1986), as well as more lime-indifferent plants such as *Anthoxanthum odoratum* and *Polygonum viviparum*. Nordhagen (1943) classifies ssp. *straminea* as a member of the Caricion atrofuscae community.

IV. Response to biotic factors

Pseudorchis albida is well-adapted to survive in open habitats and populations flourish when the vegetation is kept short, with a thin litter layer and some disturbance that creates open patches for seedling recruitment (Reinhammar et al. 2002). The positive effect of disturbance is also supported by *P. albida* occurrence in recently cleared areas such as power line corridors and alpine ski slopes (Reinhammar 1995). Disturbance provided by domesticated animals (most often sheep) can be detrimental if too great. With hard grazing pressure most flowers are eaten, inhibiting seed set and plant recruitment. Jeřábková (2006) observed damage to flowering shoots by deer and complete destruction of tubers by wild boar and rodents; she also found rare damage to capsules caused by herbivorous insects.

V. Responses to the environment
(A) GREGARIOUSNESS

Pseudorchis albida characteristically occurs in very small populations. For example, out of seven populations recorded from 1980 onwards on the island of Rum, five consisted of single plants, one of a few plants and one of four plants (Pearman et al. 2008). Nevertheless, large populations of 200 plants are also

known, e.g. two grassland populations in Šumava mountains in the Czech Republic. The average density of plants in four 16-m^2 plots laid in four *P. albida* populations in Šumava mountains ranged from 0.64 to 1.51 m^{-2} (Jeřábková 2006). The plants exhibited spatial clustering, as the density within a site ranged from 0.31 to 2.68 m^{-2}.

(B) PERFORMANCE IN VARIOUS HABITATS

Pseudorchis albida can tolerate the moderate shade of light forests, but does not grow under fully closed canopies. It prefers open habitats with regular mowing or grazing practices, which positively influence plant flowering and seedling recruitment. Reinhammar et al. (2002) studied its population dynamics over 6 years in two permanent plots (3 x 3 m), one mown and the other left to ongoing forest succession (Fig. 3). In the mown plot, the number of new individuals appearing annually was large and stable, while the unmanaged plot showed little or no recruitment.

(C) EFFECT OF FROST, DROUGHT, SHADE ETC.

Pseudorchis albida has an Ellenberg indicator value of 8 for light (i.e. it is light-loving; Hill et al. 2004). Nevertheless the species can withstand moderate shade as has been reported from birch-woodlands on limestone in Yorkshire and Durham counties in North England (Tennant 2008) and from coniferous forests in Akrhangelsk Province in European part of Russia (Vakhrameeva et al. 2008).

There are no available data related to the effects of frost and drought.

Fig. 3. Population dynamics of *Pseudorchis albida* based on the number of individuals of a particular stage recorded over a 6-year period in one mown plot (a) and one unmanaged plot with ongoing forest succession (b). All individuals were permanently marked, plot size 9 m^2. Not found, individuals without aboveground appearance in a given year; Flowering and Vegetative plants were classified into 'old' or 'new' ones, depending on whether they had been recorded previous years or for the first time in a given year, respectively. Adapted from Reinhammar et al. (2002).

VI. Structure and physiology

(A) MORPHOLOGY

Pseudorchis albida has leaves arranged in a semi-rosette on a short, vertical belowground stem attached to a brownish root tuber (Rasmussen 1995, Stern 1997, Klimešová and Klimeš 2006) with two to four tapering extensions. At maturity, *P. albida* shows sympodial growth (Blinova 2000, Tatarenko and Kondo 2003, Vakhrameeva et al. 2008), and a new whitish tuber emerges from a bud in the axil of a scale leaf on the belowground stem. The tuber, which is of root origin with a bud of stem origin, serves as a storage and regenerative organ, and only this tuber survives until the next season (Rasmussen 1995, Klimešová and Klimeš 2006; Fig. 4b). The apical bud of the mature tuber produces a new shoot that develops adventitious roots on its belowground part and eventually terminates with an inflorescence (Rasmussen 1995). The estimated life span of the shoot is 33 months belowground, from its differentiation in the bud in July of the first year until May of the third year,
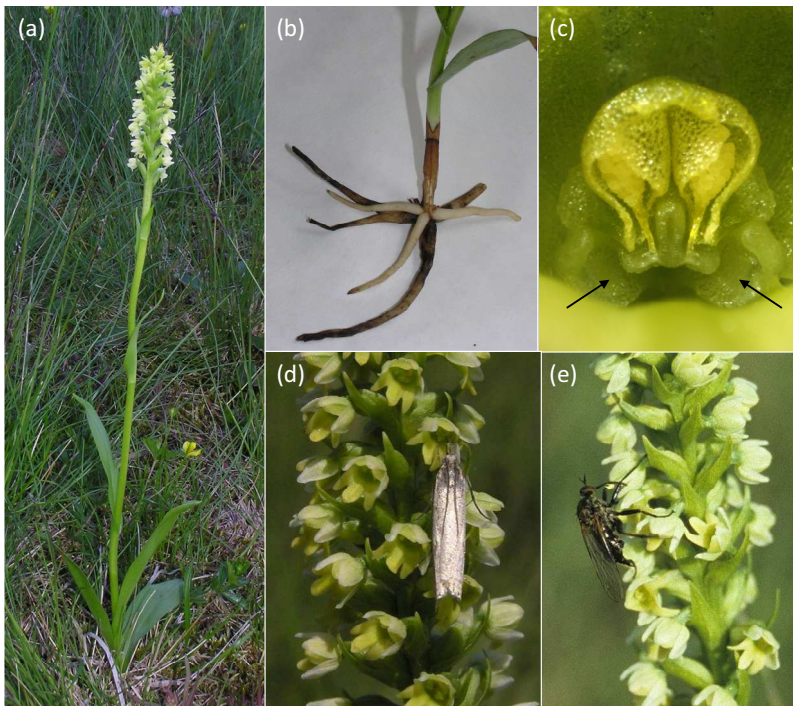
Fig. 4. (a) Flowering plants of *Pseudorchis albida* in June in Šumava mountains, Czech Republic; (b) Fusiform root tubers in June. The new, whitish tuber lasts till the next season, whereas the brownish, old tuber nourishes present shoot; (c) Detail of *P. albida* flower showing two sectile pollinia enclosed in anther sacs (arrows point at stigmatic surface; photo Jean Claessens); (d) *Crambus lathoniellus* (Pyralidae) sucking nectar; (e) Female *Empis bistortae* (Empididae) with two pollinia on the proboscis. (Photos Jana Jersáková unless otherwise stated.)

and subsequently 3 to 5 months aboveground. The roots are short-lived, surviving only for five months (Tatarenko and Kondo 2003).

There is uncertainty about the structure of underground organs: some authors have described the root system as a cluster-root life form, with thickened roots being attached to a rhizome or axis (Blinova 2000), and others as a tuberoid life-form (Tatarenko and Batalov 1999). Pridgeon and Chase (1995), however, argue that the swollen, food-storing underground structures of Orchidoideae are root tubers, not root-stem tuberoids or tuberoids, because their anatomical structure is root-like, not stem-like. Furthermore, the 'fingers' cannot act as independent tubers, since they have a common regenerative bud (Klimešová J., pers. comm.).

The root vascular bundles are radial, with only one in the adventitious roots and several in the root tuber extensions (Fig. 5a), decreasing in number towards the tip. The several steles are parts or branches (meristeles) of a singular stelar system, rather then separate vascular bundles and thus should not be termed polystele (Stern 1997).

(B) MYCORRHIZA

Despite the relatively well-described ecology of adult *P. albida* plants (Reinhammar et al. 2002), the identity of its symbiotic mycorrhizal partners has been little studied. While the wider parts of root tuber extensions store nutrient reserves, the narrow ends are heavily colonized with mycorrhizal fungi, as are the narrow, adventitious horizontal roots (Summerhayes 1968, Fig. 5a). Tatarenko and Batelov (1999) reported colonization in these parts as high as 75% in Arkhangelsk Province, whereas short young roots and young tuber extensions up to 2.5 cm in length had no colonization. Transmission electron microscopy of roots of adult plants revealed rhizoctonia-like hyphae possesing septal dolipores with imperforated parenthesomes, consisting of two electron-dense layers separated by an electron-transparent zone (Jersáková and Malinová, unpublished data; Fig. 5b). Such ultrastructure of the septal pore and parenthesome is typical for the family Tulasnellaceae (Shimura et al. 2009). A *Tulasnella*-like nrDNA ITS sequence was also detected in a two-year seedling cultivated *in situ* (GB accession no. HQ852051; Jersáková and Malinová, unpublished data). Downie (1959) had earlier concluded that the fungus was a member of a polyphyletic basidiomycete group collectively called 'rhizoctonia', which are typically found in photosynthetic orchids of open habitats (Rasmussen 2002). This assumption is further supported by the close taxonomical relatedness of *P. albida* to the genera *Platanthera*, *Amerorchis* and
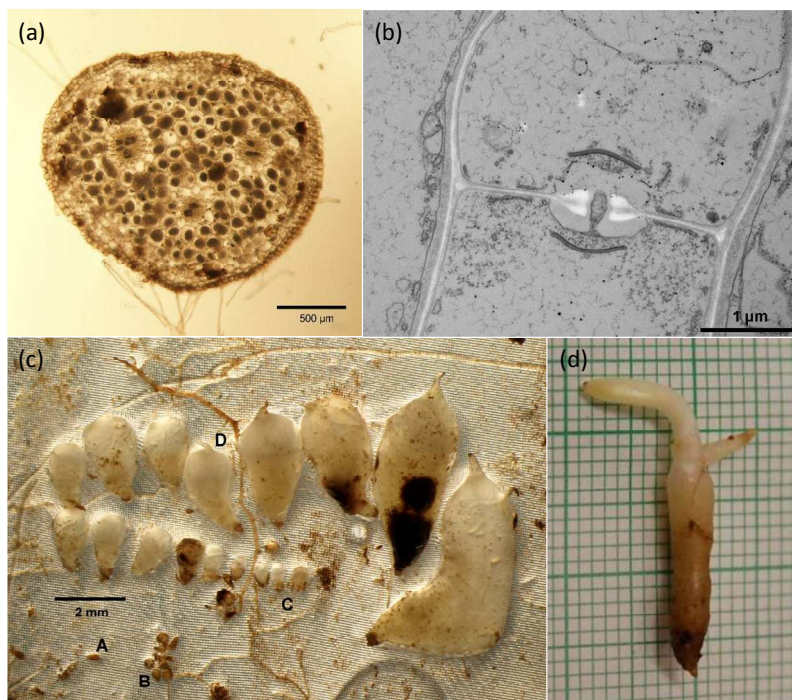
Fig. 5. (a) Cross-section of a root tuber extension of *Pseudorchis albida*; brown coils of fungal hyphae (pelotons) are visible within the cells, note three vascular bundles; (b) Dolipore septum that is composed of a pore cap (parenthesome) surrounding a septal swelling and septal pore; (c) Stages in the development of seeds into the seedling stage. A – ungerminated seed, B – non-mycorrhizal seedlings with ruptured testa and rhizoids, C – mycorrhizal pear-shaped protocorms, D – protocorms with a leaf primordium; (d) Two years old seedling with an apical shoot and adventitious root (mycorrhizome). (Photos of Jana Jersáková and Tamara Malinová.)

Galearis (Bateman et al. 2003), which typically associate with rhizoctonia (Zelmer and Currrah 1995; Rasmussen 2002).

(C) PERENNATION: REPRODUCTION

Vegetative propagation of the tubers is thought to make an insignificant contribution to population growth (Summerhayes 1968). The main perennating organ in *Pseudorchis albida* is a root tuber (Fig. 4b). Each year the tuber is wholly replaced by a new one, which gives rise to the whole functional plant in the following growing season. Although the old and new tubers remain in contact, the old one usually becomes completely exhausted in September and contributes little or nothing to the next year's growth. Adult vegetative dormancy (Shefferson 2009), i.e. the failure of a plant to produce aboveground parts in one or more growing seasons, followed by reappearance of full-sized photosynthetic plants in subsequent seasons, has been observed in *P. albida*

and lasts for one, or sometimes 2 years (Jeřábková 2006; Fig. 6). *P. albida* is able to flower in the year following a year in which it is dormant, which suggests that metabolic activity and growth continue even when above-ground parts are not produced. The transition to dormancy is triggered by the cost of flowering and fruiting (Jeřábková 2006; Fig. 6), as plants which aborted flowering stalks and did not invest into seeds had a higher probability of flowering in the following year (64%) than plants which had produced seeds (17%).
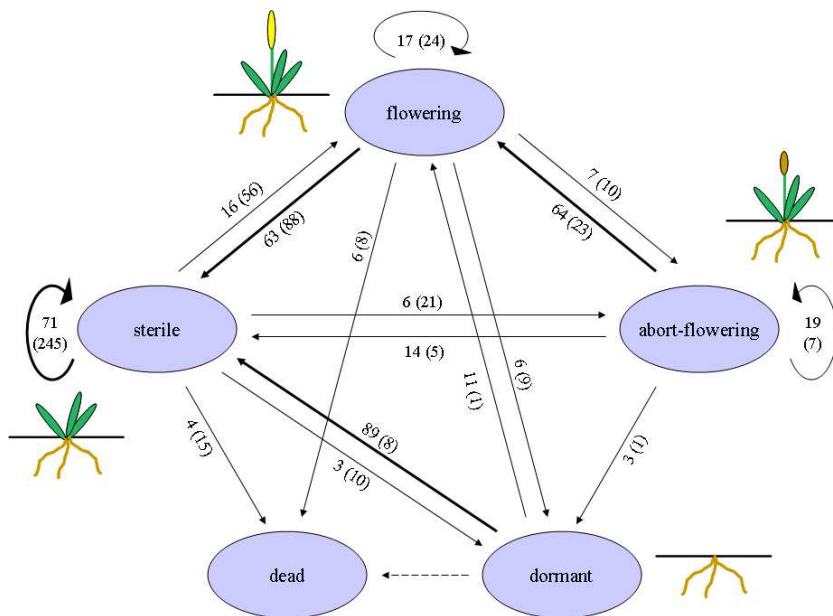
Fig. 6. Transition probabilities between stages from year *t* to year *t+1* in *Pseudorchis albida* populations in Šumava mountains (Czech Republic). Data were pooled from three populations. The values in parentheses indicate the number of plants involved in a transition. The plants were considered dead if not observed for three consecutive years (adapted from Jeřábková 2006).

(D) CHROMOSOMES

The chromosome number of all three *P. albida* subspecies has been reported consistently as *2n* = 42 (ssp. *albida*: Heusser 1938; Cauwet-Marc and Balayer 1986; ssp. *straminea*: Harmsen 1943; Jørgensen, Sørensen and Westergaard 1958; Löve and Löve 1969; Lojtnant and Jacobsen 1977; ssp. *tricuspis*: Klein 2000).

(E) PHYSIOLOGICAL DATA

The density of stomata on the lowest leaf was 47.8 mm^{-2}, of which 15% represented reduced stomata (Ziegenspeck 1936). The second and third leaves

had similar numbers of stomata to the lowest leaf, but a higher proportion of reduced ones (28%). Ziegenspeck explained these differences in the numbers of functional stomata by higher humidity near the soil surface allowing more efficient water use.

(F) BIOCHEMICAL DATA

Infection of the tubers of *P. albida* (*Gymnadenia albida* (L.) Rich.) with a strain of *Rhizoctonia repens* from *Orchis militaris* resulted in the synthesis of the phytoalexin orchinol (2,4-dimethoxy-7-hydroxy-9,10-dihydrophenanthrene) and *p*-hydroxybenzylalcohol (Nüesch 1963; Gäumann et al. 1960). These compounds function as antifungal metabolites controlling hyphal invasion of a mycorrhizal partner in orchid tissue (Rasmussen 1995).

Investigation of the flavonoid content of the leaves of *P. albida* revealed the presence of quercetin and kaempferol. These flavonol glycones were detected in acid-hydrolysed extracts of leaf tissue, indicating they originate from flavonol *O*-glycosides (Williams 1979).

Pseudorchis albida inflorescences emit a sweetish scent, and in 8 scent samples collected from 2 Czech populations (see Appendix S2 for details) by dynamic headspace and analysed by GC-MS we found in total 58 compounds, 33 of which could be identified (Table 3). The scent was rich in terpenoid compounds, and most of the dominant compounds (e.g. 4-oxoisophorone, β-myrcene, limonene, β-phellandrene, and verbenone) are frequently found in various orchid species (Kaiser 1993) and represent general floral volatiles associated with various pollination syndromes (Dobson 2006; Knudsen et al. 2006). Most compounds were found in samples collected from both of two populations, but there were some differences in the relative scent composition among populations. Some compounds were found in higher amount in one population (e.g. 4-oxoisophorone, limonene) and some in the other population (e.g. some of the unknown compounds). Samples from both populations contained several unknown compounds, and these uncommon compounds might be involved with the attraction of unusual pollinators (e.g., Pyralidae and Pterophoridae moths, Empididae flies; see below).

VII. Phenology

The phenology of *Pseudorchis albida* s.l. has been described by Jeřábková (2006) and Vakhrameeva et al. (2008). The duration of vegetative period varies from 2.5 to 5 months depending on geographic location. The appearance of leaves aboveground depends on spring humidity and temperature. In warm springs the plants appear at the beginning of May, but in colder, wetter years

Table 3. Percentage amount of floral scent compounds found in eight *Pseudorchis albida* plants in two populations (A and B) located in Šumava Mountains in the Czech Republic. Values >5.0 are printed in bold; unknown compounds <1.0 were pooled with the superscript digit giving the number of pooled compounds

	A (N = 5)			B (N = 3)		
	Median	Min	Max	Median	Min	Max
Aliphatics						
Hexanal	0.1	0.0	0.2	—	—	—
(*E*)-2-Hexenal	tr	0.0	0.4	—	—	—
Irregular Terpenes						
4-Oxoisophorone	**9.6**	**7.6**	**11.1**	4.8	3.4	**5.9**
2,2,6-Trimethyl-1,4-cyclohexanedione	0.1	0.1	0.3	—	—	—
4-Hydroxyisophorone	0.1	tr	0.1	—	—	—
Homoterpenes						
(*E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	0.0	0.0	0.1	—	—	—
Monoterpenes						
α-Thujene	2.3	2.2	5.0	—	—	—
α-Pinene	2.0	1.1	3.3	0.6	0.5	1.0
Thuja-2,4(10)-diene	0.8	0.5	2.8	0.4	0.1	0.4
β-Sabinene	2.8	2.2	4.7	0.4	0.3	0.7
β-Myrcene	**11.4**	1.5	**15.2**	3.1	1.9	**5.6**
α-Terpinene	0.1	0.1	1.5	0.2	tr	0.3
Limonene	**14.1**	**10.2**	**18.3**	2.9	1.5	3.1
β-Phellandrene	**5.7**	2.6	**8.1**	1.0	0.4	1.0
(*E*)-beta-Ocimene	0.1	0.0	0.1	—	—	—
γ-Terpinene	0.5	0.1	1.7	0.4	0.3	0.5
(*Z*)-Sabinene hydrate	3.7	3.1	4.1	1.7	1.2	2.1
Terpinolene	0.3	tr	0.9	tr	tr	0.1
Linalool	1.0	0.2	1.6	0.8	0.5	1.2
(*E*)-Sabinene hydrate	4.5	3.5	**5.5**	2.9	2.6	3.4
α-Pinene oxide	0.1	tr	0.1	0.2	0.2	0.7
Chrysanthenone	0.1	tr	0.2	0.4	0.3	0.5
(*Z*)-Verbenol	**11.1**	**7.6**	**12.8**	**5.9**	3.4	**6.2**
(*E*)-Verbenol	0.4	0.3	0.5	0.2	0.1	0.2
Phellandral	0.6	0.2	0.7	1.0	0.6	1.0
Pinocarvone	0.4	0.1	1.2	—	—	—
4-Terpineol	0.5	0.3	0.6	—	—	—
(*Z*)-Pinocarveol	0.4	0.1	0.5	1.0	0.5	1.7
α-Terpineol	0.3	0.0	0.6	0.5	0.4	0.7
(*Z*)-Carveol	0.4	0.2	0.5	0.1	tr	0.3
Verbenone	**8.6**	**7.6**	**12.2**	**7.9**	**5.8**	**9.5**
Geraniol	0.4	0.1	0.5	0.1	0.0	0.5
2-Hydroxypinane-3-one	1.2	0.6	2.5	3.1	2.5	3.9
unknown monoterpenes	0.2[2]	0.0	1.5	0.9[2]	0.7	1.6
Unknowns						
m/z:123,151,95,108,77,67	0.1	0.0	0.5	3.5	3.2	4.2
m/z:95,39,67,123,55,83 [S3_a]	**5.7**	3.0	**8.3**	**5.8**	4.4	**7.9**
m/z:83,112,39,97,55,98 [S3_b]	0.7	0.0	1.1	**26.8**	**25.3**	**28.1**
m/z:153,125,43,107,39,111 [S3_c]	0.9	0.0	2.3	**9.4**	**7.5**	**11.6**
m/z:109,137,152,79,91,81 [S3_d]	3.6	0.0	**7.8**	**8.0**	**7.9**	**9.3**
further unknowns	3.2[17]	1.1	**7.7**	5.0[18]	3.7	**8.4**

The methods of scent collection and identification are described in Appendix S2. Mass spectra of dominant unidentified compounds (indicated by superscript "S3_a" - "S3_d") are reported in Appendix S3.

appearance is postponed to the end of May. At the extreme north in Murmansk Province of Russia, leaves appear above the ground in mid-June. The leaves are fully developed throughout the time of flowering and fruit production. Differentiation of the inflorescence and flower structures in the bud has been observed in the first half of July, more than a year before the flowering of the shoot (Tatarenko and Batalov 1999). The time of flowering depends on a combination of factors, especially latitude and altitude. In Britain, the species flowers from late May to mid July; it is latest at higher altitudes in the north and earliest in Ireland, but generally peaks around mid-June. In alpine areas of the Alps the flowering ranges from mid June until August (Presser 2000). The flowers fade extremely quickly, with the lower ones turning brown before those higher up on the stem have even opened. The capsules are usually fully developed by the end of July, with seeds disseminated from August onwards. As the capsules do not open fully, the seeds can be shed gradually throughout autumn and winter. In September, the aerial parts finally die.

Reinhammar et al. (2002) reported differences in flowering phenology of *P. albida* subspecies growing sympatrically in Central Sweden: ssp. *albida* started flowering earlier, and also flowered for a shorter time period than ssp. *straminea*.

The factors that determine whether a plant will flower or not are complex and little understood. In general, the probability that a plant will flower in a given year depends on both internal and environmental variables. According to Wells et al. (1998), orchids have to reach a critical minimum size before they can flower and once that size is reached the probability of flowering increases with leaf number. Jeřábková (2006) showed that a *P. albida* plant has to reach at least four, or better five leaves to start flowering, while plants with more than 6 leaves always flower (Fig. 7). The most frequent plants in populations were those with 3 and 6 leaves. Plants with one or two leaves were always juveniles, never old senescing individuals. The probability of consecutive flowering in *P. albida* is strongly influenced by cost of flowering and fruiting (Fig. 6), as has been shown for many other orchid species (e.g. Primack and Stacy 1998; Jacquemyn and Hutchings 2010; but see Shefferson et al. 2003).

VIII. Floral and seed characters
(A) FLORAL BIOLOGY
The flowers are very small (c. 2.5 mm in diameter) with colour ranging from greenish to yellowish white (Fig. 4a). The perianth segments form a short, broad hood, although the lateral outer perianth segments are occasionally
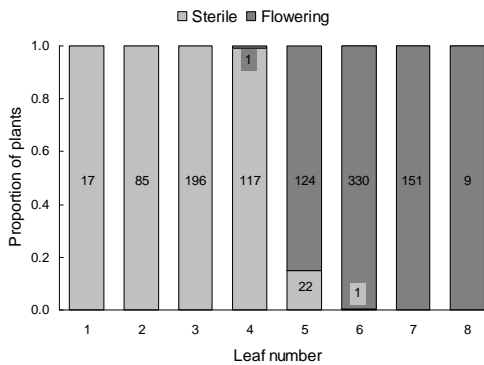
Fig. 7. Proportion of sterile and flowering plants in three *Pseudorchis albida* populations in relation to the leaf number (adapted from Jeřábková (2006), measured on 1093 plants over 4 years). The numbers depicted in columns indicate the number of plants in each leaf number category.

slightly spreading. The labellum is three-lobed with a mid lobe most often distinctly longer than the side-lobes (Fig. 4d-e). The labellum lobes are curved downward, which produces an oval, horizontally-elongated aperture that leads to the short, blunt, downcurved spur (2.5 mm long) with free nectar (mean volume $\pm$ SD = 0.096 $\pm$ 0.07 μl, n = 10). A short compact gynostemium bears two massulate pollinia, separated by the thick, fleshy, laminar projection of the midlobe of the rostellum (Fig. 4c). The pollinia have short caudicles that terminate in sticky naked viscidia set c. 0.5 mm apart. A concave stigma is situated underneath the rostellum, just at the entrance to short spur. Pollen sculpturing varies, either distinctly hamulate or ornate (Schill and Pheiffer 1977). The mean number of massulae per pollinium is 52.9 $\pm$7.4 SD (n = 25; Jeřábková 2006).

The flowers emit a sweet scent during the whole day. Müller (1881), Knuth (1899), and Ziegenspeck (1936) have, on the basis of floral morphology, suggested that *P. albida* s.s. should be pollinated by butterflies. Direct observations have reported visits of butterflies (Reinhard et. al 1991), tineid moths, along with solitary bees (Pijl and Dodson 1966), other hymenopterous insects (Summerhayes 1968), and mosquitoes (Mossberg and Nilsson 1982). Recently, Baumann and Baumann (2002) documented pollination of *P. albida* growing on alpine meadow (1750 m a.s.l.) in Metnitzer Berge (Steiermark, Austria). The insects carrying pollinia were identified as moths of the genus *Crambus* (*pascuellus* or *silvellus*: Lepidoptera, Pyralidae). Our unpublished observations at four sites in Šumava mountains (South Bohemia, Czech Republic) have confirmed this. At dusk, the flowers were abundantly visited by *Crambus lathoniellus* (Fig. 4d), *C. ericella*, *Chrysocrambus culmella* (Pyralidae), and *Hellinsia osteodactylus* and *H. didactylites* (Pterophoridae), carrying on average 1.6 pollinia (n = 8). The proboscis of these moths is 4.5 mm long with the pollinia usually attached to the middle of it. Besides the crepuscular pollinators, we recorded dance flies *Empis tesselata* and *E.*

bistortae (Diptera, Empididae; Fig. 4e) as diurnal pollinators, with a proboscis 2.3 mm long.

(B) HYBRIDS

Pseudorchis albida has been reported to hybridize with three orchid genera (hybrid name in parentheses): *Dactylorhiza* (x *Pseudorhiza*, x *Leucorhiza*, x *Dactyleucorhiza*), *Gymnadenia* (x *Leucadenia*, x *Pseudadenia*), and *Nigritella* (x *Leucotella*, x *Pseuditella*). Occurrence of hybrids with the genera *Herminium* (x *Leucinium*, x *Hermileucorchis*) and *Platanthera* (x *Leucanthera*, x *Pseudanthera*) remains doubtful.

In the British Isles, two intergeneric *P. albida* hybrids have been recorded. A hybrid with *Dactylorhiza maculata* (x *Pseudorhiza bruniana* (Brügger) P.F. Hunt) has been recorded from Orkney and Skye (Foley and Clarke 2005). It resembles *D. maculata* in stem and leaf characters, and *P. albida* in inflorescence shape, size and colour, but has intermediate floral characters (Stace 2010). Hybrids with *Gymnadenia borealis* have been recorded from several places in north Britain and are still frequent in NW Scotland with both parents (e.g. native Caledonian pinewood in east Iverness-shire, Foley and Clarke 2005); they are intermediate in size, and perianth shape (especially spur) and colour (pale pink). The binomial x *Pseudadenia schweinfurthii* (Hegelm. ex A. Kern.) P.F. Hunt (synonym x *Gymleucorchis schweinfurthii* (Hegelm. ex A. Kern.) T. and T.A. Stephenson) applies to the hybrid involving *G. conopsea* s.s., probably not recorded in British Isles (Stace 2010).

Outside the British Isles, intergeneric hybrids involving *P. albida* have been found with *Dactylorhiza alpestris, D. cordigera, D. fuchsii, D. lapponica, D. traunsteinerioides, Gymnadenia conopsea, G. frivaldii, G. odoratissima, Nigritella corneliana, N. rhellicani, N. rubra*, and *N. widderi* (Gerbaud and Schmid 1999; Schmidt 1989; Souche 2004; Arbeitskreise Heimische Orchideen 2005). Interestingly, *P. albida* hybridizes more readily with the genera *Gymnadenia* and *Nigritella*, than with the more closely related genus *Platanthera*.

Pre-mating and post-mating isolation barriers in *P. albida* have not been investigated.

(C) SEED PRODUCTION AND DISPERSAL

The breeding system of *P. albida* s.l. is poorly known. Reproduction is reported to occur by sexual means only (Harmsen 1943; Hagerup 1951, 1952; Summerhayes 1968). High fruit set, often over 90% (Detto 1905, Ziegenspeck

1936), raises the possibility that *P. albida* might be autogamous (Summerhayes 1968; Nilsson 1992; van der Cingel 1995), but outcrossing or a mixed breeding system have been also suggested (Mossberg and Nilsson 1982; Davies, Davies and Huxley 1983; Reinhammar and Hedren 1998). Hagerup (1951, 1952) proposed autogamy as being more frequent in arctic-alpine areas such as Greenland and the Faroes. Apomixis, previously reported from Orchidaceae (Catling 1982; Teppner and Klein 1989; Catling and Catling 1991), does not seem to occur in *Pseudorchis* (Harmsen 1943; Hagerup 1952). Our observations of moth behaviour on *P. albida* flowers suggest that pollinator-mediated geitonogamous self-pollination could play an important role in the *P. albida* breeding system, as an inflorescence visit by an individual moth can last up to 20 min.

Fruit set recorded in three populations of *P. albida* ssp. *albida* in the Šumava mountains, Czech Republic (Jeřábková 2006) ranged from 42 to 98% with following population means ±SD: 94.8 ± 3.2% (*n* = 19), 73.7 ± 17.4% (*n* = 13), and 77.3 ± 12.5% (*n* = 18).

Seeds are numerous (700 ±276 per capsule) and tiny (length 0.45 ±0.05 mm, width 0.19 ±0.03 mm; embryo length 0.21 ±0.02 mm, width 0.14 ±0.02), and the testa is transparent (Jeřábková 2006). Seeds are wind-dispersed, the majority falling in the close vicinity of the mother plant (Jersáková and Malinová 2007).

(D) VIABILITY OF SEEDS: GERMINATION

The period from seed germination to appearance aboveground lasts at least four years according to Fuchs and Ziegenspeck (1925). Germination of *P. albida* seeds in seed packets buried in natural conditions (Rasmussen and Whigham 1993) showed that in suitable places the seeds can develop to the protocorm stage (mycorrhizal, pear-shaped seedlings) within one year, and can reach 22 mm in two years (Jeřábková 2006; Jersáková et al. unpublished, Fig. 5d).

Asymbiotic germination guidelines are based on relatively few studies. Downie (1941) failed to germinate *P. albida* seeds in water and various liquid media, while Veyret (1969) and Harbeck (1963) succeeded with Chang medium (Rasmussen 1995). Jeřábková (2006) germinated surface-sterilized (3 min in 70% ethanol, followed by 20-40 min in 7.2% $Ca(OCl)_2$ with Tween) unripe *P. albida* seeds on Norstog medium (Rasmussen 1995) and 3 other media which were successfully used with *Dactylorhiza sambucina*, *Gymnadenia conopsea* and *Liparis loeselii* (media composition given in Jeřábková 2006). The cultures were kept at 18-20°C in darkness. The first germination (swollen seeds with rhizoids) occurred after 5 month on

'sambucina' medium reached 21%; the first protocorms appeared after 7 months. Germination on Norstog medium started after 9 months reaching only 9%. Germination on other media was negligible. After 18 months, 20 seedlings with their first green leaves were transplanted on to new media and transferred to an illuminated growth chamber, but all died within 2 months.

Jeřábková (2006) also tested viability of *P. albida* seeds using tetrazolium staining (van Waes and Debergh 1986). The optimal duration of $Ca(OCl)_2$ pretreatment was 6 hours. Seed viability (proportion of coloured embryos) in five *P. albida* populations ranged from 45 to 74% with mean ±SD 61 ±10%.

(E) SEEDLING MORPHOLOGY

Although the seeds contain minimal storage reserves, in natural conditions they germinate without an association with a mycorrhizal fungus and develop until they achieve a spherical shape with long rhizoids (Fig. 5c). Then growth continues only after fungal colonization (Jersáková and Malinová, unpublished data). Mycoheterotrophic tissue forms in the basal part of the seedling. On the apical chalazal part, a functional meristem forms and develops further into an elongated mycorrhizome, i.e. a seedling with scale leaves, elongated apical meristem and first root (Fig. 5d; Rasmussen 1995). The mycorrhizome of *P. albida* thickens to 5 – 8 mm in diameter (Tatarenko and Batelov 1999). Its monopodial shoot consists of 6 – 8 metamers with bud formation at some nodes in the axils of scale leaves. The juvenile plants have several scales, one foliage leaf 3 – 4 cm long and 0.2 – 0.4 cm wide, and one adventitious root. The autotrophic juveniles attached to the mycorrhizome may grow monopodially for 1-3 years. The transition to sympodial growth occurs in young autotrophic plants after the development of the uppermost axillary bud to form a root tuber, which becomes the storage and regenerative organ (Ziegenspeck 1936; Tatarenko and Batelov 1999). The number of foliage leaves increases from 1 to 4 – 6, leaf length increases from 3 to 7 cm, leaf width from 0.3 to 2.2 cm, number of tuber extensions from 1 to 4, and their length from 0.5 – 2 to 3 – 8 cm during ontogeny from juvenile to generative age state. The number of thin roots ranges from 0 – 1 in juvenile individual to 1 – 3 in mature individuals (Vakhrameeva et al. 2008).

IX. Herbivory and disease
(A) ANIMAL FEEDERS OR PARASITES

There are no data for phytophagous insects. In the Czech Republic, the aerial parts of plants are frequently damaged by browsing deer, while the tubers can be eaten by wild boar and rodents (Jeřábková 2006).

(B) PLANT PARASITES AND (C) PLANT DISEASES
No data available.

X. History

The nomenclature of the species has undergone taxonomic turmoil since the first description of *Satyrium albidum* by Linné in 1753, with a specific name referring to the white flowers. Subsequent authors moved the species into various genera e.g. *Orchis* (Scopoli 1772), *Habenaria* (Brown in Aiton 1813), *Gymnadenia* (Richard 1817), *Platanthera* (Lindley 1829), *Peristylus* (Lindley 1835), *Leucorchis* (Meyer in Patze et al. 1848) and *Bicchia* (Parlatore 1860). While many of them were forgotten, the name *Gymnadenia albida* can be frequently encountered in old floras and herbaria, and *Leucorchis albida* was an accepted name till late 1980s when *Pseudorchis* was given taxonomic priority. In 1754, Séguier in his work 'Plants of Verona province' mentioned '*Pseudorchis* alpina, flore herbaceo', growing in Italian mountains Monte Baldo. His description of underground organs ('radice fibrata') and floral colour ('flos subalbidus') clearly refers to *P. albida*. He points to Micheli's work 'Nova Plantarum Genera' (1729), where the genus *Pseudo-Orchis* was described, including an authentic illustration. Micheli used the name *Pseudo-Orchis* (in Greek 'false' orchid) to point at similar appearance of the plant to members of the genus *Orchis*.

The etymology referring to 'white orchid' is reflected also in British common names: Small-white Orchid (English name), Mogairlean Bàn Beag (Gaelic name), Magairlín bán (Irish name) and Tegeirian Broga Gwyn (Welsh name).

The first British record was made by John Ray in Wales in 1662 (Clarke 1900). He found 'Orchis pusilla alba odorata, radice palmata… on the back of Snowdon-hill, by the way leading from Llanberis to Carnarvan' (Ray 1670). According to Foley and Clarke (2005) the species still occurs within this general area.

XI. Conservation

Pseudorchis albida is currently classified as Vulnerable in Great Britain (Cheffings and Farrell 2005) and Endangered in Ireland (Curtis and McGough 1988). Due to a considerable decline in its whole distribution range, it is

protected also in other European countries such as Poland, Czech Republic, Slovak Republic, Austria, Germany, Switzerland, Italy and Ukraine.

The species occupied 4.7% of hectads in Britain and 3.3% of those in Ireland during the period 1987-1999, while prior 1970 the corresponding values were 7.1% and 7.1%, respectively. It is estimated that, of its total historical range between 1500 and 1999, it has been lost from 65.5% of its British sites and 70% of those in Ireland (Preston et al. 2002). The counties from which the species has disappeared are given in detail by Harrap and Harrap (2005). Populations tend to be small and scattered and even in Scotland many vice-counties have just one or two sites. Overgrazing and habitat destruction have led to the loss of many populations; the latter reason is particularly prominent in southern England (Curtis and McGough 1988). In Sussex, where the species was last seen in the 1930s, the habitat was composed of a mosaic of small grazed pastures and woodland, now long gone under housing development. Even in the last 20 years the more accessible populations in northern England appear to have declined, although the situation appears better in the Scottish Highlands (Foley and Clarke 2005).

P. albida populations have suffered from the loss and degradation of heathlands in Britain. Over the last 100 years, Britain has lost approximately 80% of its heathland, particularly in lowland areas. In the past, the lowland heaths were lost primarily to agriculture, forestry, mineral extraction and development. The main factors affecting the habitat at present are: encroachment of trees and scrub due to a lack of conservation management such as light grazing, controlled burning and cutting; nutrient enrichment, particularly deposition of nitrogen compounds emitted from intensive livestock farming; fragmentation and disturbance from developments such as housing and road constructions; and agricultural improvement including reclamation and overgrazing, especially in Northern Ireland. Holland et al. (2008) reported rapid changes in the population of *P. albida* following the removal of grazing from an area of *Calluna vulgaris – Erica cinerea* heath (H10). The population was maintained under a low level of sporadic grazing by sheep and deer, which kept the dwarf shrub short. Once grazing was removed *Calluna vulgaris* and *Erica cinerea* increased in height, as did *Molinia caerulea*. The increased competition and reduced disturbance resulted in a rapid decline from sixty individuals to the point of local extinction within six years. Clearly, small isolated populations are at significant risk of local extinction if livestock are removed from sites.

The other two important habitats of *P. albida* in Britain have encountered a similar fate. The *Anthoxanthum odoratum - Geranium*

sylvaticum community (MG3) is an upland grassland confined to areas where traditional hay meadow management has been applied in a harsh submontane climate. Although widespread in the past, it is now confined to few upland areas in northern England and Scottland. Species-rich, semi-natural *Nardus* grasslands found on sloping, acidic soils of upland areas are typically maintained by a low intensity grazing regime. Their continued existence is under threat from agricultural intensification, agricultural abandonment and afforestation, which is likely to have resulted in significant losses in the extent of this habitat in the last number of decades. For these reasons the overall conservation status of the habitat is considered unfavourable.

Both heathlands and species-rich *Nardus* grasslands are protected by the EU Habitats Directive (92/43/EEC). Heathlands and upland hay meadows (MG3) are habitats being prioritized under the UK Biodiversity Action Plan (UK BAP) to reverse the decline of the country's biodiversity.

Outside the British Isles, the reasons for the overall decline of *P. albida* populations are basically the same. According to Reinhammar et al. (2002 and references therein) the species decreased dramatically during the 20th century and became rare in Scandinavia, especially in Denmark and in the south-west of Sweden, while the orchid has not suffered the same decrease in the central provinces of Norway and Sweden. This suggests that most threatened and extinct populations occur in intensively-used lowland areas, while populations in alpine and sparsely populated areas have a better chance of survival. Comparison of managed and unmanaged *P. albida* s.l. sites in Scandinavia clearly identified the overgrowth of *P. albida* habitats as a major threat (Reinhammar et al. 2002). The cessation of traditional agricultural practices such as mowing and grazing on the one hand, and intensification of agriculture using artificial fertilizing on the other, are major reasons for the decline of *P. albida* (Nilsson and Gustafsson 1978; Olsson et al. 2000).

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Supporting information

Appendix S1. List of publications indicating the geographical distribution of *Pseudorchis albida* s.l.

Countries with *Pseudorchis albida* s.l. occurence:

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van Rompaey E, Delvosalle L (1979) *Atlas van de Belgische en Luxemburgse Flora. Pteridofyten en Spermatofyten*. Jardin Botanique National de Belgique, Meise, Belgique.

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Gejdeman TS (1975) *Opredelitel' vysšich rastenij Moldavskoj SSR: 3. izd., pererab. i dop*. Štiinca, Kišinev, Moldavit.

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Tabaka LV (1990) *Flora i rastitel'nost' Latvii: Central'no-Vidzemskij geobotanicheskij rajon.* Zinatne. Riga. Latia.

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Appendix S2. Floral scent collection and analyses of volatiles.

The scent samples were collected in two populations of *Pseudorchis albida* subsp. *albida* located in Šumava mountains in the Czech Republic (A: Kvilda: N 49°00´, E 013°34´; B: Zhůří: N 49°05´, E 013°33´). For the collection of volatiles, single inflorescences were enclosed into polyester oven bags (Toppits®) and the air was pumped from these bags through cartridges containing adsorbent polymer at a flow rate of 200 ml/min. Five samples were taken for 4.5 hours using cartridges filled with 30 mg of Porapak Q (Alltech Associates, USA) and a further 3 samples were taken for 30 min using cartridges filled with 1 mg of Tenax® TA (mesh 60-80; Supelco, Bellefonte, Pennsylvania, USA) and 1 mg of Carbotrap® B (mesh 20-40, Supelco, Bellefonte, Pennsylvania, USA). Samples were collected between 2 to 7 p.m., including an ambient air to control for background contamination. The samples taken using Porapak cartridges were eluted with 100 µl of acetone.

The composition of the floral scent was analysed on a Varian Saturn 3800 gas chromatograph (GC) fitted with a 1079 injector and a ZB-5 column (5% phenyl polysiloxane, length 60 m, inner diameter 0.25 mm, film thickness 0.25 µm, Phenomenex; Torrance, CA, USA), and a Varian Saturn 2000 mass spectrometer (MS) (Varian Inc., Palo Alto, CA, USA). To analyse the acetone samples, 1 µl thereof was placed in a quartz vial in the injector port of the Varian 3800 GC by means of the ChromatoProbe (Amirav & Dagan, 1997; Dötterl *et al.* 2005). Samples taken using tenax:carbotrap cartridges were inserted directly via Varians Chromatoprobe into the GC injector. The injector split vent was opened and the injector was heated at 40°C to flush any air from the system. After 2 min the split vent was closed and the injector heated at 200 Cmin^{-1}, then held at 200 C for 4.2 min, after which the split vent was opened and the injector cooled down. Electronic flow control was used to maintain a constant helium carrier gas flow rate (1.8 ml min^{-1}). The GC oven temperature was held for 7 min at 40 °C, then increased by 6 °C min^{-1} to 260 °C and held for 1 min at this temperature. The mass spectra were taken at 70 eV with a scanning speed of 1 scan s^{-1} from *m/z* 30 to 350.

Compounds were identified using the Saturn Software package 5.2.1. with the NIST 08, Wiley 7, Adams 2007 and MassFinder 3 mass spectral databases and verified, where possible, using retention times of authentic standards or comparison of estimated and published Kovats retention indices. Finally the contribution of the single compounds to the total scent (percentage amount) was calculated.

References:
Dötterl S, Wolfe LM, Jürgens A (2005) Qualitative and quantitative analyses of flower scent in *Silene latifolia. Phytochemistry* 66:203–213
Adams RP (2007) *Identification of Essential Oil Components by Gas Chromatography & Mass Spectrometry*. Allured Publishing Corporation: Carol Stream, Illinois, USA

Appendix S3. Mass spectra of dominant unidentified volatile compounds (see also Table 3)
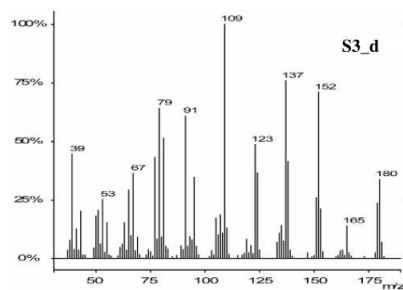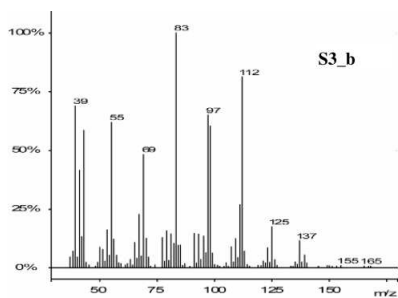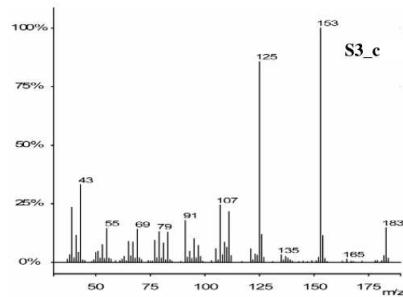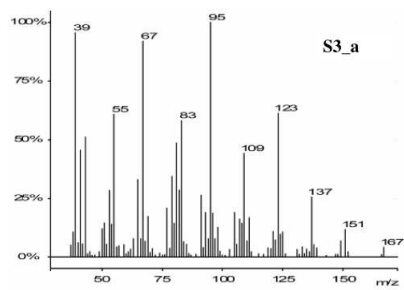
CHAPTER III

Symbiotic germination capability of four *Epipactis* species (Orchidaceae) is broader than expected from adult ecology

Těšitelová T, Těšitel J, Jersáková J, Říhová G, Selosse M-A (2012)

Symbiotic germination capability of four *Epipactis* species (Orchidaceae) is broader than expected from adult ecology

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Abstract

•   Both abiotic and biotic factors shape species distributions. Orchids produce minute seeds with few nutrient reserves, thus requiring mycorrhizal fungi for germination. Therefore, both environmental conditions and mycorrhizal fungi distribution affect their germination success, but these ecological requirements and their congruence with habitat preferences of adults remain poorly understood. We investigated the importance of these factors during germination in four forest orchid species of the genus *Epipactis*.

•   We sowed seeds of three habitat specialists and one generalist in different forest types at sites harboring adults of at least one of these ecologically diverging species. We analyzed germination pattern and identified mycorrhizal fungi of both seedlings and adults.

•   Habitat conditions had little influence on germination pattern as seedlings grew in more habitats than expected from the adults' ecology. Ectomycorrhizal fungi availability did not limit germination. Suitable mycorrhizal fungi, mostly pezizalean ascomycetes, were recruited in various forest types, though the fungal communities differed according to habitat type. Finally, orchids with divergent ecological preferences shared similar mycorrhizal fungi.

•   Limited adult distribution contrasted with successful seed germination at diverse sites and indicates existence of niche differentiation between adults and seedlings. Ecological specialization may thus be determined by factors other than mycorrhizal fungi that act later in the ontogeny, perhaps during the transition to above-ground development.

Introduction

The distribution of a species is largely determined by habitat requirements and dispersal ability (Zobel 1997). Habitat requirements, i.e., the realized species niche, results from both abiotic constraints and interactions with other organisms. Of these, the biotic interactions represent the dominant factor shaping the ecological niche of species (e.g. Michalet et al. 2006; van der Heijden et al. 2008). Their influence is especially pronounced in obligately mycorrhizal or parasitic plants requiring host fungi or plants, respectively, for germination or even whole life cycle (Selosse and Roy 2009; Westwood et al. 2010). Such assisted seedling development allows them to avoid the trade off between seed size and amount of produced seeds, because dust seeds with little nutrient reserves can be produced in large quantities. This strategy evolved independently in several angiosperm families (Eriksson and Kainulainen 2011). However, the requirement of a biotic interaction presents a constraint, and understanding this constraint is crucial for uncovering the ecological needs of these species, with obvious implications for their conservation. Here, we examine habitat requirements of two different life stages, germinating seeds and adults, in four terrestrial orchid species strongly dependent upon biotic interactions with mycorrhizal fungi.

Owing to the production of dust seeds, the orchid germination stage (the protocorm) requires mycorrhizal fungi, which provide carbon and mineral resources (Rasmussen 1995; Dearnaley et al. 2012). This heterotrophic nutritional mode is called mycoheterotrophy (Leake 1994). Orchids recruit mycorrhizal fungi mostly from saprophytic basidiomycetes of a heterogeneous, polyphyletic complex of fungi of the families Tulasnellaceae, Ceratobasidiaceae and Sebacinaceae, collectively named rhizoctonias (Dearnaley et al. 2012). The rhizoctonias typically associate with green orchids of open habitats. By contrast, orchid species that remain fully mycoheterotrophic at adult stage associate with diverse basidiomycetes or ascomycetes that usually form ectomycorrhizal (ECM) symbioses (Dearnaley et al. 2012). The association with ECM fungi also allows a mixotrophic lifestyle (i.e., mycoheterotrophy combined with autotrophy), which is typical of forest understory species of the Neottieae tribe of Orchidaceae (Gebauer and Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005; Selosse and Roy 2009).

The range of mycorrhizal fungi of adult orchids is increasingly becoming known (e.g. Shefferson et al. 2007; Roy et al. 2009), but fungal associations at an early germination stage have been less investigated and may differ from the adult stage. Recent studies mostly focus on germination in situ within a population of conspecific plants, and, when identified, the spectrum of mycorrhizal fungi in seedlings usually corresponds to that in the co-occurring adults (McKendrick et al. 2002; Bidartondo and Read 2008), with a few exceptions (McCormick et al. 2004). Furthermore, Bidartondo and Read (2008), Swarts et al. (2010) and Phillips et al. (2011) showed that seeds can germinate and are also selective for fungi that are typical of adults at sites environmentally similar to species' natural habitat, but lacking an extant population of the parent species.

Revealing the factors that affect early development presents a major challenge, but is critical for understanding the mechanisms that underlie habitat specialization in orchids. For adult plants, the ecological and geographical ranges of individual orchid species are usually well described in literature, but are very poorly defined for most of their mycorrhizal fungi. Habitat preferences of orchid species can be underpinned by (1) a direct interplay between the plant physiology and the environment (including biotic and abiotic factors) and/or (2) the impact of the environment on the presence and symbiotic capability of mycorrhizal fungi (Batty et al. 2001; Diez 2007; McCormick et al. 2006, 2012). However, studies testing the influence of these mechanisms on orchid habitat preferences are largely lacking (but see a recent study by McCormick et al. 2012). Analyses of germination ability and mycorrhizal fungi associated with seedling and adult stages under different environmental conditions represent a possible way to untangle these mechanisms.

The orchid genus *Epipactis* comprises mostly forest-dwelling perennial rhizomatous species with contrasting ecological requirements and amplitudes in their ecological range. In a few *Epipactis* species investigated for mycorrhizal fungi, molecular techniques (Bidartondo et al. 2004; Selosse et al. 2004; Ogura-Tsujita and Yukawa 2008) and microscopy (Selosse et al. 2004) revealed diverse ECM ascomycetes and basidiomycetes but very few rhizoctonias (except for the *E. palustris* clade; Bidartondo et al. 2004), indicating a mixotrophic lifestyle of adults. Although little is known about the conditions and fungi that allow germination in *Epipactis* species, Selosse et al. (2004) found identical fungi in advanced seedlings and adults of *E. microphylla*, and Bidartondo and Read (2008) found that protocorms of *E. atrorubens* depend on ECM fungal taxa that also colonize adults. Fungal

selectivity thus makes *Epipactis* species excellent models for investigating biotic requirements at germination.

To uncover the role of symbiotic factors and environmental conditions in orchid establishment and distribution, we examined the relationships between ecological preferences, germination pattern, and mycorrhizal associations in four ecologically distinct *Epipactis* species. We selected three species restricted to particular forest habitats and soil conditions, thus expected to be limited by environmental factors, and one common species growing across a wide range of habitats, including those of the three specialists. In the initial stage of our study, we analyzed data from databases of the phytosociological relevés to precisely describe ecological demands of adults. On this basis, we monitored in situ germination over 23 months at sites (1) within the ecological range of the species with and without an extant conspecific population and (2) outside the ecological range of the specialist species, at sites hosting another ecologically distinct *Epipactis* species. The experimental design allowed us to answer following questions: (1) does the germination pattern of individual species correspond to ecological range of adults? Are the habitat specialized species able to germinate also at sites with unsuitable soil characteristics and/or forest habitat? (2) Is there any difference in germination rate between sites with and without a population of conspecific adults? (3) Is there any difference in the spectrum of mycorrhizal fungi between the generalist and specialists in adult and seedling stages? (4) Is the germination supported by same fungi in and outside the ecological range of the species independently of presence of conspecific adults?

Material and methods

Model species and their ecological demands

We focused on four *Epipactis* species (Neottieae tribe), three of which grow in distinct forest types with specific soil conditions, while the fourth is an ecological generalist (Fig. 1). *Epipactis albensis* is an autogamous species derived from the *E. helleborine* aggregate (Nováková and Rydlo 1978), growing typically in floodplain forests, streamside vegetation, or poplar alleys in the vicinity of *Populus nigra* or *P. ×canadensis* (Nováková and Rydlo, 1978; Fig. 2). This species is endemic to Central Europe. *Epipactis atrorubens* is an allogamous species confined to dry, nutrient-poor, and light forests frequently with *Pinus sylvestris* (Fig. 2, Appendix S1), strictly in calcareous soils with higher pH (Delforge 2006). *Epipactis purpurata* is an allogamous species growing in beech and hornbeam forests (Delforge 2006). In contrast to *E. atrorubens*, it prefers shady, nutrient-rich, and moist sites with lower pH (Fig.

Fig. 1. Species used in the seed sowing experiment: (1, 2) *Epipactis helleborine*, (3, 4) *E. albensis*, (5, 6) *E. purpurata*, and (7, 8) *E. atrorubens*.

2). In the Eurasian part of its range, *E. helleborine* is a common allogamous species with wide ecological amplitude. It grows on nutrient-rich soils of various pH (ranging from moderately acidic to basic) in forests, shrubs, or partly disturbed vegetation ranging from lowland floodplain forests to mountain spruce forests (Delforge 2006); thus, it may also occur in forest types typical of the other species (Fig. 2).
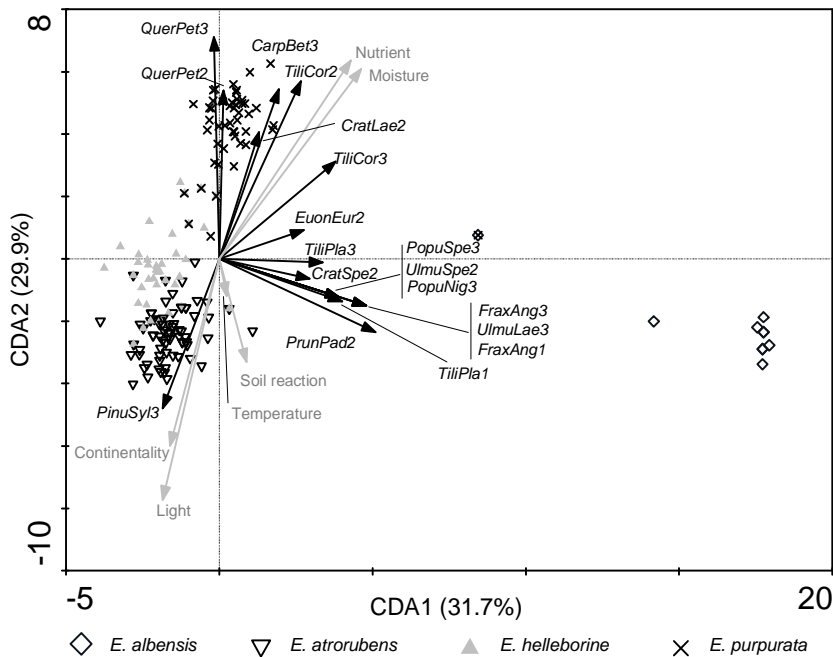
Fig. 2. Ordination plot of canonical discriminant analysis (CDA) showing differences in tree layer composition of phytosociological relevés containing *Epipactis albensis* (*N* = 13), *E. atrorubens* (*N* = 73), *E. helleborine* (*N* = 45) and *E. purpurata* (*N* = 50) from the Czech Phytosociological Database (Chytrý and Rafajová 2003). All herbs and mosses were excluded, since *Epipactis* plants depend on ECM fungi associated with trees and shrubs. Mean Ellenberg's indicator values for each relevé (with exclusion of bryophytes, species not categorized by Ellenberg et al. 1992, and *Epipactis* spp. to prevent circular reasoning) were passively projected in the CDA ordination space. The arrows denote an increase either in species abundance or in environmental factor value. All canonical axes explained 88.0% of total variability. Plant species: CarpBet, *Carpinus betulus*; CratLae, *Crataegus laevis*; CratSpe, *Crataegus* sp.; EuonEur, *Euonymus europeus*; FraxAng, *Fraxinus angustifolius*; FraxExc, *Fraxinus excelsior*; PinuSyl, *Pinus sylvestris*; PopuNig, *Populus nigra*; PopuSpe, *Populus* sp. (*P. nigra* or *P. ×canadensis*); PrunPad, *Prunus padus*; QuerPet, *Quercus petraea*; TiliCor, *Tilia cordata*; TiliPla, *Tilia platyphyllos*; UlmuSpe, *Ulmus* sp.; UlmuLae, *Ulmus laevis*. Numerals behind tree species abbreviations indicate vegetation layer: 1, herb; 2, shrub; 3, tree). Summary of a Monte-Carlo permutation test (999 permutations) testing statistical significance of all canonical CDA axes: $F = 3.03$, $P < 0.001$. See Appendix S1 for Ellenberg's indicator values of the studied species.

Sowing experiment

We selected one or two sites per habitat specialists depending on site availability throughout the Czech Republic. The sites differed in tree layer composition and soil substrate according to the species ecological preferences (Table 1). Consequently, we chose three sites of the habitat generalist, *E. helleborine* (H1 to H3) with environmental conditions mirroring the sites of the

Table 1. Description of the seven sites used in the reciprocal seed sowing experiment.

Site code	Latitude/ longitude	Elevation (m a.s.l.)	*Epipactis* sp. present	Forest type	Dominant trees	pH dH$_2$O	Ca (g.kg^{-1})	K (g.kg^{-1})	P (mg. kg^{-1})	Organic matter content (%)
Alb	50°07'N 15°09'E	190	*E. albensis*	poplar alley	*Populus xcanadensis* *Fraxinus excelsior* *Acer spp.*	7.2	6.5	5.0	35.3	6.9
Atr	49°16'N 13°35'E	488	*E. atrorubens*	pine wood	*Pinus sylvestris* *Populus tremula*	7.8	288.4	1.5	24.9	6.4
H1 [a]	48°50'N 16°42'E	218	*E. helleborine*	lime wood	*Tilia cordata*	7.9	29.8	5.3	16.2	7.3
H2 [a]	48°48'N 16°49'E	158	*E. helleborine*	poplar alley	*Populus xcanadensis* *Prunus avium* *Acer campestre*	7.9	38.2	5.9	84.7	8.8
H3	48°54'N 14°24'E	461	*E. helleborine*	conifer forest	*Picea abies* *Pinus sylvestris* *Corylus avellana*	7.1	78.1	0.7	10.1	23.9
P1 [b]	49°13'N 16°31'E	265	*E. purpurata*	hornbeam- lime forest	*Tilia cordata* *Carpinus betulus* *Populus tremula*	5.2	4.7	1.7	9.9	6.0
P2	48°50'N 16°41'E	271	*E. purpurata*	hornbeam- lime forest	*Tilia cordata* *Carpinus betulus* *Populus tremula*	5.6	2.7	3.2	7.7	5.4

[a] Presence of the Neottieae *Cephalanthera damasonium*.
[b] Presence of the Neottieae *Neottia nidus-avis*.

habitat specialists; i.e. H2 is a poplar alley near a flood-plain forest similar to an *E. albensis* site; similarly, H1 and H3 represent sites potentially suitable for growth of *E. purpurata* and *E. atrorubens*, respectively (Table 1, see Appendix S2 for location of the experimental sites). This set-up enabled us to compare the germination pattern at the sites of seed origin (called "home sites") with that of the putatively suitable sites (within ecological range of a species, with similar tree layer composition and soil conditions) and ecological range). For the generalist species *E. helleborine*, all nonhome sites were considered as putatively suitable for growth. Soil samples from each study site (a mixture of five random replicates from 5 to 10 cm soil depth) were analyzed for their chemical properties (Table 1) by standard methods at the Institute of Botany, Academy of Sciences of the Czech Republic in Třeboň.

Up to five mature seed capsules were harvested from at least eight *Epipactis* specimens per population at the time of capsule dehiscence in August and September 2004. The capsules were dried in paper bags at room temperature. Released seeds were subsequently pooled within populations and stored at 4°C until the burial of seed packets in mid-October. Approximately 300 well-developed seeds for *E. helleborine* or *E. purpurata*, 250 seeds for *E. atrorubens*, and 120 seeds for *E. albensis* were separately placed in a 1.5×3.5 cm pocket (42 µm nylon mesh; Silk and Progress Ltd., Brněnec, Czech Republic) and enclosed in 35-mm plastic slide mounts as in Rasmussen and Whigham (1993). In total, 980 seed packets were buried at seven sites with an *Epipactis* population. The packets were sown in a factorial design: 140 packets composed of 20 replicates per each *Epipactis* population were buried at each site. Within the sites, the packets were buried in 10 groups of 14 packets (two packets per each *Epipactis* population). These groups were placed vertically in the topsoil layer close to randomly chosen adult *Epipactis* plants. Because of slow germination of *E. helleborine* reported by van der Kinderen (1995), who observed development of protocorms after 15 months, the first third of the packets was examined after 12 months in soil. Because no germination was seen for *E. purpurata* and the germination of other species was sparse and slow, developing into small mycorrhizal seedlings (stage 4, protocorm, described later) at most, the remaining packets were retrieved after 23 months in soil and used for all analyses.

Evaluation of germination and root sampling
Recovered seed packets were kept moist at 4°C until processing. The packets were washed and opened under a dissecting microscope, and the length of the mycorrhizal seedlings was measured. Every seed was then categorized into one

of the six developmental stages described in Fig. 3. The mean proportion of germinated (stages 2–6) and mycorrhizal (stages 3–6) seedlings from the total amount of seeds sown, and the growth stage of the most developed seedling after 23 mo were used for detailed statistical analyses of the germination pattern. The mycorrhizal seedlings were stored in 55% ethanol up to 3 wk, then washed and kept at −20°C prior to molecular analyses; except for the seedlings from P2 site which were not used due to similarity of this site with P1.

Roots from two to four *Epipactis* adults were collected at each study site except for P2 in summer 2005 and 2006 (as well as roots of one *Cephalanthera damasonium* adult at H1 and H2. For these additional data, see Appendices S3 and S4). The roots were cleaned and cut to 1-cm long pieces. Thin cross sections of each cutting were inspected for mycorrhizal colonization. Eight to 10 randomly selected infected cross sections were pooled per plant in a single sample for molecular analyses.

Fig. 3. Developmental stages of *Epipactis helleborine* seedlings; stage 1: ungerminated seed; stage 2: swollen, nonmycorrhizal seedling; stage 3: small, oval-shaped mycorrhizal seedling (brown hyphal pelotons visible); stage 4: pear-shaped seedling longer than 0.5 mm, a protocorm; stage 5: seedling with leaf primordium longer than 1 mm; and stage 6: branched seedling, a young mycorrhizome.

Molecular identification of fungal symbionts

Molecular tools allowed the identification of fungi in the roots of the adult plants and in the mycorrhizal seedlings of *E. helleborine* and *E. atrorubens*. Seedlings of the other two *Epipactis* species were not analyzed, as their numbers were too low. To facilitate analyses we created two mycorrhizal seedling pools per *Epipactis* species (or population in case of *E. helleborine*) at each site by pooling (1) up to two small seedlings around 0.5 mm in length (stages 3 and 4) per packet and (2) up to two medium seedlings around 1 mm (smaller seedlings at stage 5) per packet if possible. In addition, up to six particularly large seedlings of stages 5 or 6 per species at each site were analyzed separately. This approach allowed testing potential narrowing or switching in fungal associates during ontogeny (McCormick et al. 2004).

Fungal DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France). Fungal internal transcribed spacer of nrDNA (ITS) was amplified as in Selosse et al. (2002) using primers ITS1F/ITS4 (White et al. 1990; Gardes and Bruns, 1993). Whenever a unique fragment occurred after amplification, it was directly sequenced from both strands. PCR products were purified by ExoSAP-IT (USB Corp., Cleveland, Ohio, USA), and a sequencing reaction was performed on an ABI3130xl sequencer (Applied Biosystems, Courtaboeuf, France), using the BigDye Terminator kit. Whenever direct sequencing failed or multiple fragments occurred, PCR products were cloned using pGEM-T Easy Vector systems kit (Promega, Charbonnières, France) and transformed into supercompetent cells XL1-Blue (Stratagene, Amsterdam, Netherlands). The clones were amplified as previously, and at least 20 positive PCR products per cloning were subjected to restriction fragment length polymorphism (RFLP) analyses using *Hinf*I + *Hae*III and *Hha*I + *Nde*II (Promega, Charbonnières, France). Four to 12 µL of PCR product was mixed with 0.5 µL of each enzyme, buffer and BSA, and incubated at 37°C for 4 h. RFLP patterns were visualized on 3% agarose gels in 0.5× TAE buffer, and up to four clones per unique RFLP pattern were sequenced. Whenever sequences from a given cloning were more than 97% identical over the ITS, a consensus was built to eliminate polymerase errors and possible chimerical sequences resulting from cloning. ITS1/ITS4-Tul primer combination (Taylor and McCormick, 2007) was used to check for potential presence of the rhizoctonia clade Tulasnellaceae in a subset of samples as in Selosse et al. (2004), but no additional fungal lineages were detected. A presumed taxonomical position and ecology was found using Blast search at the NCBI database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). To delimit fungal species, we arbitrarily grouped together sequences that were more than 97% identical in the

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ITS sequence (although there is no universally applicable threshold, this is most often used for mycorrhizal fungi and is also considered most biologically realistic; Hughes et al. 2009). Although the barcoding-based, cultivation-independent method cannot satisfy Koch's postulates, this is the predominant method used for fungal identification in cases where the obligately mycorrhizal fungi are involved in mycorrhiza formation. Their possible exclusion in a cultivation approach would introduce bias into the spectrum of associated fungi (e.g. Bidartondo et al. 2004; McCormick et al. 2004; Selosse et al. 2004; Bidartondo and Read 2008). Only fungi potentially mycorrhizal in *Epipactis* species (i.e. ECM fungi and rhizoctonias) were used for statistical analyses. Classification of fungi to genera was used in the statistical analyses, as no metric distance among fungi was available due to the highly variable and thus poorly alignable ITS sequences.

Statistical analyses

The mean proportion of germinating seeds (stages 2–6) and mycorrhizal seedlings (stages 3–6) from the total amount of seeds sown and the growth stage of the most developed seedling were used for detailed statistical analysis. The value for each combination of site and seeds from individual *Epipactis* populations was considered as an independent observation. Cases with mean zero proportions of germinated seeds were omitted from the statistical analysis of mycorrhizal seedlings. Mean proportions of germinated and mycorrhizal seedlings were subjected to angular transformation prior to the analyses. We used analyses of variance (ANOVA) to test factors affecting the germination pattern. We tested effects of conditions at sowing sites, i.e. species identity of *Epipactis* adults (ES) and forest types (FS) present at experimental sites, and origin of seeds sown, i.e. species identity of *Epipactis* seeds sown (EO) and forest type on sites of *Epipactis* seed origin (FO). The last factor was used to test whether seeds sourced from a particular forest type differed in germination from seeds of other forest types. Tukey's honestly significant difference tests were employed to test differences between individual levels of the predictors with statistically significant effects detected by ANOVA.

Differences in fungal spectra found at the sites and in different *Epipactis* species were analyzed by canonical correspondence analysis (CCA). Partial CCA (pCCA) with sites as covariates was used to test whether fungal spectra differed between seedlings of *E. atrorubens* and *E. helleborine*. Design-based Monte-Carlo permutation tests with 999 permutations were used to test significance in CCAs. We used R package v. 2.12 (R Development Core Team

2006) for the univariate statistical analyses and Canoco v. 4.53 (ter Braak and Šmilauer 2002) for the multivariate statistics.

Results

Germination at different sites

After 23 months of soil incubation, the species substantially differed in their germination pattern (Table 2, Fig. 4). *Epipactis atrorubens* and *E. helleborine* exhibited the largest proportions of germinating seeds (i.e. nonmycorrhizal and mycorrhizal seedlings of stages 2–6, Fig. 3) and the fastest development with numerous seedlings reaching stages 5 and 6. Of these, *E. atrorubens* produced the largest seedling over 1 cm long. Germination rate was lower in the other two species, in which the most developed seedlings were at stage 3 and in *E. purpurata* a few seedlings had reached even stage 4. All germination parameters tested exhibited the same pattern: *E. helleborine* and *E. atrorubens* performed significantly better than *E. purpurata* and *E. albensis*, while no difference was found within these pairs of species (Table 2, see Appendix S5 for details of the posthoc comparison tests).

At least some germination occurred in ca. 90% of all *E. atrorubens* and *E. helleborine* packets, 46% of *E. albensis*, and 27% of *E. purpurata* packets, although the proportion of germinating and mycorrhizal seedlings in packets was highly variable for each species (Appendix S6). At all sites, both habitat specialists and the generalist reached at least stage 2 (nonmycorrhizal),

Table 2. Summary of four-way analyses of variance testing the effects of conditions at sowing sites and origin of seeds sown on the proportion of germinated seeds (stages 2 to 6), proportion of mycorrhizal seedlings (stages 3 to 6), and the growth stage of the most developed seedling.

Effect	Germinated seeds			Mycorrhizal seedlings			Maximal stage		
	df	SS	*F*	df	SS	*F*	df	SS	*F*
Condition at sowing sites									
Identity of *Epipactis* adults present on site (ES)	**3, 13**	**0.27**	**6.16****	**3, 9**	**0.12**	**32.55*****	**3, 13**	**11.75**	**4.63***
Forest type on site (FS)	**2, 13**	**1.28**	**43.23*****	**2, 9**	**0.18**	**75.77*****	2, 13	1.24	0.73
Origin of seeds sown									
Identity of *Epipactis* seeds sown (EO)	**3, 13**	**5.86**	**132.27*****	**3, 9**	**0.54**	**148.62*****	**3, 13**	**76.90**	**30.29*****
Forest type on a site of seed origin (FO)	**2, 13**	**0.14**	**4.63***	2, 9	<0.01	2.00	2, 13	2.38	1.41
Interactions									
ES × EO	9, 13	0.24	1.83	**9, 9**	**0.08**	**7.58****	9, 13	4.75	0.62
ES × FO	6, 13	0.02	0.24	6, 9	0.01	0.75	6, 13	0.62	0.12
FS × EO	**6, 13**	**0.67**	**7.55****	**6, 9**	**0.16**	**21.67*****	6, 13	1.10	0.22
FS × FO	4, 13	0.03	0.49	4, 9	0.01	2.37	4, 13	1.33	0.39
Residuals	13	0.19		9	0.01		13	11.00	

Notes: *P < 0.05; **P < 0.01; ***P < 0.001

Fig. 4. Germination pattern of the four studied *Epipactis* species. (A) Scheme of the experimental design: home sites of particular *Epipactis* species (dark gray), putatively suitable sites (light gray), unsuitable sites (white); (B) proportion of germinated seeds (stages 2–6) and (C) mycorrhizal seedlings (stages 3–6) from the total number of sown seeds (note the differing scales in B and C). Values are based on the counts from 12 to 14 seed packets per population at each site, except P2, where the number decreased to 8–11 because of animal disturbance (see repetition numbers in Appendix S6). Numerals behind species names indicate sites of seed origin, e.g. *E. helleborine* 1 comes from site H1.

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and at most sites they reached stages 3–6 (mycorrhizal), showing a general capability to germinate irrespective of the presence of adult conspecifics and the type of habitat (Fig. 4). Proportions of germinating and mycorrhizal seedlings, however, did not correlate, since most sites exhibited high proportions of germinating seeds on average, but only some of them also had high proportions of mycorrhizal seedlings (stage ≥3; compare site H1 with site Atr in Fig. 4).

Further development and mycorrhization of seedlings were more frequent at home sites and putatively suitable sites for *E. albensis* and *E. purpurata*; by contrast, *E. atrorubens* and *E. helleborine* showed no site germination preferences (Fig. 4). *Epipactis albensis*, an exclusive inhabitant of floodplain forests, started germinating in various forest types, although seedling mycorrhization occurred only at sites with poplars (i.e. home site Alb and the putatively suitable site H2) and also in presumably unsuitable hornbeam–lime woods (H1 and P2; Fig. 4C). *Epipactis purpurata* successfully germinated at all sites but reached the mycorrhizal stages 3 and 4 only at home sites (P1 and P2) and the putatively suitable site H1 (two stage-3 seedlings occurred also at the unsuitable site H3). *Epipactis atrorubens* germinated well and reached stages 5 or 6 at all sites, including the poplar alleys (Alb and H2) or sites with low pH (P1 and P2) where this calcicolous species does not occur naturally. The generalist *E. helleborine* also germinated successfully to stages 5 or 6 at all sites except at site Atr (where the stage 3 was hardly reached). In general, the presence of conspecific adults or a putatively suitable habitat was not a reliable predictor of the occurrence of germination.

Despite this lack of correlation between habitat preferences of *Epipactis* species and their germination pattern, we found highly significant effects of most predictor variables on germination performance (Table 2), although the predictors were intercorrelated (forest type on site [FS] and species identity of *Epipactis* adults present on site [ES], $r = 0.7$; and identity of *Epipactis* seeds sown [EO] and forest type on a site of seed origin [FO], $r = 0.72$). Both FS and ES markedly influenced germination (Table 2). Higher proportions of both germinating and mycorrhizal seedlings were found at sites with poplars or hornbeam–lime forest compared to the conifer forests. Sites with *E. helleborine* or *E. purpurata* populations had more mycorrhizal seedlings (Appendix S5). The few significant interactions between EO and conditions at sowing sites (ES, FS) were generally caused by the fact that the two most successful species (*E. atrorubens* and *E. helleborine*) performed better at sites with the highest germination than was explainable by a linear relationship. Some habitat types (e.g. hornbeam–lime forest) thus provided more suitable conditions for the

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germination of all the *Epipactis* species, irrespective of their occurrence at adulthood.

Fungal diversity in seedlings and adults

Fungal ITS amplification was successful in all adults' roots, large seedlings, and in 23 out of 26 pooled samples of small and medium seedlings, revealing 148 unique ITS sequences after direct sequencing or cloning and 110 presumed species (GB accession numbers GU327385–GU327530, EU363516, EU363517; Appendices S3 and S4). ECM fungi represented at least 38% of all unique sentences (not considering Helotiales with dubious ecology; Vrålstad, 2004). Two possible rhizoctonia sequences belonging to *Ceratobasidium* and Sebacinales clade B, represented only 1% of all unique sequences. In five pooled seedling samples, neither ECM fungi nor rhizoctonia were detected. The other sequences belonged to usual saprobic, endophytic, and/or parasitic taxa (such as *Cladosporium*, *Tetracladium*, *Leptodontidium*, *Mortierella*, or Nectriaceae; Appendices S3 and S4) and were omitted from further analyses.

Irrespective of the forest type, ascomycetes from Tuberaceae and Pyronemataceae were the most frequently detected ECM fungi in adults (except for *E. purpurata*) and in the seedlings of *E. atrorubens* and *E. helleborine* (Table 3). In addition, some *Helvella* and *Hydnotria* were found in *E. helleborine* and *E. purpurata*, respectively. The rare ECM basidiomycetes mostly belonged to Thelephoraceae, Hymenogastraceae, Russulaceae (mainly in *E. purpurata* adults), Sebacinales clade A (sensu Weiss et al. 2004) and Tricholomataceae.

Epipactis atrorubens and *E. helleborine* seedlings showed some differences in mycorrhizal associations between the home and nonhome sites (Table 3). Seedlings of *E. helleborine* associated with similar ECM fungi as those in *E. helleborine* adults at both home and nonhome sites (e.g. *Tuber* spp., *Genea* sp.1). Having the same fungal species among seedlings and co-occurring adults was found only at the home sites (H1, H2 and H3); no identical fungi were detected between *E. helleborine* seedlings and co-occurring adults of other *Epipactis* species at nonhome sites Alb, Atr and P1. Despite presence of the mycorrhizal species *Tuber* sp.1 in adults' roots at site Atr, germination of *E. helleborine* was poor at this site and no ECM fungi were detected in seedlings. The association pattern in *E. atrorubens* is less clear. Although *Tuber* sp.1 and *Wilcoxina* sp.1, presumably mycorrhizal fungi of *E. atrorubens* adults, were found at most sites and in all forest types, they were associated with seedlings only at the unsuitable sites Alb and H2. At the remaining sites, including the home site Atr and the putatively suitable site H3,

Table 3. ECM and rhizoctonia fungal species (ITS grouped at a 97% similarity threshold) detected in adults and seedlings at the six sites (site P2 was omitted from analyses due to its similarity with the site P1, Table 1).

Site	Species	Stage
Alb	*E. albensis*	A (3)
	E. atrorubens	S (2)
	E. helleborine (H2)	S (2)
Atr	*E. atrorubens*	A (4)
	E. atrorubens	S (2)
	E. helleborine (H2)[a]	S (1)
H1	*E. helleborine*	A (3)
	E. atrorubens	S (3)
	E. helleborine (H1)	S (3)
	E. helleborine (H2)	S (1)
H2	*E. helleborine*	A (2)
	E. atrorubens	S (3)
	E. helleborine (H2)	S (2)
	E. helleborine (H3)	S (1)
H3	*E. helleborine*	A (4)
	E. atrorubens	S (2)
	E. helleborine (H2)[a]	S (2)
	E. helleborine (H3)	S (3)
P1	*E. purpurata*	A (2)
	E. atrorubens	S (3)
	E. helleborine (H2)	S (3)
	No. of occurrences	

Column headings (fungal species):
Tuber sp.1 (T), *Tuber* sp.2 (T), *Tuber* sp.3 (T), *Tuber* sp.4 (T), *Tuber* sp.5 (T), *Tuber* sp.6 (T), *Tuber* sp.7 (T), *Tuber* sp.8 (T), *Tuber* sp.9 (T), *Tuber* sp.10 (T), *Genea* sp.1 (Py), "*Genea*" sp.1 (Py), *Geospora* sp.1 (Py), *Geopyxis* sp.1 (Py), "*Geopyxis*" sp.1 (Py), *Trichophaea* sp.1 (Py), *Trichophaea* sp.2 (Py), *Wilcoxina* sp.1 (Py), Pyronemataceae sp.1, *Hydnotrya* sp.1 (Di), *Helvella* sp.1 (He), *Hymenogaster* sp.1 (Hy), *Hymenogaster* sp.3 (Hy), *Inocybe* sp.1 (Co), *Tricholoma* sp.1 (Tr), Thelephoraceae sp.1, Thelephoraceae sp.2, Thelephoraceae sp.3, Thelephoraceae sp.4, Thelephoraceae sp.6, *Russula* sp.1 (Ru), *Russula* sp.2 (Ru), *Certobasidium* sp.1 (Ce), Sebacinales (A) sp.1, Sebacinales (B) sp.1

No. of occurrences (per column): 4, 1, 1, 1, 1, 5, 1, 2, 1, 2, 1, 4, 3, 1, 3, 5, 3, 3, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 2, 1, 1, 1

Notes: Results from cloning or direct sequencing per species were pooled together. Seedlings of *Epipactis helleborine* from different source populations (seeds from H1, H2 and H3 sites) were treated separately. Numeral in parentheses shows the number of analyzed adults or seedling growth categories (small, medium, large; details in Appendix S3). A = adult (black shading), S = seedling (gray shading). T. = Tuberaceae, Py. = Pyronemataceae, Di. = Discinaceae, He. = Helvellaceae, Hy. = Hymenogastraceae, Co. = Cortinariaceae, Tr. = Tricholomataceae, Ru. = Russulaceae, Ce. = Ceratobasidiaceae. Quotation marks indicate unsure affiliation: "*Genea*" sp. 1 belongs to the *Genea-Humaria* clade, "*Geopyxis*"sp. 1 to the *Geopyxis-Stephensia* clade, *Trichophea* to *T. woolhopeia* group, clade 7 in Pyronemataceae (sensu Perry et a.., 2007). Sebacinales clade A and B are defined sensu Weiss et al. (2004).
[a] Non-ECM fungi only.

E. atrorubens seedlings associated predominantly with diverse Pyronemataceae, very often *Trichophaea* species, which we never detected in adults. The only sites where *E. atrorubens* seedlings had fungi in common with adults of local *Epipactis* species were the nonhome sites H2 and H3. In general, fungi presumably suitable for germination (i.e. those associating with adults) were present at each study site, but seedlings did not always associate with them. Thus, the presence of adults and the adults' fungi, as well as habitat suitability turned out to be a poor predictor of the fungal spectrum in seedlings.

When germinating under identical conditions at a given site, *E. atrorubens* and *E. helleborine* seedlings did not associate with significantly different ECM genera (pCCA, $F = 2.46$, $P = 0.07$). At least one ECM species or even one identical ITS sequence was shared by the seedlings of the two species within each site, except for Atr (Table 3, Appendices S3 and S4). According to the separate analysis of seedlings of different size (i.e. small, medium, and large), seedlings of diverse sizes of both *Epipactis* species shared ECM fungi at sites Alb, H1, H3 and P1, and the number of ECM species was also comparable among them (Appendix S3). ECM fungal genera associated with *E. atrorubens* and *E. helleborine* seedlings were significantly different among habitat types (Fig. 5). More similar spectra of fungal genera were found in seedlings growing at the sites with similar tree layer composition, such as hornbeam–lime forests (P1 and H1) or poplar alleys (Alb and H2; Fig. 5).
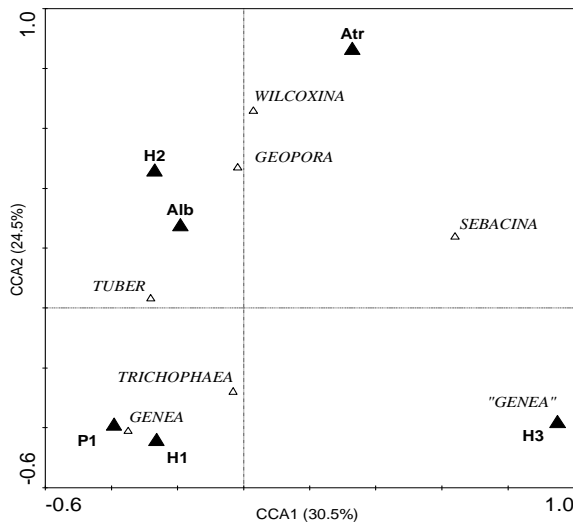
Fig. 5. Partial canonical correspondence analysis (pCCA) ordination plot displaying differences in ECM and rhizoctonia genera detected at six sites (Alb, Atr, H1-3, P1 – see Table 1) in seedlings of *Epipactis atrorubens* and *E. helleborine* (fungal genera from both species were merged); all canonical axes explained 75.8% of total variability; summary of a Monte-Carlo permutation test (999 permutations): $F = 3.13$, $P < 0.003$.

Discussion

Our study examined the influence of different habitat conditions on germination in four ecologically divergent forest-dwelling *Epipactis* species and focused on the ability of seeds to germinate and to associate with mycorrhizal fungi. The germination trials and identification of mycorhizal fungi were conducted in both occupied and vacant habitats, that did not have conspecific adults and that differed in ecological parameters (Fig. 2, Table 1). Comparison of ecological demands of above- and below-ground life stages showed that the ecological niche at germination may be broader than that observed for adults. Such a niche differentiation is often observed for plants (e.g. Eriksson 2011; Vítová and Lepš 2011), but has very rarely been tested for species such as orchids that are strongly dependent on biotic interactions (but see McCormick et al. 2012). Environmental conditions appeared to have little direct impact on seed germination or the presence of ECM fungi involved in symbiosis with *Epipactis* spp., though the particular spectrum of fungi associated with *Epipactis* seedlings differed among the habitats (Fig. 5).

Broad germination potential in Epipactis spp.

Seeds of all four *Epipactis* species – the ecological generalist *E. helleborine* and the three habitat specialists *E. albensis*, *E. atrorubens*, and *E. purpurata* (Fig. 2; Appendix S1) – germinated and reached the stage of small nonmycorrhizal seedlings at all study sites (Fig. 4), regardless of the forest type or soil conditions (Table 1). This stage, preceding establishment of mycorrhizal symbiosis, may be nonspecifically triggered by abiotic factors or diffusive fungal compounds, even from nonmycorrhizal fungi (Vujanovic et al. 2000). The range of conditions initializing germination may be thus broader than those allowing symbiosis establishment (Bruns and Read 2000), potentially leading to nonmycorrhizal seedlings at sites where no further growth could occur. However, no stagnation in the very first stage of germination without further ontogenetic development was observed – with exception of *E. albensis* and *E. purpurata* at some sites (Fig. 4), but in those cases the slow germination at all other study sites prevents any conclusion regarding developmental progress.

Conversely, the presence of mycorrhizal seedlings (stages 3–6, Fig. 3) seems to be a good predictor of population establishment: first, mycorrhizal seedlings typically occur in the vicinity of adult specimens (McKendrick et al. 2002; Diez 2007; Jacquemyn et al. 2007; McCormick et al. 2009); second, their mycorrhizal fungi are very often similar to those in adults (McCormick et al. 2004; Bidartondo and Read 2008), as at most sites in this study. The small mycorrhizal seedlings of the habitat specialists *E. albensis* and *E. purpurata*

were mostly found at sites suitable for adult growth (Fig. 4C; the ecological requirements of *E. albensis* and *E. purpurata* might partly overlap, as supported by their observed co-occurrence in one phytocenological record [Table 1; Fig. 2]). Conversely, the habitat generalist *E. helleborine* and, unexpectedly, the limestone specialist *E. atrorubens* produced stage 5 and stage 6 seedlings in all forest types, including habitats in which *E. atrorubens* adults do not occur. Although developmental abortion could take place at the stage of advanced mycorrhizal seedlings due to the absence of a fungus specifically required for growth beyond the protocorm stage, no developmental bottleneck in terms of fungal associates has been found in *E. atrorubens* yet (the present study; Bidartondo and Read 2008). Our findings support the fact that mycorrhizal seedlings of *E. atrorubens* have the potential to settle in unexpected habitats and exploit fungal carbon sources in local conditions; however, the advanced developmental stages may be limited by environmental factors other than the presence of mycorrhizal fungi itself (see below).

Mycorrhizal associations
Regardless of the forest type and soil conditions, Tuberaceae and Pyronemataceae, pezizalean ECM ascomycetes (Tedersoo et al. 2006), were the most frequently detected ECM fungi in adults of all species, which is consistent with previous studies on forest *Epipactis* species (Bidartondo et al. 2004; Selosse et al. 2004; Bidartondo and Read 2008; Ogura-Tsujita and Yukawa 2008; Ouanphanivanh et al. 2008; Shefferson et al. 2008). ECM basidiomycetes were also found, as in other studies (although no peloton formation by basidiomycete has been confirmed in *Epipactis* to date; Selosse et al. 2004), as well as a range of endophytic and/or parasitic fungi frequently revealed by cloning procedures (e.g. Julou et al. 2005). Moreover, we demonstrated that not only adults, but also seedlings of *E. atrorubens* and *E. helleborine* selectively associate with pezizalean ascomycetes while facing diverse mycorrhizal communities and heterogeneous ecological conditions, e.g. *E. helleborine* seedlings with *Tuber* and *Genea* spp. at both home and nonhome sites (Table 3). Interestingly, the sharing of fungal species was found not only between seedlings and adults within one site (H1, H2, H3), but also between seedlings at nonhome (both putatively suitable and unsuitable) sites and conspecific adults at home sites.

Genera of mycorrhizal fungi associated with seedlings of *E. helleborine* and *E. atrorubens* differed across forest types in which the seeds were cultivated (Fig. 5). Likewise, Ogura-Tsujita and Yukawa (2008) found *E. helleborine* adults growing in pine forests in an exclusive association with

pezizalean *Wilcoxina*, but with *Tuber* and *Hydnotrya* mainly in other forests. However, it is hard to distinguish whether environmental conditions influence distribution and/or availability of mycorrhizal fungi or *Epipactis* fungal selectivity.

When comparing the spectra of fungal associates between habitat specialists and a generalist, one may expect a broader spectrum of fungal associates in *E. helleborine* than in specialist species studied (Rasmussen 2002; Bonnardeaux et al. 2007; Swarts et al. 2010). Our results do not support this trend, since poplar alley-dwelling *E. albensis,* calcicolous *E. atrorubens*, and hornbeam-dwelling *E. purpurata* did not have different or more specific mycorrhizal associations, e.g. all species commonly shared *Wilcoxina* sp.1. *Epipactis purpurata* additionally associated with *Hydnotrya* (that is commonly detected in *E. helleborine* in Japan; Ogura-Tsujita and Yukawa 2008), or *Russula* (which was found in other *E. purpurata* populations; M.-A. Selosse, unpublished data). Although full conclusions on species specificity cannot be drawn due to low sampling effort on adults, the scheme fits into a rather idiosyncratic relationship between orchid rarity and mycorrhizal specialization (Bonnardeaux et al. 2007; Otero et al. 2007; Swarts et al. 2010).

Association with pezizalean ascomycetes occurs only on rare occasions in orchid species investigated so far, and *Epipactis* species show little specificity to any particular fungal genus (Selosse et al. 2004; Bidartondo and Read 2008; Ogura-Tsujita and Yukawa 2008; M.-A. Selosse, unpublished data). ECM pezizaleans colonize various tree species but form only a minor part of the whole ECM community in mature forests (ca. 5% of ECM root tips; Tedersoo et al. 2006). This relative scarcity and unpredictability of occurrence of orchid mycorrhizal ascomycetes sharply contrasts with orchids associated with ECM basidiomycetes, such as *Cephalanthera* associating with Thelephoraceae or Russulaceae, the most frequent and abundant ECM taxa (Tedersoo and Nara 2010). The rarity of pezizalean ascomycetes could have prevented *Epipactis* species from specializing toward a narrower range of mycorrhizal fungi, as a result of the low probability of finding a specific ascomycete species. This relatively low specificity may in turn contribute to finding suitable fungi in a broad range of ecological conditions. Indeed, the occurrence of related fungal taxa in all *Epipactis* species could explain why mycorrhizal communities of sites hosting one *Epipactis* species are able to support germination of congeneric species.

What are the limitations to Epipactis spp. establishment?

The ability of a plant to establish a new population in an unoccupied site is usually limited by two factors: seed dispersal and local habitat conditions (Zobel 1997; Münzbergová and Herben 2005). Long-distance seed dispersal is reported in orchids (Arditti and Ghani 2000), but as a rare event since the most frequent seed arrival occurs around mother plants (Jacquemyn et al. 2007; Jersáková and Malinová 2007). Hence, orchid distribution seems at least in part limited by dispersal, which is reflected by the fact that suitable sites for symbiotic germination are more widespread in landscape than orchid populations themselves (e.g. Bidartondo and Read 2008; Swarts et al. 2010; Phillips et al. 2011; present study). This appears also to be the case of the generalist *E. helleborine*, which reached advanced seedling stages at most putatively suitable sites (Alb, P1, P2). For habitat specialized species, the situation could be further complicated by a requirement for specific habitat conditions. However, the germination pattern observed in habitat-specialized *Epipactis* species suggests low limitation of the early stages by both the environmental conditions and the distribution of mycorrhizal fungi. If a species germinates successfully in seemingly unsuitable habitats, reaching advanced seedling stages, then what prevents its further development? Assuming that ecological conditions remain unchanged from germination to adult plant, they may entail developmental restrictions during a later seedling stage, and thus habitat filtering. For instance, soil pathogens, which could be attracted or enhanced by developing seedlings, may contribute to limited survival, since their impact over time can exclude plant regeneration from a site (Mangan et al. 2010). It is also possible that fungi suitable for initiation of seed germination may not have ability to support further growth due to local conditions (fluctuation in abundance, inadequate physiological status, or insufficient abundance; McCormick et al. 2009, 2012). It should be stressed that orchids undergo a drastic developmental transition, equivalent to the metamorphosis in animals with a larval stage, when they emerge as green shoots above ground. A major change in intrinsic physiology (such as increased transpiration or a switch to partial or full autotrophy) or in nutritional requirements may enhance habitat-dependent mortality: indeed, many organisms with complex life cycles suffer from high mortality at metamorphosis (Wilbur 1980). The impact of this metamorphosis-like transition on orchid populations and its role in limiting occurrence of adult plants has been hitherto overlooked, even in demographic analyses.

In the case of *E. atrorubens*, in spite of its mixotrophic abilities, insufficient light conditions could be limiting for survival of young recruits. In

our phytosociological analysis, this species avoids deeply shaded or nutrient-rich conditions (Fig. 2), where the intensity of plant competition for light can be expected to be much higher than on its preferred sites with dry, nutrient-poor soils of higher pH, and low vegetation cover. Isotopic measurements showed that *E. atrorubens* adults derive only 15 to 62% of carbon from its fungi (Gebauer and Meyer 2003; Bidartondo et al. 2004; Tedersoo et al. 2007), and very rarely produce chlorophyll-free shoots compared to other *Epipactis* species (Jakubska and Schmidt 2005, and references herein). Consequently, *E. atrorubens* may be less efficiently mycoheterotrophic than its congeners and thus could require higher irradiation to cover its nutritional demands.

Implications for conservation

The orchid family is one of the largest in the plant kingdom (ca. 25 000 species), but many of its species are threatened or endangered owing to habitat loss (Dixon et al. 2003). Indeed, three of the investigated species are listed as endangered on the Red list of the Czech flora (Holub and Procházka 2000). Habitats of the calcicolous *E. atrorubens* are often endangered by limestone mining, while other *Epipactis* species suffer from the transformation of natural broadleaf forests to spruce monocultures. For relatively fast-developing *E. atrorubens*, a feasible compensation for a destroyed habitat or the restoration of abandoned mining sites could lie in the introduction of seeds from local populations or from those condemned to destruction. However, in all three species and especially in the very slow-growing *E. albensis* and *E. purpurata*, strict conservation of forest habitats and vital populations is required, because seed establishment seems to have very low success. Based on the relatively common occurrence of suitable ectomycorrhizal fungi in the landscape, demonstrated also by our study, it is likely that there are more suitable sites for *Epipactis* than are actually occupied. Therefore, when preserving some of these species in regional flora is a conservation concern, sowing seeds of local origin in putatively suitable sites represents a possible option that should be considered. These conclusions, however, should not be transferred to conservation efforts regarding orchids associated with saprophytic rhizoctonias, for which germination success was shown to depend not only on the presence, but mainly on abundance of mycorrhizal fungi (McCormick et al. 2012).

Conclusions

Although early germination stages involved in our study present only first developmental step of these long-lived slow-growing orchids, understanding their ecological requirements sheds more light on the ecological needs of the

species. The in situ sowing of seeds in different forest habitats and soil conditions showed a broad germination tolerance to abiotic conditions even in species that are ecologically specialized at maturity; however, environmental conditions or other biotic interactions may have the strongest influence during the transition to aboveground phase, a shift which is technically difficult to study because of the very slow development and somewhat unpredictable growth of these orchid species. Low impact of substrate characteristics directly on plant growth but higher influence on suitable mycorrhizal fungus presence and abundance (McCormick et al. 2012) is probably a more general rule for the orchid family, as seen also for epiphytic species (Otero et al. 2007; Gowland et al. 2011). Our results suggest that this rule also fits ECM-associated mixotrophic orchids; although *Epipactis* germination was not limited by fungal presence in different forest habitats, the fungal spectra in seedlings were influenced by forest type. Other parameters, such as germination at sites devoid of adult congeners, and especially survival during the metamorphosis-like transition from mycoheterotrophic seedlings to green aerial plant, deserve further investigation.

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Supporting information

Appendix S1. Ellenberg's indicator values for *Epipactis* spp. as indicated in Ellenberg et al. (1992) and median values calculated from phytosociological relevés from the Czech Phytosociological Database (Chytrý and Rafajová, 2003) separated by a slash. *Epipactis albensis* was not characterized by Ellenberg. Values on the ordinal scale range from 1-12 for moisture and 1-9 for other characteristics. Low value indicates species preference for low level of the factor, e.g. light: 1 - deep shade, 9 - full sunlight.

	Light	Temperature	Continentality	Moisture	Soil pH*	Nutrients
E. albensis	-/4.8	-/5.5	-/3.8	-/5.8	-/6.9	-/6.4
E. atrorubens	6/6.5	-/5.5	3/4.1	3/4.1	8/6.9	2/3.4
E. helleborine	3/4.7	5/5.4	3/3.7	5/4.9	7/6.2	5/5.0
E. purpurata	2/4.5	6/5.4	4/3.7	6/5.1	8/6.5	6/5.3

* Soil pH: values on the ordinal scale: 1 - very acidic soils, 9 - very alkaline soils.

Appendix S2. Location of the experimental sites in the Czech Republic.
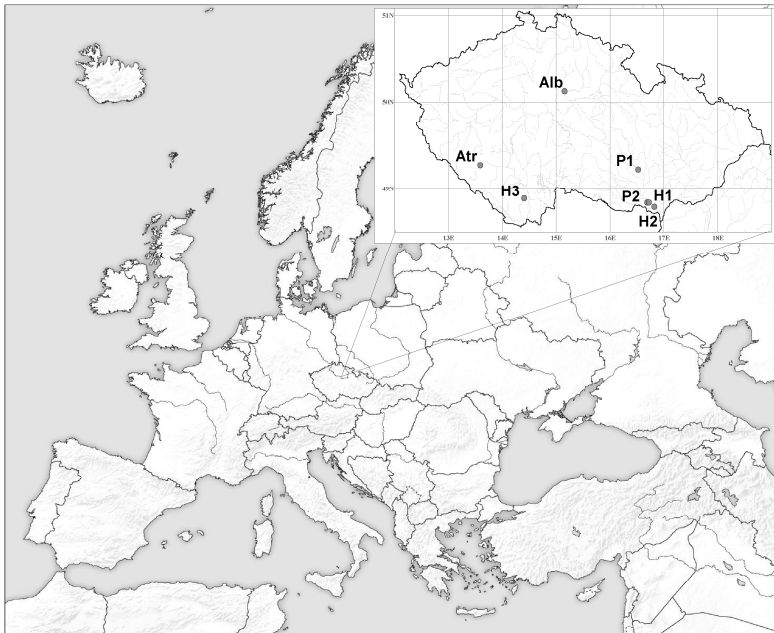
Appendix S3. Fungal species (grouped at a 97% ITS similarity threshold) detected in orchid roots and seedlings at six sites (site P2 was omitted due to its duplicity with the site P1). Presumably non-mycorrhizal fungi are italicized. Mycorrhizal fungi occurring in both seedlings and adults are typed in bold, and those shared by both ontogenetic stages within the same site are also underlined. Grey fields indicate that seedlings of this stage were not available. EAl = *E. albensis*, EAt = *E. atrorubens*, EH1, EH2, EH3 = *E. helleborine* (seeds from H1, H2 and H3 respectively), EP = *E. purpurata*, CD1, CD2 = *Cephalanthera damasonium* (from H1 and H2 respectively). Quotation marks indicate unsure affiliation: *"Genea"* belongs to the *Genea-Humaria* clade, *"Geopyxis"* to the *Geopyxis-Stephensia* clade. Numeral in parenthesis shows number of seedlings in which the fungus was detected, if more than one.

Site	Adults	Fungi in adults	Seedling species	Fungi in different sized seedlings		
				small (~0.5 mm)	medium (~1 mm)	large seedlings
Alb	EAl #1	**Wilcoxina sp.1**, "Geopyxis" sp.1, Thelephoraceae sp.2, Ceratobasidium sp.1, *Penicillium sp.1, Bionectria sp.1, Alternaria sp.2, Debaryomyces sp.1*	EAt	**Tuber sp.1**, Geopora sp.1, *Cladosporium sp.1, Nectriaceae sp.1, Volutella sp.1, Arthopyreniaceae sp.1*	Trichophaea sp.1, *Cladosporium sp.1, Aspergillus sp.1*	
	EAl #2	Tuber sp.9, "Geopyxis" sp.1, Thelephoraceae sp.2 + sp.3, *Penicillium sp.1, Capnodiales sp.1, Malasseziales sp.1, Malassezia sp.1, Dipodascaceae sp.1, Debaryomyces sp.1, Cryptococcus sp.1, Candida sp.1*	EH2	**Tuber sp.1**, Geopora sp.1, *Nectriaceae sp.1, Pleosporaceae sp.1, Plectosphaerella sp.1, Truncatella sp.1, Chaetosphaeriaceae sp.1, Mortierella sp.1+sp.2*	**Tuber sp.1 + sp.8**, *Volutella sp.1, Plectosphaerella sp.1, Leptosphaeria sp.1, Peniophora sp.1, Clitocybe sp.1, Mortierella sp.3, Chytridiomycete sp.2*	
	EAl #3	Tuber sp.3 + sp.9, Ceratobasidium sp.1, *Diaporthales sp.1, Chytridiomycete sp.1*				
Atr	EAt #1	**Wilcoxina sp.1**	EAt	Pyronemataceae sp.1, Geopora sp.1, Agaricomycete sp.1, *Cladosporium sp.1, Nectriaceae sp.3, Eudarluca sp.1, Dipodascaceae sp.1*		Sebacinales (B) sp.1
	EAt #2	**Tuber sp.1**				
	EAt #3	**Wilcoxina sp.1**	EH2	*Cladosporium sp.1, Malasseziales sp.1, Malassezia sp.3, Dipodascaceae sp.1, Trichosporon sp.1*		
	EAt #4	**Wilcoxina sp.1, Tuber sp.1**				
H1	EH1 #1	**Tuber sp.5**	EAt	Trichophaea sp.1 + sp.2, *Tetracladium sp.1*	Trichophaea sp.1 + sp.2	Trichophaea sp.1 (3) + sp.2(3)

	EH1 #2	***Tuber* sp.1 + sp.5**, *Geopyxis* sp.1, *Hymenogaster* sp.1, *Fusarium* sp.1 + sp.2	EH1	*Trichophaea* sp.1	*Penicillium* sp.2, *Nectriaceae* sp.3, *Helotiales* sp.3, *Exophiala* sp.1, *Malasseziales* sp.1, *Malassezia* sp.4, *Tremellales* sp.1, *Dipodascaceae* sp.1, *Agaricostilbomycetidae* sp.1
					Genea sp.1, *Mortierella* sp.7, *Debaryomyces* sp.1
	EH1 #3	**_Genea_ sp.1**, *Hymenogaster* sp.3, *Thelephoraceae* sp.1			
	CD1	*Hymenogaster* sp.2, *Thelephoraceae* sp.2, *Mortierella* sp.5, *Fusarium* sp.2, *Dipodascaceae* sp.2	EH2	*Trichophaea* sp.1 + sp.2, *Tuber* sp.4, *Mortierella* sp.5	(gray)
H2	**EH2 #1**	**_Tuber_ sp.5**	EAt	**_Tuber_ sp.5**, *Mortierella* sp.1+sp.4+sp.6, *Nectriaceae* sp.1	no PCR amplification
					Wilcoxina sp.1, *Tetracladium* sp.1, *Mortierella* sp.1, *Malassezia* sp.2
	EH2 #2	**_Tuber_ sp.8**, *Nectriaceae* sp.1	EH2	*Thelephoraceae* sp.4 + sp.6, *Nectriaceae* sp.3, *Fusarium* sp.2	*Tetracladium* sp.2
	CD2	*Hymenogaster* sp.4, *Thelephoraceae* sp.4 + sp.5 + sp.7, *Tetracladium* sp.2, *Helotiales* sp.4	EH3	**_Tuber_ sp.5**, *Tetracladium* sp.3, *Mortierella* sp.7, *Malassezia* sp.3	not analyzed
					not analyzed
	"_Genea_" sp.1	**"_Genea_" sp.1**	EAt	**"_Genea_" sp.1**, *Trichophaea* sp.1, *Entolomataceae* sp.1, *Dipodascaceae* sp.1, *Itersonilia* sp.1	**"_Genea_" sp.1**, *Tricholoma* sp.1, *Sebacinales* (A) sp.1, *Cladosporium* sp.1+sp.2, *Ascochyta* sp.1, *Dipodascaceae* sp.1
H3	**EH3 #2**	**_Tuber_ sp.5 + sp.7**, *Inocybe* sp.1			
	EH3 #3	**"_Genea_" sp.1**, *Tuber* sp.2 + sp.6, *Helvella* sp.1, *Coniosporium* sp.1	EH3	**"_Genea_" sp.1**, *Russula* sp.1, *Nectriaceae* sp.2	no PCR amplification
					"_Genea_" sp.1(2)
	"_Genea_" sp.1	**"_Genea_" sp.1**	EH2	no PCR amplification	*Nectriaceae* sp.2, *Dipodascaceae* sp.1
P1	**EP #1**	**_Wilcoxina_ sp.1, _Russula_ sp.1**, *Malassezia* sp.2, *Dipodascaceae* sp.2, *Alternaria* sp.1, *Trichocladium* sp.1, *Debaryomyces* sp.1, *Filobasidium* sp.1	EAt	*Trichophaea* sp.2, *Nectriaceae* sp.4	*Trichophaea* sp.2, *Cladosporium* sp.1
					Genea sp.1, *Tuber* sp.10
	EP #2	*Hydnotrya* sp.1, *Russula* sp.2, *Helotiales* sp.1	EH2	*Tetracladium* sp.2, *Nectriaceae* sp.2, *Helotiales* sp.2, *Leptodontidium* sp.1	**_Tuber_ sp.7**, *Nectriaceae* sp.2, *Leptodontidium* sp.1, *Trichoderma* sp.1, *Candida* sp.2
					Genea sp.1, _Tuber_ sp.7(2), *Nectriaceae* sp.2, *Leptodontidium* sp.1

Appendix S4. Fungal ITS amplified from *Epipactis* adults and seedlings from six forest sites and *Cephalanthera damasonium* adults from two sites.

Lineage*	Putative species†	Accession number	Isolation source‡	Origin¶	Closest matches found by BLAST (with BLAST E-value)§	Putative ecology*:
ASCOMYCOTA						
PEZIZOMYCOTINA						
Pezizales						
Tuberaceae	*Tuber* sp.1	GU327385	EAt/Atr(a), EH/H1(a), EAt/Alb(s), EH/Alb(s)	d.s., c.	AY634153 Uncultured ECM (*Tuber*) (0.0) EF362475 *Tuber rufum* (0.0)	ECM
Tuberaceae	*Tuber* sp.2	GU327386	EH/H3(a)	c.	AY634153 Uncultured ECM (*Tuber*) (0.0) EF362475 *Tuber rufum* (0.0)	ECM
Tuberaceae	*Tuber* sp.3	GU327387	EAl/Alb(a)	c.	EF362475 *Tuber rufum* (0.0)	ECM
Tuberaceae	*Tuber* sp.4	GU327388	EH/H1(s)	c.	EF644166 Uncultured ECM (*Tuber*) (3.0e-127) EF362473 *Tuber rufum* (6.0e-124)	ECM
Tuberaceae	*Tuber* sp.5	GU327389	EH/H1(a),EH/H2(a)	c.	EU202708 Uncultured *Tuber* (0.0) DQ011848 *Tuber scruposum* (0.0)	ECM
Tuberaceae	*Tuber* sp.5	GU327390	EAt/H2(s)	c.	AY940165 Uncultured ECM (*Tuber*) (0.0) DQ011847 *Tuber scruposum* (0.0)	ECM
Tuberaceae	*Tuber* sp.5	GU327391	EH/H2(s)	c.	AY940165 Uncultured ECM (*Tuber*) (0.0) DQ011848 *Tuber scruposum* (0.0)	ECM
Tuberaceae	*Tuber* sp.5	GU327392	EH/H3(a)	c.	EF644167 Uncultured ECM (*Tuber*) (0.0) DQ011848 *Tuber scruposum* (0.0)	ECM
Tuberaceae	*Tuber* sp.6	GU327393	EH/H3(a)	c.	EU668241 Uncultured *Tuber* (0.0) DQ011848 *Tuber scruposum* (0.0)	ECM
Tuberaceae	*Tuber* sp.7	GU327394	EH/H3(a)	d.s.	EU668243 Uncultured *Tuber* (0.0) AJ969625 *Tuber puberulum* (0.0)	ECM
Tuberaceae	*Tuber* sp.7	GU327395	EH/P1(s)	c.	AJ969625 *Tuber puberulum* (0.0)	ECM
Tuberaceae	*Tuber* sp.8	GU327396	EH/H2(a)	c.	EU753269 *Tuber maculatum* (0.0)	ECM
Tuberaceae	*Tuber* sp.8	GU327397	EH/Alb(s)	c.	AJ893250 Uncultured ECM (*Tuber*) (0.0) EU753269 *Tuber maculatum* (0.0)	ECM
Tuberaceae	*Tuber* sp.9	GU327398	EAl/Alb(a)	c.	AJ534706 *Tuber* sp. (0.0) AJ969627 *Tuber maculatum* (0.0)	ECM
Tuberaceae	*Tuber* sp.10	GU327399	EAt/P1(s)	d.s.	FJ013059 Uncultured ECM (*Tuber*) (0.0)	ECM

Family	Species	Accession	Type	Collection	BLAST match	
					EU753267 *Tuber borchii* (0.0)	
Pyronemataceae	**Wilcoxina sp.1**	GU327400	EAt/Atr(a), EAt/Alb(a)	d.s.,c.	EU645612 Uncultured ECM (*Wilcoxina*) (0.0) AF266708 *Wilcoxina rehmii* (0.0)	ECM
Pyronemataceae	**Wilcoxina sp.1**	GU327401	EP/P1(a)	c.	EF458013 *Wilcoxina* sp.(0.0) AF266708 *Wilcoxina rehmii* (0.0)	ECM
Pyronemataceae	**Wilcoxina sp.1**	GU327402	EAt/H2(s)	c.	EF458013 *Wilcoxina* sp.(2.0e-174) AF266708 *Wilcoxina rehmii* (4.0e-171)	ECM
Pyronemataceae	**Genea sp.1**	GU327403	EH/H1(a), EA/P1(s), EH/P1(s)	d.s.,c.	DQ206858 *Genea arenaria* (0.0)	ECM
Pyronemataceae	**Genea sp.1**	GU327404	EH/H1(s)	c.	DQ206839 *Genea arenaria* (0.0)	ECM
Pyronemataceae	**"Genea" sp.1**	GU327405	EH/H3(a,s)	d.s., c.	EU668290 Uncultured *Genea* (0.0) EU819470 *Humaria hemisphaerica* (6.0e-103)	ECM
Pyronemataceae	**"Genea" sp.1**	GU327406	EH/H3(a)	c.	EU668290 Uncultured *Genea* (0.0) DQ206851 *Genea gardnerii* (2.0e-84)	ECM
Pyronemataceae	**"Genea" sp.1**	GU327407	EAt/H3(s)	c.	EU668290 Uncultured *Genea* (0.0) DQ206851 *Genea gardnerii* (7.0e-83)	ECM
Pyronemataceae	**"Genea" sp.1**	GU327408	EAt/H3(s)	c.	EU668290 Uncultured *Genea* (0.0) DQ206851 *Genea gardnerii* (2.0e-84)	ECM
Pyronemataceae	**"Genea" sp.1**	GU327409	EH/H3(s)	c.	EU668290 Uncultured *Genea* (0.0) DQ206851 *Genea gardnerii* (7.0e-83)	ECM
Pyronemataceae	**"Genea" sp.1**	GU327410	EH/H3(s)	d.s.	EU668290 Uncultured *Genea* (0.0) DQ206851 *Genea gardnerii* (2.0e-84)	ECM
Pyronemataceae	**Trichophaea sp.1**	GU327411	EAt/H1(s), EH/H1(s), EAt/Alb(s), EAt/H3(s)	d.s.,c.	AY351623 Uncultured ECM (Pyronemataceae) (0.0) DQ200835 *Trichophaea woolhopeia* (0.0)	ECM
Pyronemataceae	**Trichophaea sp.2**	GU327412	EAt/H1(s), EH/H1(s)	d.s.,c.	DQ200835 *Trichophaea woolhopeia* (0.0)	ECM
Pyronemataceae	**Trichophaea sp.2**	GU327413	EAt/H1(s)	c.	DQ200835 *Trichophaea woolhopeia* (0.0)	ECM
Pyronemataceae	**Trichophaea sp.2**	GU327414	EAt/P1(s)	c.	DQ200835 *Trichophaea woolhopeia* (0.0)	ECM
Pyronemataceae	**Geopora sp.1**	GU327415	EAt/Alb(s)	c.	FM206417 *Geopora cervina* (0.0)	ECM
Pyronemataceae	**Geopora sp.1**	GU327416	EH/Alb(s)	c.	FM206417 *Geopora cervina* (0.0)	ECM
Pyronemataceae	**Geopora sp.1**	GU327417	EAt/Atr(s)	c.	FM206417 *Geopora cervina* (0.0)	ECM

Family	Taxon	Accession	Code		Best match (E-value)	Ecology
Pyronemataceae	*Geopyxis* **sp.1**	GU327418	EH/HI(a)	c.	Z96991 *Geopyxis rehmii* (0.0) EU837242 *Stephensia bynumii* (1.0e-142)	ECM
Pyronemataceae	**"***Geopyxis***" sp.1**	GU327419	EAl/Alb(a)	c.	EU837242 *Stephensia bynumii* (8.0e-125) Z96990 *Geopyxis carbonaria* (5.0e-113)	ECM
Pyronemataceae	**Pyronemataceae sp.1**	GU327420	EAt/Atr(s)	c.	EU668289 Uncultured *Geopora* (1.0e-168) EU669387 *Pseudaleuria quinaultiana* (6.0e-165)	ECM
Discinaceae	**Hydnotrya sp.1**	GU327421	EP/P1(a)	c.	AM261522 *Hydnotrya tulasnei* (0.0)	ECM
Helvellaceae	**Helvella sp.1**	GU327422	EH/H3(a)	c.	AF335455 *Helvella elastica* (0.0)	ECM
Capnodiales						
Davidiellaceae	**Cladosporium sp.1**	GU327423	EAt/H3(s)	c.	EU167574 *Cladosporium* sp. (0.0)	S/P
Davidiellaceae	**Cladosporium sp.1**	GU327424	EAt/Alb(s)	c.	EU759978 *Cladosporium sphaerospermum* (0.0)	S/P
Davidiellaceae	**Cladosporium sp.1**	GU327425	EAt/Alb(s)	c.	EU167574 *Cladosporium* sp. (0.0)	S/P
Davidiellaceae	**Cladosporium sp.1**	GU327426	EAt/Atr(s)	c.	EU167574 *Cladosporium* sp. (0.0)	S/P
Davidiellaceae	**Cladosporium sp.1**	GU327427	EAt/P1(s)	c.	EU167574 *Cladosporium* sp. (0.0)	S/P
Davidiellaceae	**Cladosporium sp.1**	GU327428	EH/Atr(s)	c.	EU167574 *Cladosporium* sp. (0.0)	S/P
Davidiellaceae	**Cladosporium sp.2**	GU327429	EAt/H3(s)	c.	EU272532 *Cladosporium cladosporioides* (0.0)	S/P
u.f.	**Capnodiales sp.1**	GU327430	EAl/Alb(a)	c.	FJ553951 Uncultured Mycosphaerellaceae (0.0) AY260092 *Teratosphaeria bellula* (2.0e-170)	S/P
Pleosporales						
Pleosporaceae	**Alternaria sp.1**	GU327431	EP/P1(a)	c.	FJ455502 *Alternaria alternata* (0.0)	P
Pleosporaceae	**Alternaria sp.2**	GU327432	EAl/Alb(a)	c.	FJ266475 *Alternaria conjuncta* (4.0e-158)	P
Pleosporaceae	**Pleosporaceae sp.1**	GU327433	EH/Alb(s)	c.	EU750693 *Pyrenochaeta* sp. (0.0)	S
Venturiaceae	**Eudarluca sp.1**	GU327434	EAt/Atr(s)	c.	AY607011 *Eudarluca caricis* (0.0)	M
Leptosphaeriaceae	**Leptosphaeria sp.1**	GU327435	EH/Alb(s)	c.	AY336132 *Leptosphaeria* sp. (0.0)	P
Dothideomycetes						
Arthopyreniaceae	**Arthopyreniaceae sp.1**	GU327436	EAt/Alb(s)	c.	DQ682563 Arthopyreniaceae sp. (0.0)	S
Hypocreales						
Bionectriaceae	**Bionectria sp.1**	GU327437	EAl/Alb(a)	c.	AB369487 *Bionectria ochroleuca* (0.0)	P

Nectriaceae	**Nectriaceae sp.1**	GU327438	EAt/Alb(s), EH/Alb(s)	c.	AJ875336 *Neonectria radicicola* (0.0)	P
Nectriaceae	**Nectriaceae sp.1**	GU327439	EAt/H2(s)	c.	AB369421 *Cylindrocarpon* sp. (0.0)	P
Nectriaceae	**Nectriaceae sp.1**	GU327440	EH/H2(a)	c.	AB369421 *Cylindrocarpon* sp. (0.0)	P
Nectriaceae	**Nectriaceae sp.2**	GU327441	EH/H3(s)	c.	AJ875330 *Neonectria radicicola* (0.0)	P
Nectriaceae	**Nectriaceae sp.2**	GU327442	EH/P1(s), EH/H3(s)	c.	AJ875331 *Neonectria radicicola* (0.0)	P
Nectriaceae	**Nectriaceae sp.3**	GU327443	EAt/Atr(s)	c.	DQ317342 *Nectria* sp. (0.0)	P
Nectriaceae	**Nectriaceae sp.3**	GU327444	EH/H1(s)	c.	DQ779785 *Nectria* sp. (0.0)	P
Nectriaceae	**Nectriaceae sp.3**	GU327445	EH/H2(s)	c.	DQ779785 *Nectria* sp. (5.0e-150)	P
Nectriaceae	**Nectriaceae sp.4**	GU327446	EAt/P1(s)	c.	EU754943 Uncultured Nectriaceae (0.0) AJ608955 *Cylindrocarpon magnusianum* (0.0)	P
Nectriaceae	**Volutella sp.1**	GU327447	EAt/Alb(s), EH/Alb(s)	c.	AJ301966 *Volutella ciliata* (0.0)	P
Hypocreaceae	**Trichoderma sp.1**	GU327448	EH/P1(s)	c.	EU280074 *Trichoderma longipile* (0.0)	S
u.f.	**Fusarium sp.1**	GU327449	EH/H1(a)	c.	EF495234 *Fusarium redolens* (0.0)	P
u.f.	**Fusarium sp.2**	GU327450	EH/H1(a)	c.	FJ037744 *Fusarium lateritium* (0.0)	P
u.f.	**Fusarium sp.2**	GU327451	CD/H1(a), EH/H2(s)	c.	FJ233193 *Fusarium oxysporum* (0.0)	P
Chaetosphaeriales						
Chaetosphaeriaceae	**Chaetosphaeriaceae sp.1**	GU327452	EH/Alb(s)	c.	EF488392 *Codinaeopsis* sp. (0.0)	P
Phyllachorales						
Phyllachoraceae	**Plectosphaerella sp.1**	GU327453	EH/Alb(s)	c.	FJ430715 *Plectosphaerella* sp. (0.0)	P
Xylariales						
Amphisphaeriaceae	**Truncatella sp.1**	GU327454	EH/Alb(s)	c.	AF377300 *Truncatella angustata* (0.0)	P
Diaporthales						
Diaporthales	**Diaporthales sp.1**	GU327455	EAl/Alb(a)	c.	EU003012 Uncultured ascomycete (0.0) EF110614 *Harknessia ipereniae* (3.0e-80)	S/P
Leotiomycetes						
Helotiales	**Helotiales sp.1**	GU327456	EP/P1(a)	c.	DQ497975 Uncultured ECM (Helotiales) (0.0) FM180478 Helotiales sp. (0.0)	ECM/P
Helotiales	**Helotiales sp.2**	GU327457	EH/P1(s)	c.	DQ273336 Uncultured Pezizomycotina (0.0) EF029222 *Clathrosphaerina zalewskii* (0.0)	ECM/P

Helotiales	**Helotiales sp.3**	GU327458	EH/H1(s)	c.	EF644169 Uncultured ECM (Helotiales) (0.0) AY706329 *Leohumicola minima* (0.0)	ECM/P
Helotiales	**Helotiales sp.4**	GU327459	CD/H2(a)	c.	DQ182424 Uncultured Helotiales (0.0) U57089 *Cistella grevillei* (0.0)	ECM/P
Eurotiales						
Trichocomaceae	**Penicillium sp.1**	GU327460	EAI/Alb(a)	c.	AY373906 *Penicillium corylophilum* (0.0)	S
Trichocomaceae	**Penicillium sp.2**	GU327461	EH/H1(s)	c.	AY373929 *Penicillium roquefortii* (0.0)	S
Trichocomaceae	**Aspergillus sp.1**	GU327462	EAt/Alb(s)	c.	EF652080 *Aspergillus rubrum* (0.0)	S
Chaetothyriales						
Herpotrichiellaceae	**Exophiala sp.1**	GU327463	EH/H1(s)	c.	AY213652 *Exophiala salmonis* (0.0)	P
Mitosporic Pezizomycotina						
u.f.	**Ascochyta sp.1**	GU327464	EAt/H3(s)	c.	AF520642 *Ascochyta* sp. (0.0)	P
SACCHAROMYCOTINA						
Saccharomycetales						
Dipodascaceae	**Dipodascaceae sp.1**	GU327465	EAI/Alb(a), EAt/Atr(s), EAt/H3(s), EH/Atr(s), EH/H3(s), EH/H1(s)	c.	DQ286062 *Galactomyces* sp. (2.0e-175)	P
Dipodascaceae	**Dipodascaceae sp.2**	GU327466	EP/P1(a)	c.	AY787702 *Geotrichum* sp. (5.0e-143)	P
Dipodascaceae	**Dipodascaceae sp.2**	GU327467	CD/H1(a)	c.	DQ668351 *Galactomyces geotrichum* (4.0e-171)	P
Saccharomycetaceae	**Debaryomyces sp.1**	GU327468	EAI/Alb(a), EP/P1(a), EH/H1(s)	c.	EU569039 *Debaryomyces hansenii* (0.0)	S
Saccharomycetaceae	**Debaryomyces sp.1**	GU327469	EAI/Alb(a)	c.	EF643593 *Debaryomyces hansenii* (0.0)	S
u.f.	**Candida sp.1**	GU327470	EAI/Alb(a)	c.	AM117818 *Candida diddensiae* (0.0)	P
u.f.	**Candida sp.2**	GU327471	EH/P1(s)	c.	DQ269921 *Candida* sp. (2.0e-161)	P
Mitosporic Ascomycota						
u.f.	**Tetracladium sp.1**	GU327472	EAt/H2(s)	c.	EU883431 *Tetracladium breve* (0.0)	P
u.f.	**Tetracladium sp.1**	GU327473	EAt/H1(s)	c.	EU883432 *Tetracladium furcatum* (0.0)	P
u.f.	**Tetracladium sp.2**	EU363517	CD/H2(a)	c.	DQ068996 *Tetracladium maxilliforme* (0.0)	P
u.f.	**Tetracladium sp.2**	EU363516	EH/H2(s)	d.s.	DQ068996 *Tetracladium maxilliforme* (0.0)	P
u.f.	**Tetracladium sp.2**	GU327474	EH/P1(s)	c.	FJ000375 *Tetracladium furcatum* (0.0)	P
u.f.	**Tetracladium sp.3**	GU327475	EH/H2(s)	c.	DQ068996 *Tetracladium maxilliforme* (0.0)	P

u.f.	*Coniosporium* sp.1	GU327476	EH/H3(a)	c.	AJ972792 *Coniosporium* sp. (0.0)	S
u.f.	*Leptodontidium* sp.1	GU327477	EH/P1(s)	c.	AF486133 *Leptodontidium orchidicola* (0.0)	P
u.f.	*Trichocladium* sp.1	GU327478	EP/P1(a)	c.	EU754970 Uncultured *Trichocladium* (0.0) AM292049 *Trichocladium opacum* (0.0)	S/P
BASIDIOMYCOTA AGARICOMYCOTINA Agaricales						
Hymenogastraceae	*Hymenogaster* sp.1	GU327479	EH/H1(a)	c.	AY634136 Uncultured ECM (Hymenogastraceae) (0.0) AF325642 *Hymenogaster olivaceus* (0.0)	ECM
Hymenogastraceae	*Hymenogaster* sp.2	GU327480	CD/H1(a)	c.	AY634136 Uncultured ECM (Hymenogastraceae) (0.0) AF325642 *Hymenogaster olivaceus* (0.0)	ECM
Hymenogastraceae	*Hymenogaster* sp.3	GU327481	EH/H1(a)	c.	AF325636 *Hymenogaster griseus* (0.0)	ECM
Hymenogastraceae	*Hymenogaster* sp.4	GU327482	CD/H2(a)	c.	AY351629 Uncultured ECM (Hymenogastraceae) (0.0) AF325641 *Hymenogaster bulliardii* (0.0)	ECM
Cortinariaceae	*Inocybe* sp.1	GU327483	EH/H3(a)	c.	AM882888 *Inocybe fuscidula* (0.0)	ECM
Tricholomataceae	*Tricholoma* sp.1	GU327484	EAt/H3(s)	c.	DQ822835 Uncultured ECM (*Tricholoma*) (0.0) AF377238 *Tricholoma fracticum* (0.0)	ECM
Tricholomataceae	*Clitocybe* sp.1	GU327485	EH/Alb(s)	c.	EU669216 *Clitocybe subditopoda* (0.0)	S
Entolomataceae	*Entolomataceae* sp.1	GU327486	EAt/H3(s)	c.	DQ974695 *Entoloma* sp. (0.0)	S
Thelephorales						
Thelephoraceae	**Thelephoraceae sp.1**	GU327487	EH/H1(a)	c.	EF644157 Uncultured ECM (*Tomentella*) (0.0) EF644116 *Tomentella* sp. (0.0)	ECM
Thelephoraceae	**Thelephoraceae sp.2**	GU327488	EAl/Alb(a)	c.	EF218826 Uncultured ECM (*Tomentella*) (0.0) U83482 *Tomentella* sp. (0.0)	ECM
Thelephoraceae	**Thelephoraceae sp.2**	GU327489	CD/H1(a)	c.	EF218826 Uncultured ECM (*Tomentella*) (0.0) DQ974780 *Tomentella* sp. (0.0)	ECM

Family	Species	Accession	Sample		Best match	Status
Thelephoraceae	**Thelephoraceae sp.3**	GU327490	EAl/Alb(a)	c.	EU668199 Uncultured *Tomentella* (0.0) U83482 *Tomentella* sp. (0.0)	ECM
Thelephoraceae	**Thelephoraceae sp.4**	GU327491	CD/H2(a), EH/H2(s)	c.	EU668199 Uncultured *Tomentella* (0.0) U83482 *Tomentella* sp. (0.0)	ECM
Thelephoraceae	**Thelephoraceae sp.5**	GU327492	CD/H2(a)	c.	FJ210768 Uncultured ECM (*Tomentella*) (0.0) U83482 *Tomentella* sp. (0.0)	ECM
Thelephoraceae	**Thelephoraceae sp.6**	GU327493	EH/H2(s)	c.	EF655687 Uncultured ECM (*Thelephora*) (0.0) AJ889980 *Thelephora caryophyllea* (0.0)	ECM
Thelephoraceae	**Thelephoraceae sp.7**	GU327494	CD/H2(a)	c.	EU563503 Uncultured ECM (*Pseudotomentella*) (0.0) AF274771 *Pseudotomentella tristis* (0.0)	ECM
Russulales						
Russulaceae	***Russula* sp.1**	GU327495	EP/P1(a)	c.	EF218804 Uncultured ECM (*Russula*) (0.0) EU819428 *Russula nigricans* (0.0)	ECM
Russulaceae	***Russula* sp.1**	GU327496	EH/H3(s)	c.	EF218804 Uncultured ECM (*Russula*) (0.0) EU819428 *Russula nigricans* (0.0)	ECM
Russulaceae	***Russula* sp.2**	GU327497	EP/P1(a)	c.	AY061660 *Russula azurea* (0.0)	ECM
Cantharellales						
Ceratobasidiaceae	***Ceratobasidium* sp.1**	GU327498	EAl/Alb(a)	c.	EU002954 Uncultured *Ceratobasidium* (0.0) EU273525 *Ceratobasidium cornigerum* (0.0)	R R
Sebacinales						
Sebacinaceae	**Sebacinales (A) sp.1**	GU327499	EAt/H3(s)	c.	AM161532 Uncultured ECM (Sebacinaceae) (0.0) AF490393 *Sebacina* aff. *epigaea* (0.0)	R/ECM
Sebacinaceae	**Sebacinales (B) sp.1**	GU327500	EAt/Atr(s)	c.	EF127237 Uncultured Sebacinales (0.0) DQ520096 *Serendipita vermifera* (2.0e-140)	R
Polyporales						
Lachnocladiaceae	***Peniophora* sp.1**	GU327501	EH/Alb(s)	c.	AF210825 *Peniophora aurantiaca* (0.0)	S

Agaricomycetes						
u.f.	**Agaricomycete sp.1**	GU327502	EAt/Atr(s)	c.	U85799 *Athelia pellicularis* (0.0)	?
Filobasidiales						
u.f.	***Cryptococcus* sp.1**	GU327503	EAl/Alb(a)	c.	AF145327 *Cryptococcus kuetzingii* (0.0)	S
Filobasidiaceae	***Filobasidium* sp.1**	GU327504	EP/P1(a)	c.	AF190007 *Filobasidium floriforme* (0.0)	P
Cystofilobasidiales						
u.f.	***Itersonilia* sp.1**	GU327505	EAt/H3(s)	c.	AB072233 *Itersonilia perplexans* (0.0)	S
Tremellales						
u.f.	***Trichosporon* sp.1**	GU327506	EH/Atr(s)	c.	EU559346 *Trichosporon asahii* (0.0)	S
u.f.	***Tremellales* sp.1**	GU327507	EH/H1(s)	c.	AF042453 *Tremella giraffa* (5.0e-127)	P
PUCCINIOMYCOTINA						
Agaricostilbomycetes	**Agaricostilbomycetidae sp.1**	GU327508	EH/H1(s)	c.	AF444519 *Bensingtonia ingoldii* (9.0e-144)	S
USTILAGOMYCOTINA						
Malasseziales						
u.f.	**Malasseziales sp.1**	GU327509	EAl/Alb(a)	c.	EU915323 Uncultured *Malassezia* (0.0) AY743657 *Malassezia sympodialis* (2.0e-145)	P
u.f.	**Malasseziales sp.1**	GU327510	EH/Atr(s)	c.	EU915323 Uncultured *Malassezia* (0.0) AY743657 *Malassezia sympodialis* (1.0e-148)	P
u.f.	**Malasseziales sp.1**	GU327511	EH/H1(s)	c.	EU915323 Uncultured *Malassezia* (0.0) AY743657 *Malassezia sympodialis* (1.0e-148)	P
u.f.	***Malassezia* sp.1**	GU327512	EAl/Alb(a)	c.	AY743636 *Malassezia restricta* (0.0)	P
u.f.	***Malassezia* sp.2**	GU327513	EP/P1(a)	c.	AY743636 *Malassezia restricta* (0.0)	P
u.f.	***Malassezia* sp.2**	GU327514	EAt/H2(s)	c.	AY743636 *Malassezia restricta* (0.0)	P
u.f.	***Malassezia* sp.3**	GU327515	EH/Atr(s)	c.	EU915456 *Malassezia restricta* (0.0)	P
u.f.	***Malassezia* sp.3**	GU327516	EH/H2(s)	c.	EU915456 *Malassezia restricta* (0.0)	P
u.f.	***Malassezia* sp.4**	GU327517	EH/H1(s)	c.	AY743640 *Malassezia sympodialis* (0.0)	P
ZYGOMYCOTA						
MUCOROMYCOTINA						
Mortierellales††						

Mortierellaceae	**Mortierella sp.1**	GU327518	EAt/H2(s)	c.	EU877758 *Mortierella* sp. (0.0)	S
Mortierellaceae	**Mortierella sp.1**	GU327519	EAt/H2(s)	c.	DQ093723 *Mortierella gamsii* (0.0)	S
Mortierellaceae	**Mortierella sp.1**	GU327520	EH/Alb(s)	c.	DQ093723 *Mortierella gamsii* (0.0)	S
Mortierellaceae	**Mortierella sp.2**	GU327521	EH/Alb(s)	c.	AJ890432 *Mortierella* sp. (0.0)	S
Mortierellaceae	**Mortierella sp.3**	GU327522	EH/Alb(s)	c.	EU877758 *Mortierella* sp. (0.0)	S
Mortierellaceae	**Mortierella sp.4**	GU327523	EAt/H2(s)	c.	DQ888725 *Mortierella* sp. (0.0)	S
Mortierellaceae	**Mortierella sp.5**	GU327524	EH/H1(s)	c.	AJ271630 *Mortierella alpina* (0.0)	S
Mortierellaceae	**Mortierella sp.5**	GU327525	CD/H1(a)	c.	AY310443 *Mortierella alpina* (0.0)	S
Mortierellaceae	**Mortierella sp.6**	GU327526	EAt/H2(s)	c.	EU918703 *Mortierella alpina* (0.0)	S
Mortierellaceae	**Mortierella sp.7**	GU327527	EH/H2(s)	c.	EU754996 Uncultured Mortierellaceae (0.0) EU877758 *Mortierella* sp. (0.0)	S
Mortierellaceae	**Mortierella sp.7**	GU327528	EH/H1(s)	d.s.	EU754996 Uncultured Mortierellaceae (0.0) EU877758 *Mortierella* sp. (0.0)	S
CHYTRIDIOMYCOTA						
u.f.	**chytridiomycete sp.1**	GU327529	EAl/Alb(a)	c.	AY999095 *Synchytrium macrosporum* (7.0e–56)	P
u.f.	**chytridiomycete sp.2**	GU327530	EH/Alb(s)	c.	AY997082 *Rhizophydium sphaerotheca* (8.0e–42)	P

* Taxonomic classification; order and family level denoted whenever possible; u.f.: unknown family.

† Putative species assembling >97% similar sequences.

‡ Orchid species / site with developmental stage in parentheses; EAl = *Epipactis albensis*, EAt = *E. atrorubens*, EH = *E. helleborine*, EP = *E. purpurata*, CD = *Cephalanthera damasonium*; s = seedling, a = adult.

¶ Origin of sequences referring to cloning (c.) or direct sequencing (d.s.).

§ Only the closest BLAST informative for taxonomy is denoted. In case the closest match did not belong to a vouchered specimen, the closest sequence from a herbarium specimen or culture is added. The BLAST E-value represents the number of sequence matches expected by random chance (the smaller the value, the better the match between our sample sequence and those in the NCBI database).

** Trophic strategy of the most similar fungal strains (we assume that the strategy is similar for the sequenced species): ECM = ectomycorrhizal; R = rhizoctonia; P = parasite or endophyte; S = saprophytic; M = mycoparasitic.

†† Our BLAST results sometime retrieved a mix of *Verticillium* and *Mortierella* hits, suggesting some taxonomical confusions in GenBank for at least one of these taxa. Analysis of the best taxonomically documented sequences suggested that some *Mortierella* were mislabelled as *Verticillium* – we provisionally considered the dubious sequences as *Mortierella*.

Appendix S5. Overview of Tukey HSD post-hoc comparisons from four-way ANOVA model (Table 2). *$P< 0.05$; **$P< 0.01$; ***$P< 0.001$; n.s. non-significant effect of the predictor in the ANOVA model.

Epipactis **present on site (ES)**	Germinated		Mycorrhizal		Stage	
particular site(s) indicated in brackets	Difference		Difference		Difference	
E. atrorubens (Atr) - *E. albensis* (Alb)	**0.221**	*	-0.040		-0.714	
E. helleborine (H1-3) - *E. albensis* (Alb)	**0.208**	**	**0.093**	**	0.476	
E. purpurata (P1-2) - *E. albensis* (Alb)	0.122		**0.093**	**	0.786	
E. helleborine (H1-3) - *E. atrorubens* (Atr)	-0.014		**0.134**	***	**1.190**	*
E. purpurata (P1-2) - *E. atrorubens* (Atr)	-0.099		**0.134**	***	**1.500**	*
E. purpurata (P1-2) - *E. helleborine* (H1-3)	-0.086		< 0.001		0.310	

Forest type on site (FS)	Germinated		Mycorrhizal		Stage
particular site(s) indicated in brackets	Difference		Difference		Difference
hornbeam-lime (P1, P2, H1) - pine (Atr, H3)	**0.220**	***	**0.103**	***	n.s.
poplar (Alb, H2) - pine (Atr, H3)	**0.277**	***	**0.061**	**	
poplar (Alb, H2) - hornbeam-lime (P1, P2, H1)	0.057		**-0.040**	*	

Epipactis **species sown (EO)**	Germinated		Mycorrhizal		Stage	
	Difference		Difference		Difference	
E. atrorubens - E. albensis	**0.529**	***	**0.203**	***	**2.714**	***
E. helleborine - E. albensis	**0.680**	***	**0.207**	***	**2.095**	***
E. purpurata - E. albensis	-0.072		-0.032		-0.357	
E. helleborine - E. atrorubens	0.151		0.003		-0.619	
E. purpurata - E. atrorubens	**-0.601**	***	**-0.235**	***	**-3.071**	***
E. purpurata - E. helleborine	**-0.752**	***	**-0.239**	***	**-2.452**	***

Forest type on site of seed origin (FO)	Germinated	Mycorrhizal	Stage
particular *Epipactis* populations indicated in brackets†	Difference	Difference	Difference
hornbeam-lime (EP1-2, EH1) – pine (EAt, EH3)	-0.045	n.s.	n.s.
poplar (EAl, EH2) – pine (EAt, EH3)	0.035		
poplar (EAl, EH2) – hornbeam-lime (EP1-2, EH1)	0.080		

† EAl = *E. albensis;* EAt = *E. atrorubens*; EH1, EH2, EH3 = *E. helleborine* (seeds from H1, H2 and H3 sites, respectively); EP1, EP2 = *E. purpurata* (seeds from P1 and P2 sites).

Appendix S6. Germination pattern of *Epipactis* species at seven sites. (A) proportion of germinated seeds, and (B) mycorrhizal seedlings. Median, lower and upper quartile and minimal and maximal values are denoted. N indicates number of packets analyzed. EAl = *E. albensis*, EAt = *E. atrorubens*, EH1-3 = *E. helleborine* (seeds from H1-3 sites), EP1-2 = *E. purpurata* (seeds from P1-2 sites).

A.		*Epipactis* species						
Site		EAl	EAt	EH1	EH2	EH3	EP1	EP2
Alb	median(N)	3(14)	17(14)	7(14)	37(14)	20(14)	0(14)	0(14)
	Q25-Q75	0-4	9-38	2-14	29-47	6-36	0-0	0-0
	min-max	0-7	0-79	0-72	19-84	1-48	0-0.4	0-0
Atr	median(N)	0(13)	58(13)	62(13)	66(13)	79(13)	0.4(13)	0(13)
	Q25-Q75	0-1	42-69	46-75	61-80	67-87	0.3-1	0-0
	min-max	0-2	13-95	3-83	26-90	37-91	0-4	0-0
H1	median(N)	1(13)	78(13)	79(13)	86(13)	90(13)	1(13)	0(13)
	Q25-Q75	0-2	74-83	73-83	79-87	84-93	1-1	0-0
	min-max	0-3	64-86	61-92	51-96	42-95	0-3	0-0.5
H2	median(N)	2(14)	77(14)	86(14)	95(14)	94(13)	0(14)	0(14)
	Q25-Q75	0-9	66-86	72-92	88-97	87-97	0-4	0-0
	min-max	0-74	35-94	4-98	80-100	58-99	0-8	0-1
H3	median(N)	0(12)	0(12)	0(12)	6(12)	3(12)	0(12)	0(12)
	Q25-Q75	0-0	0-5	0-6	3-45	0-9	0-0	0-0
	min-max	0-1	0-17	0-28	0-87	0-30	0-2	0-0
P1	median(N)	0(13)	8(13)	16(12)	32(13)	48(13)	0(13)	0(13)
	Q25-Q75	0-0	3-32	7-29	18-73	16-75	0-1	0-0
	min-max	0-1	2-50	2-52	2-88	0-86	0-3	0-0
P2	median(N)	0(11)	20(9)	61(10)	77(8)	69(10)	1(9)	0(11)
	Q25-Q75	0-2	14-49	46-72	68-90	34-84	0-8	0-2
	min-max	0-10	0-61	8-96	25-93	0-89	0-22	0-3

B.		*Epipactis* species						
Site		EAl	EAt	EH1	EH2	EH3	EP1	EP2
Alb	median(N)	0(14)	1(14)	0.4(14)	1(14)	0(14)	0(14)	0(14)
	Q25-Q75	0-0	0-3	0-1	0-2	0-1	0-0	0-0
	min-max	0-3	0-10	0-7	0-7	0-9	0-0	0-0
Atr	median(N)	0(13)	1(13)	0(13)	0(13)	0(13)	0(13)	0(13)
	Q25-Q75	0-0	1-2	0-1	0-0	0-0.3	0-0	0-0
	min-max	0-0	0-6	0-2	0-2	0-2	0-0	0-0
H1	median(N)	0(13)	34(13)	14(13)	11(13)	16(13)	0(13)	0(13)
	Q25-Q75	0-0	28-46	10-33	6-20	7-34	0-0.4	0-0
	min-max	0-1	5-58	1-56	0.4-47	2-61	0-1	0-0
H2	median(N)	0(14)	2(14)	6(14)	4(14)	9(13)	0(14)	0(14)
	Q25-Q75	0-0	0-3	1-10	2-12	2-13	0-0	0-0
	min-max	0-1	0-19	0.2-32	0-49	0-79	0-0	0-0
H3	median(N)	0(12)	0(12)	0(12)	0.4(12)	0(12)	0(12)	0(12)
	Q25-Q75	0-0	0-0	0-1	0-3	0-1	0-0	0-0
	min-max	0-0	0-2	0-3	0-11	0-3	0-1	0-0
P1	median(N)	0(13)	2(13)	6(12)	3(13)	7(13)	0(13)	0(13)
	Q25-Q75	0-0	0-7	1-11	0.4-8	1-27	0-1	0-0
	min-max	0-0	0-23	0-20	0-16	0-59	0-1	0-0
P2	median(N)	0(11)	3(9)	4(10)	8(8)	3(10)	0(9)	0(11)
	Q25-Q75	0-0	0-4	0-16	2-12	0-10	0-0.3	0-0
	min-max	0-3	0-34	0-17	0-25	0-44	0-2	0-0.3

CHAPTER IV

A diverse fungal community associated with *Pseudorchis albida* (Orchidaceae) roots

Diverse fungal community associated with *Pseudorchis albida* (Orchidaceae) roots

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Abstract

- In addition to orchid mycorrhizal fungi (OrMF), the roots of orchid harbour plant fungal endophytes termed root-associated fungi (RAF).

- In the present study, the endangered photosynthetic orchid *Pseudorchis albida* was screened for OrMF and RAF using culture-dependent (isolation from root sections and pelotons) and culture-independent (cloning from root sections) techniques. The efficiency of the different approaches for detecting the fungi and the effect of the sampling season (summer or autumn) were evaluated.

- In total, 66 distinct OTUs of mycorrhizal and non-mycorrhizal fungi were found, which, to our knoewledge, is the highest diversity of RAF that has yet been detected in a single orchid species. The OrMF community was dominated by *Tulasnella* species, which were mainly detected by isolation from pelotons or cloning from root sections. The roots and tubers showed higher mycorrhizal colonization in summer, corroborating the frequent reports of *Tulasnella* from pelotons in this season. In contrast, two helotialean fungi, *Leohumicola* sp. and *Varicosporium elodeae*, the latter of which was repeatedly isolated from pelotons, were significantly more abundant in the autumn.

Key words

Ceratobasidium, dark septate endophytes (DSE), ecology, fungal diversity, Helotiales, methods, seasonality

Introduction

The Orchidaceae, which contains > 25 000 species, is the world's largest plant family (Jones 2006). Because of the minute size of orchid seeds, which contain few storage nutrients, an association with mycorrhizal fungi is essential for their germination and the development of seedlings (Dearnaley 2007). Orchid mycorrhizas are characterized by the formation of fungal hyphal coils, called pelotons inside an orchid's cortex cells (Smith and Read 2008). While all orchids are fully dependent on their fungal partners during early ontogenetic stages, most species become autotrophic at adulthood and only a few obtain their carbon exclusively (i.e. mycoheterotrophic nutrition) or partially (mixotrophic nutrition) from symbiotic fungi for the rest of their life cycle (Selosse and Roy 2009). Mycorrhizal fungi thus represent one of the most important factors in the evolution and ecology of this diverse plant family.

Orchids form mycorrhizal symbioses with a wide range of basidiomycetes and rarely with ascomycetes. In general, photosynthetic orchids growing in open habitats usually associate with members of the *Rhizoctonia*-like group of fungi (Dearnaley 2007), a polyphyletic complex of predominantly saprotrophic species from the genera *Tulasnella*, *Ceratobasidium* and *Sebacina*, all of which have been isolated and described from cultures. On the other hand, PCR-based studies have shown non-photosynthetic orchids of shady forest habitats interact frequently with ectomycorrhizal fungi (Taylor et al. 2002). It has been suggested that the fully autotrophic orchids associate with a broader spectrum of orchid mycorrhizal fungi (OrMF) than the non-green species (Taylor et al. 2002). However, it seems that the situation is more complex than this, and that autotrophic orchids can also be highly specific in their interaction with OrMF (e.g. McCormick et al. 2004).

Approximately 86 % of vascular plant species host mycorrhizal fungi (Brundrett 2009), but even more species may interact with a miscellaneous group of non-mycorrhizal root-associated fungi (RAF), which are also collectively termed fungal endophytes or dark septate endophytes (DSE). The term RAF refers to symbionts colonizing plant tissues without forming anatomical features typical of mycorrhizas or pathogenic fungi (Addy et al. 2005). Endophytic fungi are a polyphyletic group of diverse species often belonging to orders that also accommodate mycorrhizal fungi, e.g. the Helotiales (Vrålstad et al. 2002a) or Sebacinales (Selosse et al. 2009). Hitherto, we lack key information on how ecological conditions, such as host species, climate or season shape RAF diversity and community composition.

Despite their ubiquitous and cosmopolitan distribution, RAF in orchid roots have rarely been investigated. Moreover, techniques such as isolation or

DNA extraction directly from pelotons fail to detect such RAF. Several studies on terrestrial and epiphytic orchids discovered RAF without phylogenetic affinities to any known OrMF taxa (Bayman et al. 1997; Stark et al. 2009). In temperate terrestrial orchids, the RAF spectra are dominated mainly by helotialean fungi (Stark et al. 2009). The Helotiales comprise a large number of RAF and its various subgroups, such as the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex (PAC; Grünig et al. 2007) or the *Rhizoscyphus ericae* aggregate (REA; Vrålstad et al. 2002b), which are known as common mycobionts in the roots of terrestrial plants from a wide range of habitats (Mandyam and Jumpponen 2005).

Studies on other plant groups have shown that endophytic fungi are able to enhance plant performance (e.g. Jumpponen 2001; Zijlstra et al. 2005). It has also been observed that endophytic fungi gain part of their carbon from host plants (Usuki and Narisawa 2007), and it hence appears that the interaction between endophytic fungi and plants may be beneficial, at least under certain circumstances (Newsham 2011). To better understand the role of RAF in the orchid life cycle, the first critical step is to elucidate their diversity and community assemblage in orchid roots.

Techniques used for studying community assemblages of OrMF as well as RAF have previously been focused on culture-dependent approaches. Two main methods are used for the isolation of mycobionts from orchid roots on agar media: (i) direct isolation from single pelotons (Rasmussen 1990) and (ii) isolation from root sections (Currah et al. 1987). The obvious advantage of the first approach is a higher yield of OrMF, whereas the latter method should detect RAF that do not form pelotons. The major disadvantage of culture-dependent techniques is their poor ability to detect slow-growing or unculturable fungi, which may constitute an important and diverse part of the root fungal community (Vandenkoornhuyse et al. 2002). With the advent of PCR in recent years, the most widespread techniques used for the study of these communities are based on direct DNA extraction from roots and molecular identification of fungi by DNA barcoding (Kristiansen et al. 2001). Studies using culture-independent techniques usually obtain a wider spectrum of RAF (Stark et al. 2009), but data from forensic science suggests amplifiable DNA may still persist on root surfaces even after prolonged exposure to concentrated solutions of peroxide or bleach (Kemp and Smith 2005).

In the present study, we screened OrMF and RAF in the endangered, photosynthetic orchid *Pseudorchis albida* growing in nutrient-poor mountain meadows (Jersáková et al. 2011). We aimed to: (i) compare the efficiency of two culture-dependent techniques (isolation from pelotons (IP) vs. root sections

(IRS)) and a culture-independent technique (direct molecular identification of mycobionts from root sections); (ii) study the effect of sampling season on the occurrence of OrMF and RAF; and (iii) determine and compare the diversity and community assemblages of fungi obtained from pelotons with those in root sections.

Material and methods

Plant collection

Root samples were collected in the Šumava National Park (Czech Republic, Central Europe) from five mountain meadows that were at least 3 km apart: Kvilda (49°0'58.7"N, 13°33'59.9"E; 1100 m a.s.l.); Zhůří (49°4'52.0"N, 13°33'51.3"E; 1170 m a.s.l.); Horní Antýgl (49°2'41.5"N, 13°32'41.8"E; 1100 m a.s.l.); Filipova Huť (49°1'25.2"N, 13°30'34.4"E; 1055 m a.s.l.); and Vchynice (49°2'09.0"N, 13°29'22.7"E; 1010 m a.s.l.). We collected adventitious roots and/or distal parts of the tuber extensions of *P. albida* twice during 2010 (in June, shortly before flowering, and in September, during shoot senescence), from all sites except Vchynice and Filipova Huť at the second sampling. In September, the roots and tubers were already withering and being replaced by new tubers that survive until the next season (Jersáková et al. 2011); both older and new organs were collected from all but one plant, on which only young organs were found. We chose the pre-flowering period because the roots of terrestrial orchids usually posses the highest colonization rates and diversity of OrMF at this time (Huynh et al. 2009), and we selected autumn in which to sample because of the higher abundance of hyphae of DSE in older plant roots (Mandyam and Jumpponen 2008).

At each site, we randomly selected 2–3 plants (except at Filipova Huť, where only one plant was selected) from each of which we collected 2–5 root or tuber pieces (30–90 mm length). In total, 17 plants with 55 root or tuber pieces were sampled (Supporting information Table S1). The roots or tubers with adhering soil were placed in sterile plastic bags and transferred to the laboratory. During transport and prior to processing, samples were kept moist and stored in the dark at 4°C. In the laboratory, they were thoroughly washed with tap water and processed within 3 days of sampling.

Roots and tubers were treated in the same way:

1. Each was divided into a maximum of three segments (5–20 mm length), depending on the original root length.
2. The proportion of mycorrhizal colonization in each segment was evaluated using light microscopy.

3. A smaller part of each segment with mycorrhizal colonization was used for IP.
4. Adjacent remaining parts of mycorrhizal segments as well as non-mycorrhizal segments were surface sterilized in a 100% solution of household bleach (5% chlorine) for 30 s. The segments were then immediately washed three times in autoclaved distilled water and divided into two parts, one of which was used for IRS, and the other of which was frozen at -20°C for cloning.

Culture-dependent screening of endophytic fungi

Pelotons for IP were extracted from transverse sections of mycorrhizal segments using a needle and forceps and transferred with a micropipette into water. Each peloton was washed successively in seven drops of autoclaved water to avoid contamination by RAF and rapidly growing fungi. In total, we plated 15–20 pelotons per mycorrhizal root or tuber (i.e. 6–15 pelotons from each segment, depending on the number of mycorrhizal segments per root) on Petri dishes with an agar medium containing a low level of carbon (see below). Each surface sterilized root segment for IS was cut with a razor into 8–15 pieces (1-mm length), which were then aseptically plated into Petri dishes, again containing a medium with a low level of carbon.

Two different media were used for both IRS and IP: (i) modified Melin Norkrans (MMN) medium with 1 g l^{-1} of glucose and 0.3 g l^{-1} of malt extract; and (ii) E-medium with 1 g l^{-1} of glucose (modified from Caldwell et al. 1991). To suppress bacterial growth, 50 mg l^{-1} of Novobiocin was added to both cultivation media. The dishes were incubated in the dark at 20°C and examined every other day for 9 weeks. Individual fungal colonies were transferred to separate Petri dishes containing MMN or E-medium with regular carbon levels. All obtained isolates were categorized into morphological groups according to colony color, morphology and extension rate. Between three and 15 isolates from each morphological group were used for molecular identification, except isolates with dark mycelia from the PAC-like morphological group, which were all sequenced owing to their morphological similarity.

Molecular identification of the isolates

DNA was extracted using either an Extract-N-Amp™ Plant Kit (Sigma–Aldrich, USA) following the manufacturer's instructions, or by DNeasy Plant Mini extraction kit (Qiagen GmbH, Hilden, Germany). The internal transcribed spacer (ITS) region of nuclear rDNA was amplified with the universal

eukaryotic primer pair ITS1-ITS4 (White et al. 1990). If the amount of DNA obtained after the first PCR was not sufficient for direct sequencing, we used a semi-nested PCR, where the first PCR was performed using the fungal-specific primer ITS1F (Gardes and Bruns 1993) and ITS4. These PCR products were diluted 1:100 with sterile double distilled water (ddH$_2$O) and 2 ml was used as a template in a second round of amplification with ITS1-ITS4. Although we were able to amplify the DNA of almost all isolates with these primers, they failed to amplify the DNA of a group of fungi with white colonies. We therefore amplified these isolates with the primer pair ITS1-ITS4Tul (Taylor and McCormick 2008). The PCR mix and thermal cycling parameters were as used by Kohout et al. (2011). The length, quality and quantity of the PCR products were checked by gel electrophoresis (1 % agarose). Each sample was sequenced separately with the primer ITS1 by Macrogen Inc. (Seoul, Korea).

Culture-independent screening

DNA from surface sterilized root parts assigned to direct molecular analyses was extracted using DNeasy Plant Mini extraction kits (Qiagen). The segments from the same roots or tubers were extracted together, resulting in 2–3 samples of extracted DNA per plant. Extracted DNA was diluted 1:10 with autoclaved ddH$_2$O and used as a template for semi-nested PCR reactions, as described above. Three independent PCR reactions were run for each sample to avoid PCR bias. All positive PCR products per individual plant were pooled together and were cloned with the TOPO-TA cloning kit (Invitrogen). Thirty randomly selected positive clones per cloning were re-amplified with the primer pair ITS1-ITS4 and were sequenced with ITS1 at Macrogen. To check for the presence of *Tulasnella* species, the DNA of which is poorly amplifiable with ITS1F-ITS4 (Taylor and McCormick 2008), each extracted sample was amplified with ITS1-ITS4Tul and was directly sequenced with ITS1, or was cloned as described above. Only two plants collected in June from the sites at Kvilda, Zhůří and Horní Antýgl were used for direct cloning with ITS1F-ITS4, but all plants from this sampling were subjected to direct amplification with ITS1-ITS4Tul.

Sequence and phylogenetic analyses

All high quality fungal ITS sequences were edited using FinchTV 1.4.0. (Geospiza Inc.) and were used for taxonomic identification and delimitation of operational taxonomic units (OTUs) based on 97 % similarity for Basidiomycota and 99 % for Ascomycota using TOPALi 2 (http://www.topali.org/). A different threshold for Ascomycota was used

because the vast majority of endophytic ascomycetes belong to the order Helotiales, for which a 99 % threshold has been shown to be more suitable (Hambleton and Sigler 2005; Grünig et al. 2009). Primary identification of all OTUs was achieved by conducting a BLASTn search in the GenBank and PlutoF (Abarenkov et al. 2010) sequence databases. Representative sequences from each OTU were deposited in the International Nucleotide Sequence Database (INSD; http://www.insdc.org/) under accession numbers JN655560-JN655663.

Helotialean sequences (>95 % similarity to known Helotiales) were subjected to phylogenetic analyses for improved taxonomic discrimination. Sequences with lower similarity were not used because the whole ITS region is not suitable for deep phylogenetic analyses of the Helotiales (Wang et al. 2006). Forty-five helotialean ITS sequences were automatically aligned with database sequences (retrieved from INSD) using MAFFT 6.6 (http://mafft.cbrc.jp/alignment/server/index.html). Because of the great diversity of *Acephala*/*Phialocephala* species, the sequences were aligned separately. This resulted in an alignment of 20 ITS sequences from our study with related sequences from public databases.

Phylogenetic trees were primarily obtained by neighbourjoining analyses in MEGA 5 (Tamura et al. 2011) employing the LogDet (Tamura-Kumar) model using all sites in the alignments. Posterior probabilities were estimated with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the same model (parameters: lset nst = 6, rates = invgamma). An MCMC analysis was performed, initiated with random starting trees, and was run for 10 000 000 generations. Every 100th generation was sampled, and the first 10 000 trees were discarded as burn-in.

Statistical analyses

We used a linear mixed-effect model to test for the effect of sampling season (summer/autumn) on the number of isolates of each OTU obtained per plant. Sites were considered as a random factor. The number of isolates per plant was log-transformed prior to the analysis. Rare species (present in <3 plants) were excluded from the analysis. We used a generalized linear mixed effect model (GLMM) to test for the effect of sampling season on the proportion of mycorrhizal colonization per root or tuber, with sites and plant individuals nested within sites as random factors. Data from Filipova Huť and Vchynice were excluded from the analysis because of the missing autumn replicates. The analyses were performed in R, v. 2.12 (R Development Core Team 2010),

package nlme, v. 3.1 (Pinheiro et al. 2011) and the lme4 package (Bates et al. 2011).

To study the differences in species richness between plant individuals, sampling season and culture-dependent and -independent methods, we calculated rarefaction curves using EstimateS 8.20 (Colwell 2006). The calculations were based on the number of isolates of each OTU obtained from the culture-dependent method (with data from IP and IRS being pooled) and on the number of sequences of each OTU obtained from the culture-independent method. Chao estimates were computed in R, to assess the expected number of OTUs from rarefaction curves.

Results

From the 17 adult plants of *P. albida* collected at two samplings using two culture-dependent techniques, we isolated OrMF and RAF from ca. 1500 surface sterilized root sections and 545 pelotons. In total, 440 fungal isolates were obtained from the root sections and 76 from the pelotons. There were no differences in isolation efficiency between the two cultivation media. From six plants the culture-independent technique generated 155 fungal sequences. Overall, we detected 66 OTUs of putative OrMF and RAF, whose best BLASTn matches and putative ecology are provided in Table 1.

Isolations from pelotons

Mycorrhizal colonization of the underground organs was significantly higher in summer than in autumn (GLMM; Chi Sq = 12.76, df = 1; P < 0.001; Table S1), resulting in all 10 plants in summer, but only three in autumn, being used for IP. In total, we used 465 and 80 pelotons from the summer and autumn samplings, which resulted in 36 and 40 fungal isolates, respectively, belonging to eight OTUs (Table 1). Sequencing of the ITS rDNA regions showed that 94 % of the summer peloton isolates matched three *Tulasnella* species (OTUs 62, 64 and 65). In addition, a species of *Mycosphaerella* (OTU 52) and a member of the Orbiliomycetes (OTU 60) were isolated (Fig. 1), but there was only one isolate of each species. IP during autumn resulted in a quite different fungal community assemblage. *Tulasnella* species were absent and the fungal spectrum was dominated by *Varicosporium* spp. (35 isolates of OTU 39 and three isolates of OTU 34; Fig. 2).

Isolations from root sections

All plant roots were subjected to IRS. Fungal diversity in root sections was much higher than in pelotons (26 v. eight OTUs, respectively) and the species

Table 1. Identification of obtained OTUs by culture-dependent (isolations from root sections or fungal pelotons) and culture-independent techniques (cloning with ITS1F-ITS4 and amplification with ITS1-ITS4TuI), based on the BLASTn search of the INSD and the massBLASTer in the PlutoF database.

OTU	Det[a]	Species	Order	Putative ecology[b]	Best matches	Similarity (%)	MisM[c]	No. of plants where OTU was detected by different methods		
								Sections	Pelotons	Cloning
1	B+P	*Acephala* sp. 4	Helotiales	RAF	Uncultured Phialocephala FJ378719	98.96	5	4	0	2
					Acephala sp. 4 EU434831	99.56	1			
2	B+P	Acephala sp. 7	Helotiales	RAF	Uncultured fungus AM260913	99.59	2	3	0	1
					Acephala sp.7 HQ713749	100.0	0			
4	B+P	*Acephala* sp. 8	Helotiales	RAF	Acephala sp. W2 5 HQ889709	98.55	6	1	0	0
					Acephala sp.7 HQ713749	97.79	5			
5	B+P	*Acephala* sp. 9	Helotiales	RAF	Acephala sp. W2 5 HQ889709	98.74	5	1	0	1
					Acephala sp.7 HQ713749	97.76	7			
6	B+P	*Acephala* sp. 10	Helotiales	RAF	Acephala sp. W2 5 HQ889709	98.14	8	0	0	1
					Acephala sp.7 HQ713749	97.54	8			
8	B	Helotiales sp. 1	Helotiales	RAF	Mycorrhizal fungal sp. shylmf10 EU880589	99.22	4	1	0	0
					Mollisia melaleuca AY259136	93.49	20			
9	B	Ascomycota sp. 1	Ascomycota sp.	Unknown	Ascomycete sp. olrim19 AY781241	95.55	17	3	0	0
					Mollisia melaleuca AY259136	85.82	33			
10	B+P	Helotiales sp. 2	Helotiales	RAF	Fungal endophyte sp DF38 EU314712	99.65	1	2	0	0
					Trimmatostroma salicis EU019300	95.74	11			
11	B+P	Helotiales sp. 3	Helotiales	Unknown	Uncultured fungus HQ446084	97.08	13	1	0	2
					Trimmatostroma salicis EU019300	96.61	8			
12	B+P	Helotiales sp. 4	Helotiales	Unknown	Uncultured fungus HQ446084	96.75	14	0	0	1
					Trimmatostroma salicis EU019300	95.35	12			

No.		Taxon	Order	Type	Closest GenBank match	%	N			
15	B+P	Helotiales sp. 5	Helotiales	Unknown	uncultured sordariomyceta HQ211578	94.9	18	0	0	1
					Meliniomyces variabilis EF093177	92.64	19			
16	B+P	*Leptodontidium orchidicola*	Asomycota	RAF	Leptodontidium sp. Papoch1 07 HQ731635	100.0	0	1	0	0
					Leptodontidium orchidicola AF486133	99.82	1			
17	B+P	Helotiales sp. 6	Helotiales	Unknown	Uncultured Helotiales GU998700	95.51	14	0	0	1
					Meliniomyces sp. SM7 2 EF093175	93.44	24			
18	B+P	Helotiales sp. 7	Helotiales	Unknown	Uncultured Helotiales GU998700	92.15	25	0	0	1
					Articulospora tetracladia GQ152144	91.22	26			
19	B+P	Helotiales sp. 8	Helotiales	Unknown	Uncultured Helotiales GU998700	94.78	12	0	0	1
					Varicosporium elodeae GQ152148	91.42	20			
20	B+P	Helotiales sp. 9	Helotiales	Unknown	Uncultured Helotiales GU998700	95.38	12	0	0	1
					Gyoerffyella rotula AY729937	91.04	21			
21	B+P	Helotiales sp. 10	Helotiales	Unknown	Uncultured Leotiomycetes HQ211778	97.03	12	1	0	0
					Rhizoscyphus ericae EF658750	93.51	14			
22	B+P	Helotiales sp. 11	Helotiales	RAF	Uncultured Leotiomycetes HQ211778	99.38	3	1	0	0
					Rhizoscyphus ericae EF658750	93.53	15			
23	B+P	Helotiales sp. 12	Helotiales	Unknown	Uncultured Leotiomycetes HQ211778	96.03	12	0	0	1
					Rhizoscyphus ericae AM887700	93.0	29			
26	B+P	Helotiales sp. 13	Helotiales	RAF	uncultured sordariomyceta HQ211728	98.98	4	3	0	0
					Meliniomyces sp. SM7 2 EF093175	96.26	13			
27	B+P	*Meliniomyces variabilis*	Helotiales	ErM/RAF	Meliniomyces variabilis EF093171	99.39	3	0	0	1
28	B+P	Helotiales sp. 14	Helotiales	Unknown	Uncultured sordariomyceta HQ211828	98.23	8	0	0	1
					Meliniomyces variabilis EF093177	93.58	18			
29	B+P	Helotiales sp. 15	Helotiales	Unknown	Uncultured fungus GU174363	97.36	9	1	0	1
					Rhizoscyphus sp. ZK40/08 FR837915	94.95	18			
30	B+P	Helotiales sp. 16	Helotiales	Unknown	Uncultured fungus GU174363	99.19	3	1	0	0

No.	Treatment	OTU / Species	Order	Ecology	Closest BLAST match	Identity (%)	bp			
32	B+P	Helotiales sp. 17	Helotiales	Unknown	Rhizoscyphus sp. ZK40/08 FR837915	95.40	15			
					Uncultured fungus GU174363	97.98	8	0	0	2
33	B+P	Helotiales sp. 18	Helotiales	Unknown	Rhizoscyphus sp. ZK40/08 FR837915	95.42	12			
					Uncultured fungus GU174363	97.98	8	0	0	1
34	B+P	Helotiales sp. 19	Helotiales	Unknown	Melinomyces variabilis AY394902	94.18	19			
					Uncultured Helotiales HQ021839	98.57	6	1	0	1
35	B+P	Helotiales sp. 20	Helotiales	Unknown	Varicosporium elodeae GQ152148	95.93	14			
					Uncultured sordariomyceta HQ211596	95.10	12	0	0	1
36	B+P	Helotiales sp. 21	Helotiales	Unknown	Melinomyces variabilis EF093171	93.40	14			
					Uncultured sordariomyceta HQ211596	95.64	12	0	0	1
38	B+P	Helotiales sp. 22	Helotiales	RAF/Aquatic	Varicosporium elodeae GQ152148	97.43	8			
					Uncultured Helotiales FJ827182	99.40	3	0	0	1
					Articulospora tetracladia FJ000383	96.99	9			
39	B+P	Varicosporium elodeae	Helotiales	RAF/Aquatic	Varicosporium elodeae GQ152148	100.0	0	2	5	3
40	B+P	Varicosporium sp. 1	Helotiales	RAF/Aquatic	Uncultured Gyoerffyella JF519227	98.98	4			
					Varicosporium elodeae GQ152148	98.37	7	0	0	1
42	B+P	Helotiales sp. 23	Helotiales	Unknown	Phialocephala cf fortinii CSP09 AY078131	95.98	10	0	0	1
43	B+P	Phialocephala sp. 1	Helotiales (PAC)	RAF	Phialocephala turiciensis AY078129	98.80	5	0	0	1
44	B+P	Phialocephala sp. 2	Helotiales (PAC)	RAF	Phialocephala subalpina EF446148	100.0	0	0	5	2
45	B	Helotiales sp. 24	Helotiales	Unknown	Uncultured Phialocephala GU998610	95.46	14	0	0	1
					Acephala applanata AY078151	93.33	17			
46	B	Helotiales sp. 25	Helotiales	Unknown	Uncultured Phialocephala HQ211584	95.44	17	0	0	1
					Acephala applanata AY078151	94.49	15			
48	B	Helotiales sp. 26	Helotiales	Unknown	Uncultured Phialocephala GU998610	92.67	21	0	0	1
					Phialocephala cf fortinii CSP08 AY078132	91.54	19			

No.	Code	Taxon	Class/Order	Ecological role	Closest BLAST match	Identity (%)			
49	B	Sordariomycetes sp. 1	Sordariomycetes	Unknown	Uncultured Ascomycota AY394669	94.63	14	0	0	1
					Lecythophora mutabilis HQ157861	92.23	16		0	0
50	B	Oidiodendron maius	Helotiales	ErM/RAF	Oidiodendron maius AF307772	100.0	0	1	0	0
51	B	Helotiales sp. 27	Helotiales	RAF	Uncultured ectomycorrhizal EU529971	99.39	1	1	0	0
					Hyaloscyphaceae sp BDI GU393951	94.79	15		1	0
52	B	Mycosphaerella punctiformis	Dothideomycetes	Pathogenic	Mycosphaerella punctiformis EU343242	99.11	4	0	1	0
54	B	Neonectria radicicola	Hypocreales	Pathogenic	Neonectria radicicola GQ922913	100.0	0	1	0	0
55	B	Menispora ciliata	Sordariomycetes	Saprophytic	Menispora ciliata EU488737	99.38	2	0	0	1
56	B	Sordariomycetes sp. 2	Sordariomycetes	Unknown	Uncultured fungus AM260818	94.64	17	0	0	1
					Sordariomycetes sp 11330 GQ153239	86.03	24			
57	B	Sordariomycetes sp. 3	Sordariomycetes	Unknown	Uncultured fungus AM260818	94.28	19	0	0	1
					Podospora sp GU166446	81.0	85			
59	B	Exophiala oligosperma	Chaetothyriales	Pathogenic	Exophiala oligosperma DQ836797	100.0	0	0	1	0
60	B	Orbiliomycetes sp. 1	Orbiliomycetes	Unknown	Uncultured fungus GU174352	99.64	1	0	1	1
					Dactylaria appendiculata AY265339	92.55	28			
62	B	Tulasnella sp. 1	Tulasnellaceae	OrM	Uncultured Tulasnella mycobiont EU909339	97.03	15	0	3	4
					Tulasnella sp 186 AY373307	93.75	30			
64	B	Tulasnella sp. 2	Tulasnellaceae	OrM	Uncultured Tulasnellaceae HQ852051	99.62	1	3	3	9
					Tulasnella tomaculum AY373296	93.69	8			
65	B	Tulasnella sp. 3	Tulasnellaceae	OrM	Uncultured Tulasnellaceae HQ852051	88.40	60	0	1	1
					Tulasnella tomaculum AY373296	84.68	36			
66	B	Tulasnella sp. 4	Tulasnellaceae	OrM	Tulasnella sp. 128 AY373300	97.88	9	0	0	1
					Tulasnella asymmetrica DQ388047	78.0	56			
68	B	Galactomyces sp. 1	Saccharomycetales	Saprophytic	Galactomyces geotrichum DQ907937	98.59	4	0	0	1
69	B	Galactomyces geotrichum	Saccharomycetales	Saprophytic	Galactomyces geotrichum DQ907937	99.34	1	0	0	1

No.	Analysis**	Species*	Order	Type**	Closest BLAST match	Similarity (%)	MisM***			
70	B	Mortierellales sp. 1	Mortierellales	Unknown	Uncultured fungus AB521986	97.02	11	0	0	1
					Mortierellales sp GF5V1a FN678837	93.61	22	0	0	1
71	B	*Ceratobasidium* sp. 1	Cantharellales	OrM/RAF	Uncultured Ceratobasidiaceae FJ554171	99.65	2	0	0	1
					Ceratobasidium sp AB290022	92.0	40	1	0	0
73	B	Helotiales sp. 28	Helotiales	Unknown	Uncultured fungus FN610892	100.0	0	1	0	0
					Rhizoscyphus ericae UBCtra141 AF149067	91.0	32	1	0	0
74	B	Helotiales sp. 29	Helotiales	Unknown	*Meliniomyces bicolor* EF517302	84.32	37	0	1	0
76	B+P	*Lachnum* sp. 1	Helotiales	RAF	Uncultured *Lachnum* FJ440910	99.39	3	1	0	0
					Lachnum brevipilosum UDB003074	97.58	11			
77	B+P	Helotiales sp. 30	Helotiales	Unknown	*Cudoniella acicularis* DQ202512	94.88	16	0	0	1
78	B+P	Helotiales sp. 31	Helotiales	Unknown	Uncultured Ascomycota HM141054	98.0	10	1	1	0
79	B+P	Helotiales sp. 32	Helotiales	ErM/RAF	*Cistella spicicola* CBS 731.97 GU727553	90.0	41			
					Epacrid root endophyte sp AY279188	100.0	0	1	1	0
80	B+P	Helotiales sp. 33	Helotiales	ErM/RAF	*Cistella spicicola* CBS 731.97 GU727553	94.0	24			
					Mycorrhizal fungal sp shylhs03 EU880595	99.36	3	0	0	1
81	B+P	*Leohumicola* sp. 1	Helotiales	RAF	*Cistella spicicola* CBS 731.97 GU727553	94.0	22			
					Fungal endophyte sp ECD 2008 EU686205	99.19	4	3	0	0
					Leohumicola minima AY706329	98.73	6			
82	B+P	*Cryptosporiopsis ericae*	Helotiales	ErM/RAF	*Cryptosporiopsis ericae* GU934585	100.0	0	2	0	0
83	B+P	*Cryptosporiopsis rhizophila*	Helotiales	ErM/RAF	*Cryptosporiopsis rhizophila* HQ623447	100.0	0	17	0	0

* Species identified using BLAST search (B) or BLAST search and phylogenetic analyses (B+P)

** ErM - ericoid mycorrhizal; OrM - orchid mycorrhizal; RAF – root associated fungi

*** MisM - The number of mismatches over the alignment

111
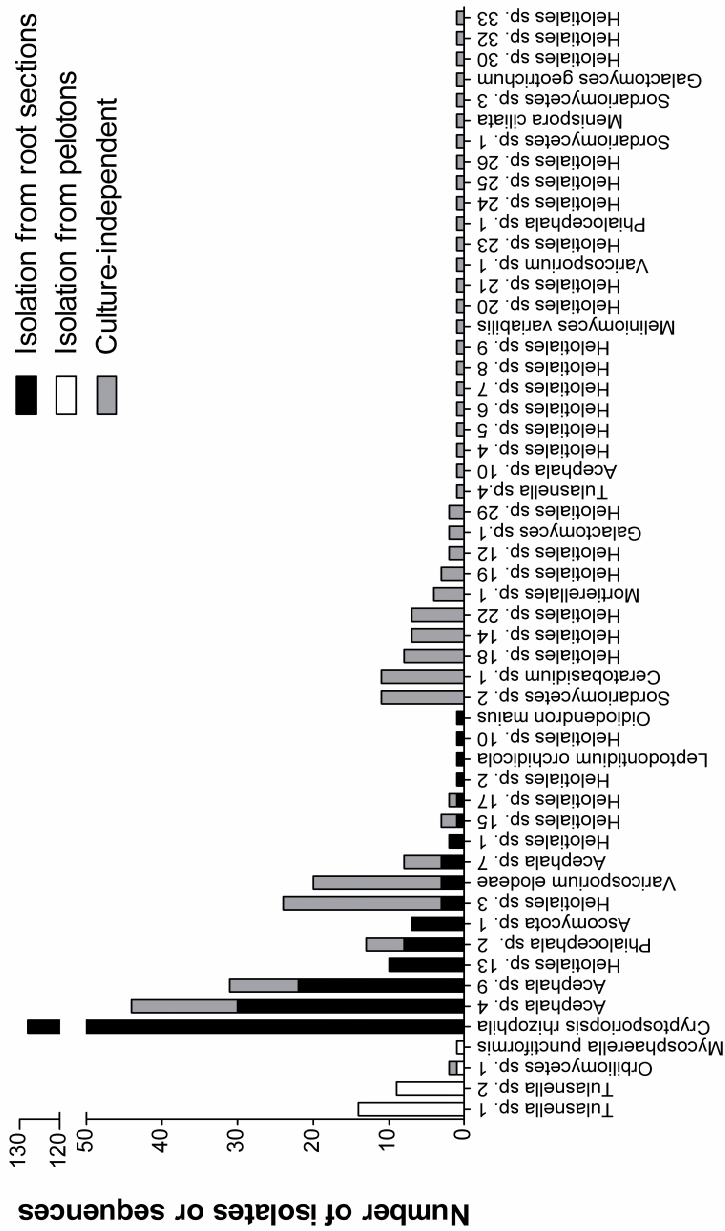
Fig. 1. Identification of obtained OTUs by culture-dependent (isolations from root sections or fungal pelotons) and culture-independent techniques (cloning with ITS1F-ITS4 and amplification with ITS1-ITS4Tul), based on the BLASTn search of the INSD and the massBLASTer in the PlutoF database.
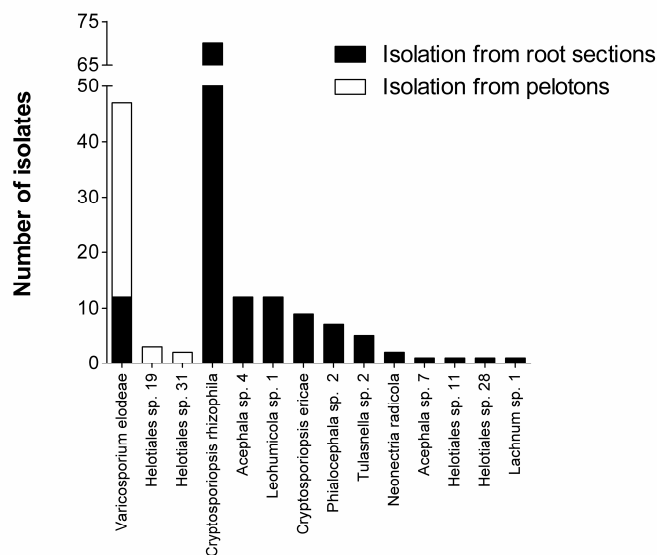
Fig. 2. Occurrence of isolates from root sections and pelotons at the autumn sampling from Kvilda, Horní Antýgl, and Zhůří.

composition in roots was different. We obtained 307 and 133 isolates from the summer and autumn samplings, respectively. Isolates from the summer sampling were classified into 20 OTUs, while 12 OTUs were detected in autumn. Both sampling times were dominated by isolates of *Cryptosporiopsis rhizophila* (OTU 83), the most common fungus in the overall study. The second most frequent isolate was *Acephala* sp. 4 (OTU 1). In general, members of the Helotiales comprised the vast majority (90 %) of all OTUs obtained by IRS, irrespective of the sampling season (Figs 1 and 2). The IRS method proved to be less suitable for the isolation of OrMF than IP, because we obtained only seven isolates of *Tulasnella* sp. 2 (OTU 64); two isolates from one plant collected in summer from Vchynice, and five isolates from two plants collected in autumn at sites Kvilda and Horní Antýgl.

Culture-independent screening

We obtained 155 fungal sequences belonging to 42 OTUs from cloning and direct sequencing. Cloning yielded 67 % of all detected taxa, and, compared with the other techniques, produced the widest spectrum of RAF (Fig. 1). Only 10 OTUs (15 % of all detected OTUs) were found using both the culture-independent and -dependent techniques (Fig. 1). The most obvious difference between the two approaches was the incidence of *C. rhizophila*, which was detected as the most abundant symbiont by IRS, but was absent from all clone

libraries. Similarly, two relatively abundant Helotiales species (OTUs 26 and 9) were exclusively detected by IRS. On the other hand, the culture-independent technique detected 32 OTUs of uncultured fungi.

Tulasnella species were only detected with the specific primers ITS1-ITS4Tul. *Tulasnella* sp. 2 (OTU 64), which was isolated from the pelotons of nine out of 10 plants that were collected in summer, was the most commonly recorded *Tulasnella* species. Direct amplification yielded the same OTUs as IP, with the addition of one rare species (*Tulasnella* sp. 4, Table 2).

Table 2. Occurrence of *Tulasnella* spp. in the roots and tubers of *Pseudorchis albida* recovered by the culture-independent method only (grey fields), or by both the culture-independent and -dependent approaches (black fields).

Site	Plant	*Tulasnella* sp. 1 OTU 62	*Tulasnella* sp. 2 OTU 64	*Tulasnella* sp. 3 OTU 65	*Tulasnella* sp. 4 OTU 66
Kvilda	K1	grey	black		
	K2	black	black		
	K3	black	grey		grey
Zhůří	Z1		black		
	Z2	black	grey		
Horní Antýgl	A1	black	grey		
	A2		grey		
Filipova Huť	F1		grey		
Vchynice	V1			black	
	V2		black		

Comparison of fungal diversity between methods

Based on Chao statistics, the observed number of OTUs was in the range of expected number of species for IP (10 observed OTUs, with 12.7 ± 3.5 expected OTUs), but not for IRS (27 observed OTUs, with 69.7 ± 33.2 expected OTUs). For the culture-independent technique, the expected number of species was 223.5 ± 108.4, which was much more than the 43 OTUs that were observed.
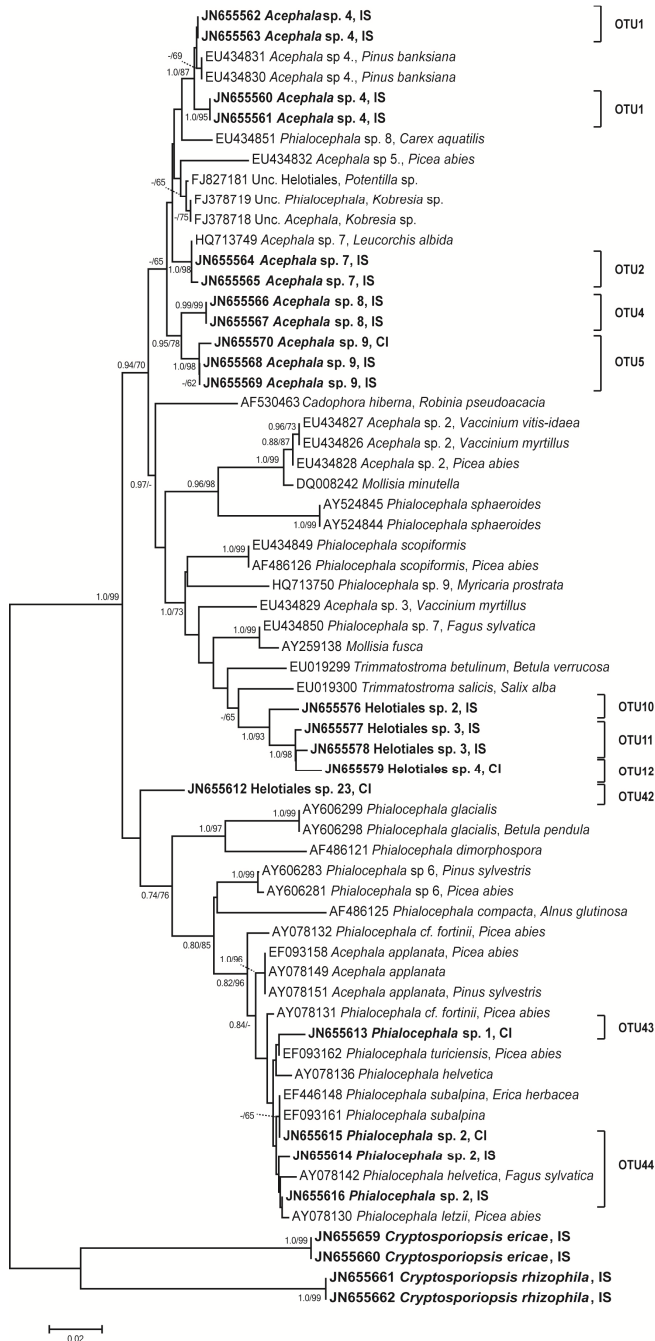
Phylogenetic analyses of Helotiales

Members of the Helotiales were subjected to two different independent phylogenetic analyses. The first focused on the PAC and related species (Fig. 3). The dataset comprised 76 sequences and 519 characters. The second, and much broader, analysis was conducted for all of the Helotiales without the taxa related to PAC (Fig. 4), and comprised 104 sequences and 519 characters.

The phylogenetic analysis of the PAC and related taxa (Fig. 3) revealed some potentially new species (e.g. OTUs 4–6 and 10–12). OTUs 2 and 4–6 were similar to the previously described taxa *Acephala* sp. 7 and *Acephala* sp. 4 (Grünig et al. 2009; Fig 3). Sequences of OTUs 10–12 were, on the other hand, similar to *Acephala* sp. 3 and *Phialocephala* sp. 7 (Grünig et al. 2009; Fig 3). However, the isolates belonging to the PAC were determined only using ITS sequences, which is not a suitable region for delimiting cryptic species

Fig. 3. Phylogenetic tree of a part of the order Helotiales, focused on the *Phialocephala fortinii* s.l.–*Acephala applanata* complex and similar fungal taxa, based on a neighbour-joining analysis of ITS1, 5.8S and partial ITS2 rDNA sequences. The numbers above the branches denote probability from Bayesian analyses/neighbour-joining bootstrap analysis values. The tree was rooted using sequences of *Cryptosporiopsis* spp. Sequences obtained in the present study are shown in bold. They are labelled with the database accession number, name of the fungal taxon and method (CI – culture-independent, IRS – isolation from sections).
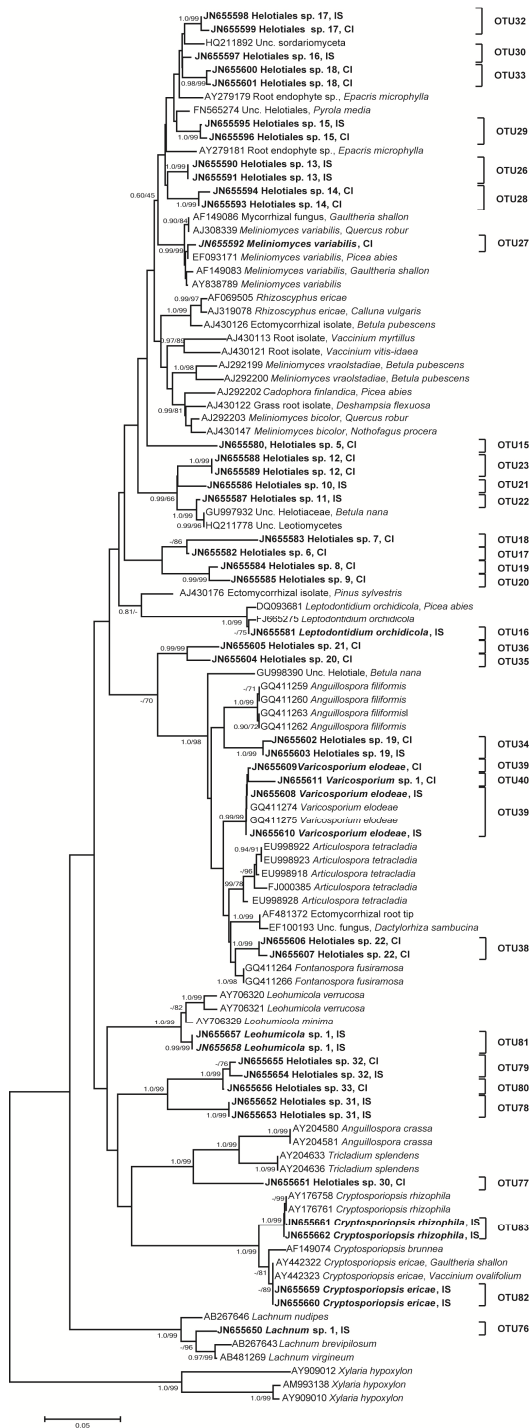
Fig. 4. Phylogenetic tree of a part of the order Helotiales based on a neighbour-joining analysis of ITS1, 5.8S and partial ITS2 rDNA sequences. The numbers above branches denote probability from Bayesian analyses/neighbour-joining bootstrap analysis values. The tree was rooted using a sequence of *Xylaria* spp. Sequences obtained in the present study are shown in bold. Labelling as for Fig. 3.

within the complex (Grünig et al. 2007), and so there could be some undescribed diversity of the PAC species remaining.

Similarly, the phylogenetic analysis of the Helotiales revealed some possibly new fungal taxa. One group of RAF (OTUs 26, 28e30, 32 and 33) matched with members of the REA, in which clustered *Meliniomyces variabilis*, but the supports for this complex were low. The second most diverse clade comprised known aquatic hyphomycetes (e.g. *Varicosporium elodea* and *Articulospora tetracladium*). Some possibly novel taxa, which were similar to an aquatic hyphomycete (OTUs 34 and 38), resided in this clade. Other sequences were placed within the genera *Leptodontidium*, *Leohumicola*, *Tricladium*, *Cryptosporiopsis* and *Lachnum* (Fig. 4).

Seasonal changes in fungal spectra

RAF, as well as OrMF, from both IP and IRS showed pronounced seasonal changes in diversity and community assemblages. In summer and autumn, we isolated 18 and 13 OTUs of RAF, and three and one OTUs of OrMF, respectively. Only six RAF species (OTUs 1, 2, 39, 44, 64 and 83) were shared between the two samplings.

Of the nine most abundant RAF and OrMF, four differed significantly in their abundance between the summer and autumn sampling period (Table 3). *Tulasnella* sp. 1 (OTU 62) and Ascomycota sp. 1 (OTU9) were more frequent in summer than in autumn, whereas *V. elodea* (OTU 39) and *Leohumicola* sp. 1 (OTU 81) were more abundant in the autumn (Table 3).

Table 3. Summary of linear mixed effects models describing differences in OTU distribution between the two sampling times. Likelihood ratios (LRs) and P-values were obtained from comparisons of the model with the null model containing no fixed-effect term. Rare species (in <3 plants) were excluded from the analyses.

Species	Putative ecology[a]	LR (df=4)	p-value	occurrence[b]
Acephala sp. 4 (OTU1)	RAF	1.21	0.271	ns
Acephala sp. 7 (OTU2)	RAF	0.76	0.384	ns
Ascomycota sp. 1 (OTU9)	Unknown	4.15	0.042	A<S
Varicosporium elodeae (OTU39)	RAF/Aquatic	3.84	0.049	A>S
Phialocephala sp. 2 (OTU44)	RAF	0.01	0.985	ns
Tulasnella sp. 1 (OTU62)	OrM	6.27	0.012	A<S
Tulasnella sp. 2 (OTU64)	OrM	0.55	0.459	ns
Leohumicola sp. 1 (OTU81)	RAF	4.18	0.041	A>S
Cryptosporiopsis rhizophila (OTU83)	ErM/RAF	0.01	0.904	Ns

a ErM – ericoid mycorrhizal; OrM – orchid mycorrhizal; RAF – root-associated fungi.
b Ns – non-significant; A > S – higher occurrence in autumn; A < S – higher occurrence in summer.

Across all sites at both samplings, the diversity of the orchid's mycobionts accumulated at more or less similar rates with increasing sampling effort. The species accumulation curves showed non-saturated shapes for the cloning approach and flatter, more saturated shapes for isolations in both summer and autumn (Fig. S2).

Discussion

Although OrMF play a crucial role in orchid ontogenesis and evolution, their diversity within the roots of individual plants is relatively low (Dearnaley 2007), especially when compared to plants with other types of mycorrhizas. In contrast, as for other mycotrophic species (Vandenkoornhuyse et al. 2002), orchids may host a diverse spectrum of RAF (Tao et al. 2008; Stark et al. 2009; Yuan et al. 2009). In the present study, using three different methodological approaches, we describe to our knowledge the highest RAF diversity ever detected in a single orchid species. In total, we recorded 66 distinct mycorrhizal and non-mycorrhizal OTUs.

Comparison of methodological approaches

We found different fungal groups using cultivation from pelotons, cultivation from root sections and direct DNA amplification from roots. The first approach detected isolates similar to *Tulasnella* (three species and 34 isolates), which are known as OrMF (Dearnaley 2007), and species of the genus *Varicosporium*, which is known to encompass aquatic hyphomycetes and endophytic fungi (Sridhar and Barlocher 1992; Goh and Hyde 1996). However, the efficiency of recovery of *Tulasnella* spp. was low, with <7 % of pelotons producing viable isolates of this genus. In orchid roots, both living and dead pelotons were present (Kristiansen et al. 2001; Huynh et al. 2004), and the proportion of living pelotons was highly variable, ranging between 6.6 % and 27.9 %, depending on age of the root or root cortex layer from which the pelotons are isolated (Zhu et al. 2008). In our study, it is possible that loose viable pelotons, which can easily disentangle, were less stable during the washing procedure and were hence less likely to be plated. However, we cannot exclude the possibility that some of the pelotons were formed by fungi that were unculturable on our media or were not amplifiable with the primers that we used.

Tulasnella spp. were even rarer among the isolates from root sections, despite the fact that 50 % of all segments used for IRS were colonized by mycorrhizas (Table S1). Out of a total of 440 isolates from root sections, only seven *Tulasnella* isolates were obtained. Besides the hypotheses mentioned

above, the low number of OrMF isolates obtained might also be due to mycorrhizal fungi being killed by the surface sterilization method. Another scenario, the overgrowing of OrMF by RAF or fast-growing contaminants, is less likely, since many sections did not yield rapidly growing fungi. In general, our results support the current view that isolation from a large number of pelotons, with focus on living pelotons, is the best way to obtain true mycorrhizal fungi from orchid roots (Zhu et al. 2008).

It has been suggested that culture-dependent techniques underestimate total OrMF diversity (e.g. Kristiansen et al. 2001; Porras-Alfaro and Bayman 2007); accordingly, we often isolated fewer *Tulasnella* OTUs per individual compared with culture-independent screening (Table 2). In addition, two rare OrMF (*Tulasnella* sp. 4 and *Ceratobasidium* sp. 1) were completely absent from the isolates. Therefore, studies addressing the diversity and community composition of OrMF should consider using a range of primer sets in the culture-independent approach instead of a combination of isolation and direct sequencing.

The cultivation-based approach is, however, valuable for studies of RAF diversity. Although the Chao statistics show that the culture-independent method revealed higher RAF diversity than cultivation (with 32 unique OTUs derived from the cloning approach), three of the seven most commonly isolated fungal OTUs from the root sections were not detected using cloning, corroborating data from other studies (e.g. Allen et al. 2003; Porras-Alfaro and Bayman 2007). A combination of isolation and cloning thus appears to be the most efficient way to describe communities of RAF in orchid roots.

Temporal dynamics of OrMF and RAF

Sampling season plays an important role in determining orchid OrMF and RAF diversity and abundance. In our study, Tulasnellaceae tended to be more abundant and diverse in summer, while some RAF (*Leohumicola* sp. and *Varicosporium elodeae*) were more abundant in autumn (Figs 1 and 2, Table 3). This fits well with their putative ecology e orchid mycorrhizal Tulasnellaceae take advantage of active nutrient and carbon exchange with fully developed green adult orchids (Cameron et al. 2008), while RAF more efficiently colonize senescing roots. Therefore, studies focussing on OrMF communities should target the pre-flowering stages (Huynh et al. 2009). However, when the whole fungal community associated with orchid roots is investigated, repeated sampling during a season is likely to reveal more diverse fungal spectra than single collections.

Mycorrhizal associations of **P. albida**

While the mycorrhizal specificity of terrestrial orchids may vary, *P. albida* features rather lower specificity, which is comparable to several other photosynthetic orchid species (McCormick et al. 2004). In 17 plants collected from five sites we found three peloton-forming species of *Tulasnella*, corroborating data from Jersáková et al. (2011), who also reported peloton formation by tulasnelloid fungi in *P. albida*. The two rare potentially mycorrhizal species (*Tulasnella* sp. 4 and *Ceratobasidium* sp. 1) were found only by cloning, but their function is unclear. The most common isolate was *Tulasnella* sp. 1 (OTU 62), which matched well with uncultured species from two liverworts, *Aneura pinguis* and *Riccardia multifida*, from Scotland (Bidartondo and Duckett 2010; Krause et al. unpublished), but which had low affinity to any described taxon (max. 93 % similarity to *Tulasnella calospora*). Two similar *Tulasnella* (OTUs 64 and 65) were distantly related to *Tulasnella tomaculum* and their closest matches were the most similar to uncultured species from green orchids from Ecuador (Herrera et al. unpublished). The most commonly isolated member of this genus, *Tulasnella* sp. 1 (OTU 62), was found at only three sites by both isolation and direct amplification (Table 2). This species always co-occurred with *Tulasnella* sp. 2 (OTU 64), which was isolated less frequently, but was more widespread (being detected at three sites by isolation and at all sites by direct amplification). *Tulasnella* sp. 3 (OTU 65) was found only in a single plant.

Community assemblages of RAF

Although mycorrhizal fungal symbionts play a key role in the orchid life cycle, very little is known about their interactions with potentially diverse communities of other RAF. Previous studies focussing on green terrestrial orchids from temperate or Mediterranean regions described non-mycorrhizal endophytic fungal communities dominated mainly by helotialean species, e.g. *Leptodontidium orchidicola*, *Tetracladium* sp. and many uncultured Helotiales taxa (Stark et al. 2009). This corroborates our results and also studies targeting different non-orchid host species within the same geographical region (e.g. Vrålstad et al. 2002a; Tedersoo et al. 2009; Kernaghan and Partiquin 2011). In contrast, non-mycorrhizal RAF communities in tropical orchids are dominated by species of Xylariales (Bayman et al. 1997; Yuan et al. 2009). In contrast with helotialean fungi, Xylariales are common and abundant members of root as well as shoot endophytic communities at lower latitudes (Rogers 2000). In particular, members of the Xylariales interact with roots of *Rhododendron lochiae* (Ericaceae) in an Australian tropical cloud forest (Bougoure and

Cairney 2005) or *Bouteloua gracilis* (Poaceae) in American deserts (Porras-Alfaro et al. 2008). RAF communities may thus be shaped by latitudinal gradients and/or other related environmental variables, such as temperature or soil chemistry.

Ecology of orchid non-mycorrhizal root fungi

The vast majority of RAF species detected in the present study belonged to the Helotiales. Members of this order have a very heterogeneous ecology, acting either as pathogens (Queloz et al. 2011) or mutualistic symbionts (Grelet et al. 2009). They inhabit various environments, including soil and freshwaters (Piercey et al. 2002; Shearer et al. 2007). RAF from *P. albida* with affinity to the Helotiales matched well with fungi known as common endophytes, aquatic hyphomycetes, or ericoid mycorrhizal fungi (Tedersoo et al. 2011).

According to the phylogenetic analyses, the helotialean species recorded in the present study belonged to several lineages throughout the Helotiales. The most diverse and abundant ones were closely related to PAC. Because of the global distribution of PAC species, their possible role in plant physiology is often discussed (Jumpponen 2001; Newsham 2011), but still remains largely unresolved. In the present study, their diversity and species composition was not influenced by the sampling season. PAC-related species were never isolated from pelotons, indicating that they do not form pelotons and do not inhabit mycorrhizal interfaces in the root, supporting current views about these fungi (Addy et al. 2005; Newsham 2011). They are more likely to colonize intercellular spaces or cortical cells without pelotons. Although PAC and their congeneric taxa are generally considered non-mycorrhizal, the recently described *Acephala macrosclerotiorum* acts as an ectomycorrhizal fungus in pine (Munzenberger et al. 2009), suggesting that a true mycorrhizal lifestyle can also occur in this mostly endophytic clade.

The most common species detected in the present study, *C. rhizophila*, was only detected using isolation from root sections. *C. rhizophila* is a well known root endophytic fungus from both terrestrial and aquatic environments (Verkley et al. 2003; Kohout et al. 2012), which was recently isolated from the roots of the Tibetan orchid *Spiranthes sinensis* (Chen et al. 2010). Zijlstra et al. (2005) reported a positive effect of *C. rhizophila* on shoot nitrogen content in *Calluna vulgaris* (Ericaceae) and *Avenella flexuosa* (Poaceae). Therefore, it appears that in addition to forming functional ErM, *C. rhizophila* may also colonize non-ErM plants and potentially influence their nutrition. According to the comparison of methodological approaches reported here, it seems that this

widespread and potentially significant RAF could be overlooked in studies using only direct sequencing with commonly used fungal-specific primers.

The second most diverse lineage of the order Helotiales was the *R. ericae* aggregate (REA) and its related species. Although bootstrap support for the entire REA was low, all taxa recorded matched well with *M. variabilis*. REA is one of the most intriguing groups of root-associated fungi, because it contains fungi forming ericoid mycorrhizal and ectomycorrhizal symbioses (Vrålstad et al. 2000; Vrålstad et al. 2002b) or those acting as root endophytes. Similarly to PAC, REA species have never been isolated from pelotons.

The third large group of Helotiales was related to known aquatic hyphomycetes. These fungi are also known as common root inhabitants (Selosse et al. 2008), but there is no evidence that they are able to form any type of mycorrhizal symbioses. Interestingly, although we isolated *V. elodea* only from root sections during the summer sampling, in the autumn it was the most abundant isolate from pelotons. In contrast, all other non-mycorrhizal RAF (10 OTUs in total) from the autumn sampling were only detected using IRS. It is probable that *V. elodeae* was not acting as an orchid mycorrhizal peloton-forming fungus in the peloton-colonized root sections in autumn, but was instead a secondary colonizer of ageing pelotons. The possibility that *V. elodea* may be able to utilize nutrients available from degrading mycorrhizal tissues requires further investigation.

Interestingly, *Leohumicola minima* was only isolated from the plants collected in the autumn. Originally, this species was described from volcanic ash soil in Chile (Hambleton et al. 2005), and similar fungal species were recently obtained from the roots of *Pyrola* spp. (Hynson and Bruns 2009) and *Epacris microphylla* (Williams et al. unpublished). These observations, together with the work of Hambleton et al. (2005), suggest that some *Leohumicola* species may be able to form ericoid mycorrhiza while others probably possess an endophytic lifestyle (Vrålstad et al. 2002a; this study).

In summary, our study, which used several methodological approaches, demonstrates a diverse community of fungi present in the roots and pelotons of *P. albida*. We demonstrate advantages and drawbacks of each method, which mainly differed in the spectra of fungi obtained and the efficiency of detection. Future research should focus on the function of the often overlooked endophytes in orchid physiology, as well as on the formation of common mycelial networks among coexisting plants.

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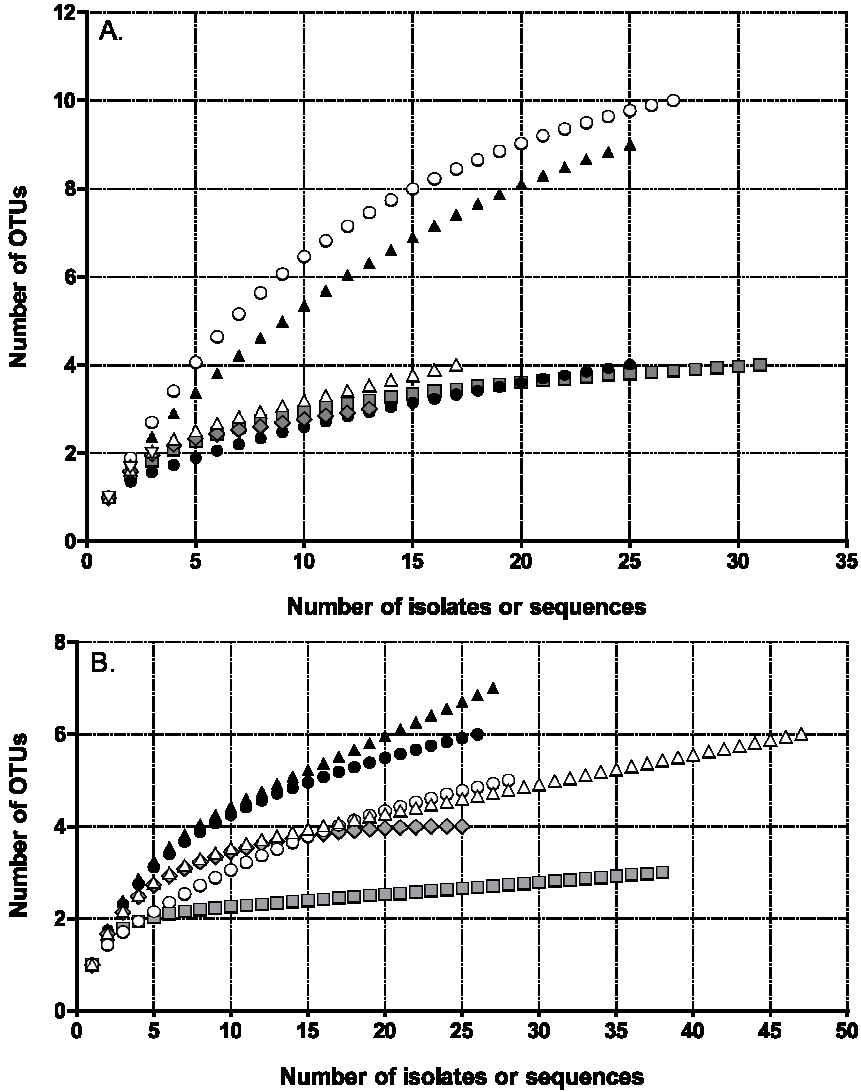
Supporting information

Table S1. Summary of sampling design and proportion of mycorrhizal colonization of *P. albida* roots or tubers; IRS – isolation from root sections, IP – isolation from fungal pelotons.
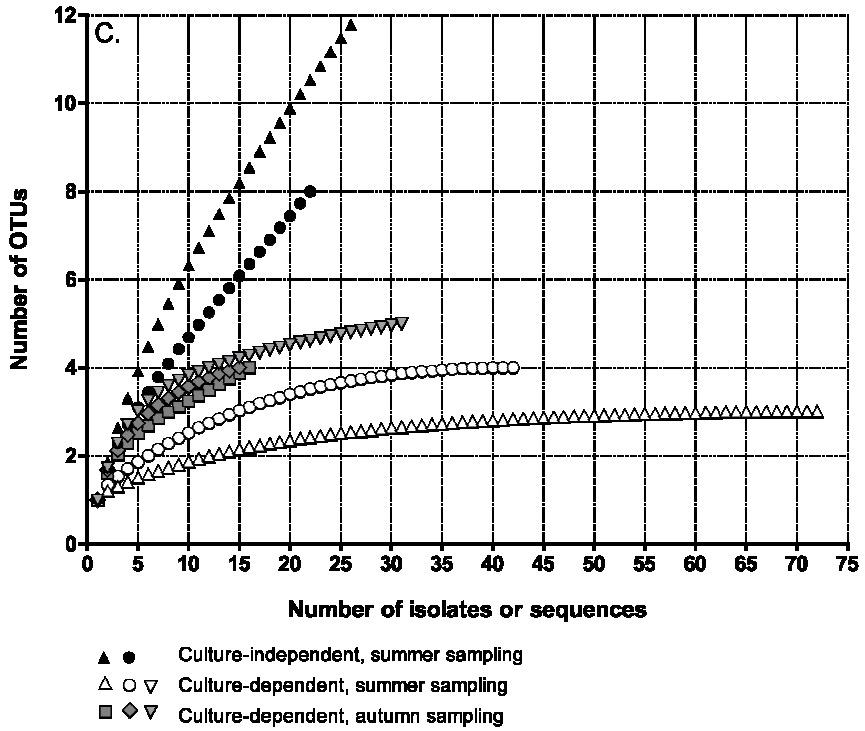
* white – summer sampling, grey – autumn sampling

Site	Plant*	Tuber (T) /root (R)	Mycorrhizal colonization	No. of segments used for IRS	No. of segments used for IP	No. of pelotons used for IP
Kvilda	K1	T1	0	3	0	0
		T2	0.5	3	1	15
	K2	T1	1	3	3	18
		R1	1	3	3	18
		R2	1	3	3	18
	K3	T1	1	3	3	18
		R1	1	2	2	20
		R2	0.5	2	2	20
	K4	T1	0	3	0	0
		R1	0	3	0	0
		R2	0	3	0	0
	K5	T1	0.25	3	1	15
		T2	0	3	0	0
		T3	0	3	0	0
		R1	1	0	1	15
		R2	0	1	0	0
Zhůří	Z1	T1	0.5	3	3	18
		T2	0.75	3	3	18
		R1	1	2	2	20
	Z2	T1	0.5	3	3	18
		T2	1	3	3	18
		R1	1	1	1	15
	Z3	T1	0	3	0	0
		R1	1	2	2	20
	Z4	T1	0	3	0	0
		T2	0.33	2	0	0
	Z5	T1	0	2	0	0
		T2	0	1	0	0
		T3	0	1	0	0
		T4	0	3	0	0
Horní Antýgl	A1	T1	0.5	3	2	20
		R1	0.75	3	3	18
		R2	1	1	1	15
	A2	T1	0	2	0	0
		T2	0	1	0	0
		R1	1	3	3	15
		R2	1	3	3	15
	A3	T1	0.33	3	0	0
		T2	0.33	3	1	15
		R1	0.5	2	1	15
	A4	T1	0	3	0	0
		T2	0	3	0	0
		T3	0	1	0	0
		T4	0	1	0	0
		R1	0	2	0	0
Filipova Huť	F1	T1	0.5	3	2	20
		R1	0.75	1	1	15
		R2	1	1	1	15
Vchynice	V1	T1	0.5	2	1	15
		R1	1	3	3	18
		R2	1	2	2	20
	V2	T1	0	1	0	0
		R1	1	1	1	15
		R2	1	1	1	15
		R3	1	1	1	15

Fig. S2. Species accumulation curves of orchid mycobionts from single plants at the Kvilda (K; Fig. S2A), Horní Antýgl (A; Fig. S2B) and Zhůří (Z; Fig. S2C) sites. Curves were calculated based on the number of isolates obtained from the culture-dependent (we pooled results from IP and IRS) and the number of obtained sequences from culture-independent method (cloning with ITS1F-ITS4). Symbols with the same shape (circle or triangle) indicate the same individual plant analyzed by both approaches.

Number of OTUs

Number of isolates or sequences

▲ ●	Culture-independent, summer sampling
△ ○ ▽	Culture-dependent, summer sampling
■ ◆ ▽	Culture-dependent, autumn sampling

CHAPTER V

**Ploidy-specific symbiotic interactions: divergence of
mycorrhizal fungi between cytotypes of the
Gymnadenia conopsea group (Orchidaceae)**

Těšitelová T, Jersáková J, Roy M, Kubátová B, Těšitel J, Urfus T, Trávníček P,
Suda J (2013)

Ploidy-specific symbiotic interactions: divergence of mycorrhizal fungi between cytotypes of the *Gymnadenia conopsea* group (Orchidaceae)

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Abstract

• Polyploidy is widely recognized as a major mechanism of sympatric speciation in plants, yet little is known about its effects on interactions with other organisms. Mycorrhizal fungi are among the most common plant symbionts and play an important role in plant nutrient supply. It remains to be understood whether mycorrhizal associations of ploidy-variable plants can be ploidy-specific.

• We examined mycorrhizal associations in three cytotypes (2x, 3x, 4x) of the *Gymnadenia conopsea* group (Orchidaceae), involving *G. conopsea* s.str. and *G. densiflora*, at different spatial scales and during different ontogenetic stages. We analyzed (i) adults from mixed- and single-ploidy populations at a regional scale, (ii) closely-spaced adults within a mixed-ploidy site, and (iii) mycorrhizal seedlings.

• All *Gymnadenia* cytotypes associated mainly with saprotrophic Tulasnellaceae (Basidiomycota). Nonetheless, both adults and seedlings of diploids and their autotetraploid derivatives significantly differed in the identity of their mycorrhizal symbionts. Inter-ploidy segregation of mycorrhizal symbionts was most pronounced within a site with closely-spaced adults.

• This study provides the first evidence that polyploidization of a plant species can be associated with a shift in mycorrhizal symbionts. This divergence may contribute to niche partitioning and facilitate establishment and co-existence of different cytotypes.

Introduction

Biological diversity is strongly shaped by multifaceted interactions among different groups of organisms, including those among plants and herbivores, pathogens or mutualistic fungi (van der Heijden et al. 1998; Wardle et al. 2004). Recently, polyploidization, that is the multiplication of the entire chromosome sets above the diploid state, has been recognized as an ubiquitous and dynamic evolutionary driver (Soltis et al. 2009), which can significantly increase intraspecific and/or intrapopulation variation in many plant species and eventually leads to speciation (Soltis and Soltis 1999; Wood et al. 2009; Husband et al. 2013). Two modes of polyploidization are traditionally distinguished; whereas allopolyploids are formed by hybridization and multiplication of chromosome sets originating from two or more different progenitors, their autopolyploid counterparts arise within a species, from the chromosome doubling of the same genome (Soltis and Soltis 1999).

When several cytotypes occur within the same species, zones of ploidy contact are usually formed. Contact zones provide unique opportunities to address the role of different evolutionary mechanisms involved in the establishment and further fate of new polyploid lineages (Petit et al. 1999; Coyne and Orr 2004). The co-existence of different ploidy races may represent a transitional stage, with the rarer ploidy finally outcompeted due to reproductive disadvantages caused by the receipt of unsuitable pollen from the other, more common, cytotype (e.g. the minority cytotype exclusion: Levin 1975). However, there are several evolutionary mechanisms to avoid minority cytotype exclusion and maintain long-term ploidy mixtures, including interploidy phenotypic and phenological divergences, and shifts in ecological niche or pollinator composition (Parisod et al. 2010; Husband et al. 2013).

Despite the growing evidence that polyploidization can notably alter host – plant interactions with both antagonistic and mutualistic insect communities (Segraves and Thompson 1999; Münzbergová 2006; Arvanitis et al. 2010), its effect on symbiotic microorganisms remains to be understood. Mycorrhizal symbioses are formed by > 80% of land plants (Brundrett 2009). In general, photosynthetic plants provide the mycorrhizal fungi with photoassimilates and receive water and mineral nutrients in return. Although mycorrhizal symbioses are generally regarded as rather nonspecific associations, in which a plant individual can associate with multiple fungal

species and vice versa (Selosse et al. 2006), the importance of distinct mycorrhizal communities for resource allocation and nonrandom spatial distribution of co-existing plant species has also been repeatedly documented (van der Heijden et al. 2003; Vandenkoornhuyse et al. 2003; Jacquemyn et al. 2012b; Montesinos-Navarro et al. 2012). Surprisingly, no attention has been paid to potential divergence in mycorrhizal symbionts between different cytotypes of the same species. Recently, Sudová et al. (2010) found ploidy-specific growth response to mycorrhizal colonization in one of the three investigated diploid–polyploid plant species under experimental conditions. These results open the possibility that genome duplication can also modify plant–mycobiont interactions *in situ*.

Due to their obligate dependence on mycorrhizal symbiosis, orchids are an excellent model for addressing interactions between ploidy level of a plant species and mycorrhizal fungi. Orchids produce tiny seeds with insufficient nutrient reserves, and therefore critically require carbon, water and mineral nutrient supply by their mycorrhizal symbionts during germination (Rasmussen and Rasmussen 2009). Most species become autotrophic at maturity, although to a varying degree still depend on fungal nutrition (Cameron et al. 2006; Selosse and Roy 2009). The specificity of orchids to mycorrhizal fungi varies greatly, ranging from loose to highly specific associations, even within a single orchid genus (Shefferson et al. 2007; Jacquemyn et al. 2010). However, at a particular site, orchids usually associate only with a subset of their potential mycorrhizal fungi (Jacquemyn et al. 2012b; Těšitelová et al. 2012). The association is usually stable during ontogenesis, because seedlings mostly associate with similar mycorrhizal fungi to those associated with adult plants of the same species (McKendrick et al. 2002; McCormick et al. 2004; Phillips et al. 2011).

Genome duplication is a common evolutionary event in Orchidaceae, with polyploid species occurring in nearly 30% of orchid genera (Pridgeon et al. 1999). Recent years have also seen a growing number of studies revealing intraspecific and intrapopulation ploidy variation, particularly in temperate orchid species (Ståhlberg 2009; Trávníček et al. 2011, 2012). Although virtually nothing is known about the nutrient demands of diploids vs. polyploids, we can hypothesize that the larger nuclei of polyploid orchids will require higher amounts of phosphorus (P) and nitrogen (N) (assuming a 1 : 1 ratio for purines and pyridines, nucleobases by mass are approximate 39% N and nearly 9% P; Sterner and Elser 2002). On the same conceptual basis, Leitch and Leitch (2012) assumed that available nutrients can influence distribution and abundance of plant species, depending on their genome sizes. In

mycorrhizal species, a significant amount of N and P uptake can be mediated by symbiotic fungi. Because different fungal species and strains can vary dramatically in their nutrient uptake efficiency (van der Heijden et al. 2003), it is possible that different cytotypes will preferentially associate with specific mycorrhizal fungi.

The most salient case of intraspecific and intrapopulation ploidy variation recorded among temperate orchids is the fragrant orchid, the *Gymnadenia conopsea* group. Recent surveys at different spatial scales (Trávníček et al. 2011, 2012) revealed several mixed-ploidy populations, consisting of different combinations of two majority (2x, 4x) and three minority (3x, 5x, 6x) cytotypes. Within a site, individuals of the same ploidy level tend to aggregate together, while the two majority cytotypes mostly show negative spatial association (Trávníček et al. 2011). Fragrant orchids are known to associate with a relatively broad variety of mycorrhizal fungi (Stark et al. 2009; Jacquemyn et al. 2012b). Because fungi are likely to be patchily distributed in soil (Masuhara and Katsuya 1994; McKendrick et al. 2002; Jacquemyn et al. 2012b), a distinct spatial distribution of *Gymnadenia* cytotypes can be caused by interploidy segregation of mycorrhizal fungi leading to aggregation of individuals with similar mycorrhizal symbionts.

In this study, we hypothesize that individual cytotypes of the same plant species associate with different mycorrhizal symbionts. To examine the specificity of mycorrhizal association, we investigated mycorrhizal fungi associated with different cytotypes of the *G. conopsea* group at three spatio-temporal scales: regional – using multiple mixed-ploidy and single-ploidy populations in the broader area of the White Carpathian Mountains in the Czech and the Slovak Republic; microsite – within a mixed-ploidy population harbouring closely spaced adult cytotypes (to control for spatial variation in soil fungi); and in early mycorrhizal seedlings (to control for potential shift in mycorrhizal symbionts during ontogenesis).

Materials and Methods
Study species
In the studied region (the White Carpathian Mountains and surrounding area, Czech and Slovak Republics), the *Gymnadenia conopsea* group encompasses two taxa (following Bateman et al. 2003): *G. conopsea* (L.) R.Br. (= *G. conopsea* s.s.), comprising two majority cytotypes based on x = 20, diploid (2xGc) and tetraploid (4xGc), and *G. densiflora* A. Dietr., comprising only diploids (2xGd) (Fig. 1a). Similarities in scent composition, genetic variation and monoploid genome size between 2xGc and 4xGc point to the autopolyploid
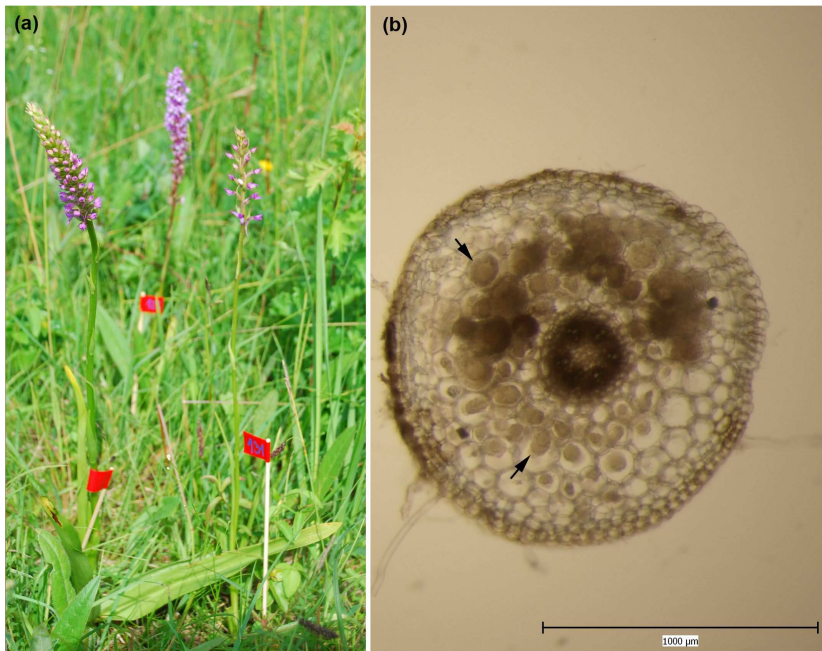
Fig. 1. (a) Three majority cytotypes/taxa growing in close proximity at microsite 14 at Zahrady pod Hájem site. Diploid *Gymnadenia densiflora* (on the left) and 20-cm distant 2x *G. conopsea* (in the background) were associated with operational taxonomic unit (OTU) 1, whereas 10-cm distant 4x *G. conopsea* (on the right) was associated with OTU 2. (b) Root section of diploid *G. conopsea* with intracellular mycorrhizal structures (arrows). Bar, 1000 µm.

origin of the latter cytotype (Jersáková et al. 2010; Trávníček et al. 2012). In addition to the majority cytotypes, three minority (3x, 5x, 6x) cytotypes were also recorded in the studied region, accounting for *ca* 2.7% of all *Gymnadenia* samples (Trávníček et al. 2011). Both the production of unreduced gametes and hybridization among the majority cytotypes/taxa is most likely involved in the origin of minority cytotypes (Trávníček et al. 2012). The most reliable way for the recognition of fragrant orchids turned out to be flow cytometry, which yielded species- and ploidy-specific fluorescence profiles (Trávníček et al. 2012). *Gymnadenia conopsea* s.s. forms mycorrhiza with saprotrophic basidiomycetes from Tulasnellaceae and Ceratobasidiaceae (Stark et al. 2009; Jacquemyn et al. 2012b; Meekers et al. 2012). Several ectomycorrhizal and endophytic basidiomycete and ascomycete taxa have also been recorded (Stark et al. 2009).

Regional sampling
The regional sampling was conducted in June 2008–2012 at 10 sites (Supporting Information Fig. S1), seven of which were surveyed for cytotype

variation by Trávníček et al. (2011) and three were surveyed in the present study (Table 1), using the same flow-cytometric protocol. Both single- and mixed-ploidy populations were included. In each population, three plants of each majority cytotype and up to seven plants of minority cytotypes were selected for mycorrhiza sampling, resulting in a total number of 51 individuals (Table 1). The plants were selected randomly, and three roots per plant were collected and processed as described below.

Table 1. Site locations, ploidy composition, and the number of adult plants per cytotype investigated for mycorrhizal symbionts.

Site	Geographic coordinates	Abundance of cytotypes (2xGd/2xGc/3x/4xGc)	Number of sampled *Gymnadenia* plants (regional + microsite sampling)			
			2xGd	2xGc	3x	4xGc
Zahrady pod Hájem[a] (CZ)	48°53'04.0" N 17°31'51.6" E	152/295/28/386	3+6	3+19	7+7	3+19
Jazevčí[a] (CZ)	48°52'27.8" N 17°34'14.4" E	0/328/4/137		3		3
Babiratka (CZ)	48°49'23.2" N 17°33'5.5" E	0/20/0/5 [b]		3		3
Machová (CZ)	48°49'45.1" N 17°32'22.7" E	58/0/2/0	3			
Strání (CZ)	48°54'30.7" N 17°40'34.2" E	100/0/0/0 [b]	3			
Čertoryje (CZ)	48°51'25.2" N 17°24'36.9" E	0/50/0/0		3		
Pod Lipinkou (CZ)	48°54'8.7" N 17°35'41.0" E	1/20/0/0 [b]		3		
Velká Javořina (CZ)	48°51'13.8" N 17°40'05.1" E	0/56/5/1		3	2	
Hustopeče (CZ)	48°57'49.8" N 16°45'21.7" E	0/0/0/66				3
Veterník (SK)	48°49'9.4" N 17°13'59.3" E	0/0/0/60				3

CZ, Czech Republic; SK, Slovak Republic.
[a]Sites used for seed sowing.
[b]Ploidy abundances screened in this study; otherwise, they were inferred from Trávníček et al. (2011). See Supporting Information Fig. S1 for a map showing the location of the sites.

Microsite sampling

Mycorrhizal fungi associated with different adult cytotypes at the microsite scale were investigated at the site Zahrady pod Hájem (Table 1), hereafter called Zahrady. The site is a mosaic of traditionally managed xerophytic and mesophytic grasslands and hosts the most ploidy-diverse population of fragrant orchids currently known in Central Europe, with a sympatric occurrence of all

five *G. conopsea* cytotypes, in addition to *G. densiflora* (Trávníček et al. 2011). Although 4xGc shows a negative spatial association with diploids also at Zahrady (Trávníček et al. 2011), patches of intermingled cytotypes can be found here, contrary to other mixed-ploidy sites.

The sampling was conducted within 5 days at the beginning of June 2011 during the flowering time of 2xGc and at the onset of flowering of 2xGd and 4xGc. Within a 0.3 ha area, we first selected 20 microsites (< 1 m² and at least 2 m apart) with putative occurrences of different cytotypes growing in close proximity (spaced by 8–100 cm; Fig. 1a). At each microsite, all vegetative and flowering individuals were labelled using a permanent marker and flagged skewers. One leaf per individual was wrapped in moist cotton tissue, placed in a plastic bag, and transported rapidly to the laboratory of flow cytometry where its ploidy level was determined following Trávníček et al. (2011). The analysis of 183 leaf samples revealed 33 individuals of 2xGd, 84 individuals of 2xGc, 55 individuals of 4xGc and 11 triploids. Finally, 11 microsites with the highest ploidy diversity were selected. At each microsite, roots from up to three individuals of 2xGd, 2xGc, 3x and 4xGc were collected whenever possible, totalling 51 plants (Table 2). Three adventitious roots per plant were taken to minimize damage to the individual. The roots were washed by distilled water immediately after their collection and stored in 60% ethanol until processing within a month. The roots were then surface sterilized (submerged for 30 s in 4.5% sodium hypochlorite, 15–30 s in 70% ethanol, followed by three 15 s washes in distilled water) and inspected for mycorrhizal colonization by light microscopy (Fig. 1b). One 1–2 mm thick cross-section per mycorrhizal root was used for molecular identification of mycorrhizal fungi.

Table 2. Summary of the microsite sampling at Zahrady pod Hájem site (Czech Republic) and mean numbers of fungal operational taxonomic units (OTUs) detected per microsite.

	Within-plot distance (cm)		Mean no. of plants	Mean (min-max) no. of
	Median	Mean (± SD)	per plot / no. of plots	fungal OTUs per plot
All individuals	30	38 (± 23)	4 / 11	2.82 (2 – 5)
2xGd	-	-	1 / 6	1
2xGc	30	34 (± 19)	1.55 / 11	1.55 (1 – 4)
3x	40	40 (± 0)	1.25 / 4	1.5 (1 – 2)
4xGc	30	27.1 (± 13.8)	1.6 / 10	1.4 (1 – 2)
2xGc vs. 4xGc	30	35.2 (± 20)		

Only *Gymnadenia* individuals successfully analyzed for mycorrhizal fungi were used for the calculation of their spatial distances.

Seed sowing

To investigate the associations in mycorrhizal seedlings (protocorms), the seeds were sown in mixed-ploidy populations at Zahrady site in 2006 and at Zahrady and Jazevčí sites in 2007 (Table 1). Only seeds of local origin were used at each site. Ripe seeds were collected in July/August and buried at the end of September. For the seed burial in 2006, seeds from 10 naturally pollinated individuals per majority cytotype/taxon (i.e. 2xGd, 2xGc, and 4xGc) were pooled separately. Although we could not entirely avoid the possibility of inter-ploidy pollination, this was limited either by phenological barriers (i.e. 2xGc individuals flowering before the other cytotypes) or by the selection of co-flowering individuals of 2xGd and 4xGc from large homoploid clumps. Approximately 300 seeds with well-developed embryos were placed on a 42 μm nylon mesh (Silk and Progress Ltd., Brněnec, Czech Republic) and enclosed in 35-mm plastic slide mounts as in Rasmussen and Whigham (1993). Six packets of each of the three majority cytotypes were placed at eight microsites, totalling 144 packets, plus 6 trial packets. For the sowing in 2007, seeds arising from hand-pollination were used. Three flowers from six to eight mother plants of each majority cytotype per site were cross-pollinated either with homoploid or heteroploid pollen. Seeds from different crosses of each mother plant were separately enclosed in a compartmented nylon bag as in Bidartondo and Bruns (2005) produced in three replicates. One bag of each mother plant was buried per three microsites at both sites (in total 48 bags at Jazevčí and 66 bags at Zahrady). Both packets and bags were buried 5 cm deep in soil in patches with at least one majority cytotype growing in abundance within a 1 m radius (Table S1). After 24 months of soil incubation, the seed packets and bags were retrieved, and protocorm formation was evaluated. However, seeds experienced very high mortality during the soil incubation. Only 13% of seed packets sown at Zahrady in 2006 contained healthy (non-decayed) protocorms suitable for molecular analyses. The germination outcome of seeds sown in 2007 was even worse, and only 0.6% and 2.4% of compartments contained protocorms at Zahrady and Jazevčí sites, respectively. In total, 32 protocorms were analyzed; these included one to three protocorms from 4, 10, 1, and 6 packets of 2xGd, 2xGc, 3x, and 4xGc, respectively, from Zahrady, and from 1 and 5 packets of 2xGc and 3x, respectively, from Jazevčí (Table S1). This small dataset did not allow a separate statistical analysis of mycorrhizal symbionts; thus the data were analyzed together with the dataset obtained from the regional sampling.

Molecular identification of fungal symbionts

Total DNA was extracted using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany). Fungal internal transcribed spacer (ITS) of nrDNA was amplified by primer set ITS1OF/ITS4OF specific to a broad variety of basidiomycetes, including most fungi known to form orchid mycorrhiza (Taylor and McCormick 2008). When the amount of amplified DNA was not sufficient for direct sequencing, 1 µL of the PCR product was used for second amplification with primer pairs ITS1OF/ITS4 or ITS1/ITS4OF (White et al. 1990; Taylor and McCormick 2008). Further, the primer pair ITS1/ITS4Tul specific to *Tulasnella* (Taylor and McCormick 2008) was used to search for additional *Tulasnella* lineages. When the amplification with the two primer pairs failed, fungi-universal primer pair ITS1F/ITS4 (Gardes and Bruns 1993) was used to check for the presence of other potentially mycorrhizal fungi. PCR reactions were performed in 20 µL volume containing 1x Plain PP Master Mix (Top-Bio, Prague, Czech Republic), 0.3 µM of each primer, 0.3–3 µL of the template DNA, and complementary volume of sterile ddH$_2$O. The thermal cycling profile was: initial denaturation at 95 °C for 4 min, followed by 35–45 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C (for ITS1F/ITS4) or 55 °C (for ITS1OF/ITS4OF and ITS1/ITS4Tul) for 60 s and elongation at 72 °C for 60 s, followed by final elongation at 72 °C for 10 min. The PCR products were visualized on 1.5% agarose gel in 1x TBE buffer. Unique PCR fragments were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and directly sequenced. Double-banded PCR products were separately excised out of the gel and purified using the Jetquick Gel Extraction Spin Kit (Genomed, Löhne, Germany). All purified products were sequenced unidirectionally and selected representatives from each operational taxonomic unit (OTU) (see later) were sequenced bidirectionally by Macrogen Inc. (Seoul, Korea). A few PCR products, in which direct sequencing or gel excision failed, were cloned using the Topo TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Sixteen positive colonies were re-amplified using the same primers as before cloning. The clones were visualized on 1% agarose gel, and six clones with different fragment lengths were sequenced.

To assess the presence of ascomycetes potentially mycorrhizal in *Gymnadenia* (Stark et al. 2009), a subset of plants collected at Zahrady site in 2008 (the regional sampling), namely two individuals of each 2xGd, 2xGc and 4xGc, and three triploids, was analyzed with primer pair ITS1F/ITS4Asco [5'-CGTTACTRRGGCAATCCCTGTTG-3'; Nikolcheva and Bärlocher (2004)] specific to ascomycetes. DNA from individual root sections was pooled per

individual and amplified following the conditions for ITS1OF/ITS4OF primer pair. The PCR products were cloned, and six clones per cloning were sequenced as described above.

Preliminary taxonomic assignments of sequences were done by BLASTn search of the NCBI database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequences of Agaricomycetes (which involve Tulasnellaceae, Ceratobasidiaceae, and also other fungal taxa of which most orchid mycorrhizal fungi are recruited; Dearnaley et al. 2013), Pezizales (mycorrhizal in some orchid genera; Dearnaley et al. 2013), and ascomycetes originating from cloning with ITS1F/ITS4Asco were grouped into operational taxonomic units (OTUs) based on 97% similarity threshold over the whole ITS region, using clustering algorithm in TOPALi v.2.5 (Milne et al. 2004). Although arbitrary, this threshold is commonly used for fungi (Hughes et al. 2009). In some studies, the threshold of 95% for Tulasnellaceae was adopted, due to their rapidly evolving DNA (Jacquemyn et al. 2012c). In our case, decreasing the threshold from 97% to 95% did not merge any OTU. OTUs affiliated to Agaricomycetes and Pezizales were considered potentially mycorrhizal in *Gymnadenia* (Stark et al. 2009) and used for statistical analyses. The representatives of each OTU have been deposited in GenBank (NCBI) under accession numbers listed in Table 3 and Table S2.

Data analysis

We used a Bayesian inference to build a phylogenetic tree of a representative of each OTU assigned by BLAST search to Tulasnellaceae, closest BLAST matches of the OTUs (Table 3), voucher specimens of Tulasnellaceae deposited in GenBank, and outgroup taxa from Cantharellales (in total 44 sequences). The sequences were aligned using algorithm E-INS-i in MAFFT v. 6 (Katoh et al. 2005) and manually adjusted in Bioedit v. 7.0.5.3 (Hall 1999). Because the ITS1 and ITS2 regions of the whole dataset were highly divergent and thus not alignable, only conserved regions of 5.8S and flanking part of 28S were used for the analysis, yielding a 288 bp long alignment. Model HKY85 with autocorrelated discrete gamma (Yang 1995) was selected as the best substitution model in Kakusan4 v. 2 (Tanabe 2011) by evaluating AIC of different models and implemented to MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). Two independent runs were conducted, each for 5×10^6 generations with sampling every 1000th generation. First 25% of trees were excluded as burn-in, and the remaining trees were used for the construction of a

Table 3 List of fungal operational taxonomic units (OTUs; defined by a 97% similarity threshold) detected in adults and seedlings of the *Gymnadenia conopsea* group.

OTU	Accession number	Taxonomic position	Closest informative match in GenBank (Accession no.)	Maximum identity (%)	E-value
OTU 1	KC243932	Tulasnellaceae	Uncultured mycorrhiza (Tulasnellaceae) 4065 (AY634130)	99	0
OTU 2	KC243933	Tulasnellaceae	Uncultured Tulasnellaceae clone GcoK03-aab45b12 (HQ330998)	97	0
OTU 3	KC243934	Tulasnellaceae	Uncultured Tulasnellaceae clone OmiAW01-aac01g07 (HQ330992)	99	0
OTU 4	KC243935	Tulasnellaceae	Uncultured *Tulasnella* clone 1251a (FJ788887)	99	0
OTU 5	KC243936	Tulasnellaceae	Uncultured *Tulasnella* clone RW12 (HM802323)	75	$5e^{-151}$
OTU 6	KC243937	Tulasnellaceae	Uncultured Tulasnellaceae clone CF361 (GQ241750)	92	0
OTU 7	KC243938	Tulasnellaceae	Uncultured Tulasnellaceae clone Dmac_Aga_9H10 (JX024733)	98	0
OTU 8	KC243939	Tulasnellaceae	Uncultured Tulasnellaceae isolate 514 (DQ925606)	99	0
OTU 9	KC243940	Ceratobasidiaceae	Uncultured ectomycorrhiza (Ceratobasidiaceae) 4074 (AY634127)	100	0
OTU 10	KC243941	Ceratobasidiaceae	Uncultured Ceratobasidium clone FDBC66 (JQ247397)	95	0
OTU 11	KC243942	Tulasnellaceae	*Epulorhiza anaticula* (EU218891)	99	0
OTU 12	KC243943	Ceratobasidiaceae	*Ceratobasidium* sp. L9Rh-col6 (HM117643)	96	0
OTU 13	KC243944	Tulasnellaceae	Uncultured *Tulasnella* clone RW12 (HM802323)	75	$1e^{-95}$
OTU 14	KC243945	Ceratobasidiaceae	*Ceratobasidium* sp. AG-I (AB290022)	98	0
OTU 15	KC243946	Tulasnellaceae	Uncultured *Tulasnella* clone SV11 (JF926495)	99	0
OTU 16	KC243947	Tulasnellaceae	Uncultured Tulasnellaceae isolate P50 (DQ925601)	97	0
OTU 17	KC243948	Tulasnellaceae	Uncultured *Tulasnella* mycobiont of *Aneura pinguis* clone 9764 (EU909268)	97	0
OTU 18	KC243949	Pezizaceae	Uncultured Pezizaceae clone 1171a (FJ788730)	88	$2e^{-113}$
OTU 19	KC243950	Tulasnellaceae	Uncultured *Tulasnella* clone RW12 (HM802323)	77	0
OTU 20	KC243951	Pezizaceae	Uncultured Pezizaceae clone 1145a (FJ788739)	98	0
OTU 21	KC243952	Pezizaceae	Uncultured *Peziza* genomic DNA (FR852086)	77	$5e^{-91}$
OTU 22	KC243953	Lyophyllaceae	*Calocybe gambosa* voucher 8064 (JF907775)	99	0
OTU 23	KC243954	Pezizaceae	*Peziza fimeti* KH 00.012 (C) (AF491605)	99	0
OTU 24	KC243955	Tulasnellaceae	Uncultured mycorrhiza (Tulasnellaceae)(AY634131)	92	0
OTU 25	KC243956	Tulasnellaceae	Uncultured *Tulasnella* clone RW12 (HM802323)	76	$3e^{-110}$
OTU 26	KC243957	Tulasnellaceae	Uncultured *Tulasnella* clone RW12 (HM802323)	76	$1e^{-103}$
OTU 27	KC243958	Serpulaceae	*Serpula himantioides* MUCL:30528 (GU187545)	99	0
OTU 28	KC243959	Hymenochaetaceae	*Fuscoporia ferruginosa* voucher JV8909/74 (JQ794573)	99	0

Taxonomic position refers to GenBank taxonomy.

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majority-rule consensus tree. Branches with Bayesian posterior probabilities below 0.95 were regarded as poorly supported.

Species composition of mycorrhizal fungi (OTUs) was analyzed by multivariate ordination methods using Canoco v. 4.53 (Ter Braak and Šmilauer 2002) and R-package vegan v. 1.17-7 (Oksanen et al. 2011). A non-metric multidimensional scaling (NMDS) was used to visualize variation in mycorrhizal species composition within and between different cytotypes/taxa. A canonical correspondence analysis (CCA) was used to test for differences in mycorrhizal composition at the regional scale (involving either adults or both adults and seedlings); each combination of cytotype, ontogenetic stage, and site was considered as an independent sample. Significance of all constrained ordination axes was tested by a Monte-Carlo permutation test with 999 permutations. A partial CCA (pCCA) with microsites as covariates was used to test for differences at the microsite scale (involving adult plants only). Each combination of cytotype and microsite was considered as an independent sample (OTUs of all plants of a given cytotype within a microsite were pooled), and the dataset was analyzed as incomplete (all cytotypes/taxa but 3x) or complete (2xGc vs. 4xGc) randomized blocks. Permutations of the Monte-Carlo test were restricted by the covariates, thus the mycorrhizal species composition was permuted only within the microsites. In (p)CCAs, all singular occurrences of OTUs were omitted prior to analysis, as were the triploids due to their uncertain origin (i.e. inter-ploidy hybridization or fusion of reduced and unreduced gametes of diploid parents).

Results

Identity of fungal symbionts

Roots of all 102 adult *Gymnadenia* plants but three 4xGc adults from the microsite sampling, and 32 protocorms showed signs of mycorrhizal colonization (Fig. 1b). Molecular analyses were successful in 50 adults from the regional sampling, 44 adults from the microsite sampling, and 31 protocorms, revealing 318 sequences of potentially mycorrhizal fungi (i.e. Agaricomycetes and Pezizales). They mostly belonged to Tulasnellaceae (86.5% of all sequences), Ceratobasidiaceae (7.5%), and Pezizaceae (5%), but rarely also to Lyophyllaceae, Serpulaceae, and Hymenochaetaceae, and grouped into 28 OTUs, based on the 97% similarity cut-off (Table 3). All OTUs were well-defined; decreasing the similarity threshold to 90% resulted in a merger of only two OTUs of Ceratobasidiaceae (OTUs 9 and 14) and three OTUs of Tulasnellaceae (OTUs 4, 7, and 17). The three most frequently recorded OTUs (OTUs 1, 2, and 7) accounted for 61% of all sequences. Only

one OTU was found in 72% and 93% of adults and protocorms, respectively, while the maximum number of OTUs observed in adults and protocorms reached three and two, respectively. Most OTUs were amplified with primer pairs ITS1OF/ITS4OF and/or ITS1/ITS4Tul, except for the uniquely occurring OTUs 21, 22, 27, and 28 that were detected only with fungal universal primers ITS1F/ITS4.

The phylogenetic analysis supported the monophyly of all Tulasnellaceae detected in *Gymnadenia* roots (Fig. S2). Twelve OTUs (OTUs 4, 5, 6, 7, 11, 13, 15, 17, 19, 24, 25, and 26) formed a well-supported clade with known *Tulasnella* species, although only OTU 11 showed more than 97% similarity to a described taxon. All of these OTUs amplified well with ITS1/ITS4Tul primer and most of them also with ITS1OF/ITS4OF, except for OTU 7 which yielded only weak PCR products and OTU 17 which did not amplify at all (Fig. S2). Five other OTUs (OTUs 1, 2, 3, 8, and 16) were placed at the base of the first clade. No described *Tulasnella* species were related to these OTUs, and their closest BLAST matches were all isolated from terrestrial orchids, including *Cypripedium*, *Dactylorhiza*, *Gymnadenia*, and *Orchis*. These OTUs amplified well with primer pairs ITS1OF/ITS4OF and ITS1F/ITS4, but no PCR amplicons were obtained with ITS1/ITS4Tul primer set.

Additional amplification with ITS1F/ITS4Asco primers on nine adult plants revealed 54 ascomycete sequences clustered into 18 OTUs (Table S2). The mean ($\pm$ SD) and maximum numbers of OTUs per individual were 3.6 ($\pm$ 1.1) and 6, respectively. Twelve OTUs were detected only once, the others were shared by different cytotypes. An additional Pezizaceae OTU 29 was detected in one 2xGc. The remaining OTUs belonged to plant pathogens, endophytes or saprobes mostly from Cordycipitaceae, Nectriaceae or Chaetothyriales, in addition to common orchid endophytes such as *Leptodontidium*, *Tetracladium*, and *Exophiala* (Table S2). These sequences were occasionally obtained also by amplification with ITS1OF/ITS4OF and ITS1F/ITS4. Similarly, two ascomycetes from Hypocreales (*Hypocrea* and *Verticillium*) were once found in two protocorms, along with Tulasnellaceae OTUs.

Mycorrhizal associations at the regional scale
The analysis of mycorrhizal fungi at ten single- or mixed-ploidy sites (Table 1) revealed 18 different OTUs associated with adults (Table S3; see Fig. S3 for rarefaction curves). The number of fungal OTUs recorded at each site was highly variable and ranged from one to five OTUs in three studied individuals per cytotype/taxon. Mycorrhizal seedlings cultivated in two mixed-ploidy

populations harboured nine OTUs (Table S1), four of which (OTUs 1, 2, 6, and 10) were shared with local adults, three (OTU 4, 15, and 24) were found in adults at other sites, and only two (OTUs 8 and 25) were undetected in any adult plant, confirming the mycorrhizal status of most OTUs frequently recorded in adults. No other potentially mycorrhizal families than Tulasnellaceae and Ceratobasidiaceae were found in seedlings.

Species composition of mycorrhizal fungi detected in seedlings and adults showed distinct ploidy-specific patterns irrespective of their origin from single- or mixed-ploidy sites (Fig. 2). Whereas mycorrhizal symbionts of both diploid taxa (2xGc and 2xGd) were quite similar, a clear shift was seen in tetraploids. Mycorrhizal symbionts of triploids overlapped with either those of diploids or tetraploids. The differences in mycorrhizal symbionts were statistically significant both among all majority cytotypes/taxa (CCA, $F = 3.41$, $P = 0.001$) and between 2xGc and its autopolyploid 4xGc ($F = 2.71$, $P = 0.009$). The same was true when only mycorrhizal associations in adults (i.e. excluding the seedling dataset) were analyzed ($F = 2.96$, $P = 0.002$ for the entire dataset and
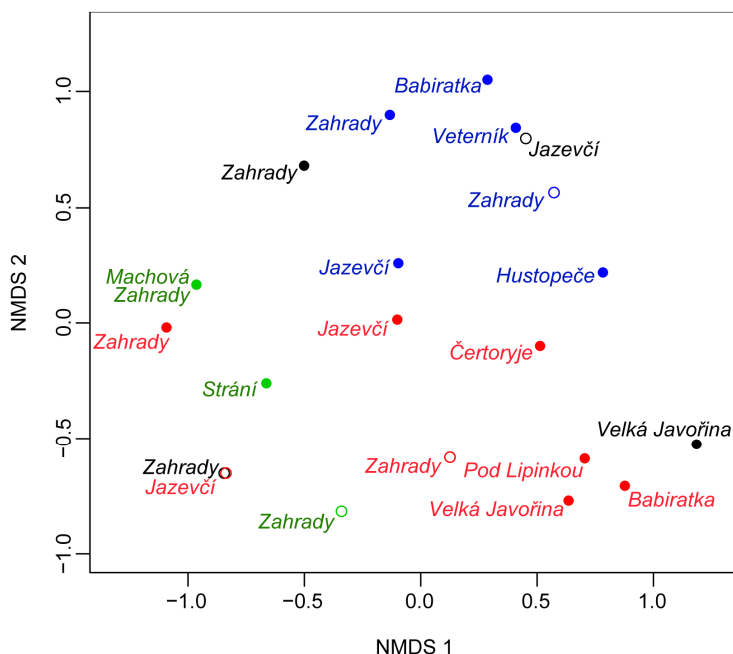
Fig. 2. A nonmetric multidimensional scaling (NMDS) plot of mycorrhizal fungi detected in adults and seedlings of four cytotypes/taxa of the *Gymnadenia conopsea* group sampled at 10 sites in the broader area of the White Carpathian Mountains (Central Europe). Green, diploid *G. densiflora*; red, diploid, and blue, tetraploid *G. conopsea*; black, triploids. Open symbols, seedlings; closed symbols, adults. The symbol denoting diploid seedlings of *G. conopsea* from Jazevčí was slightly shifted along the NMDS 1 axis because of its overlap with that of triploids from Zahrady.

$F = 2.43$, $P = 0.048$ for 2xGc versus 4xGc). The exclusive association of OTUs 1 and 24 with both protocorms and adults of diploid taxa, and OTU 2 with 4xGc underpinned the observed differences; these were further supported by the dominant association of OTUs 6 and 7 with 2xGc (Fig. 3).
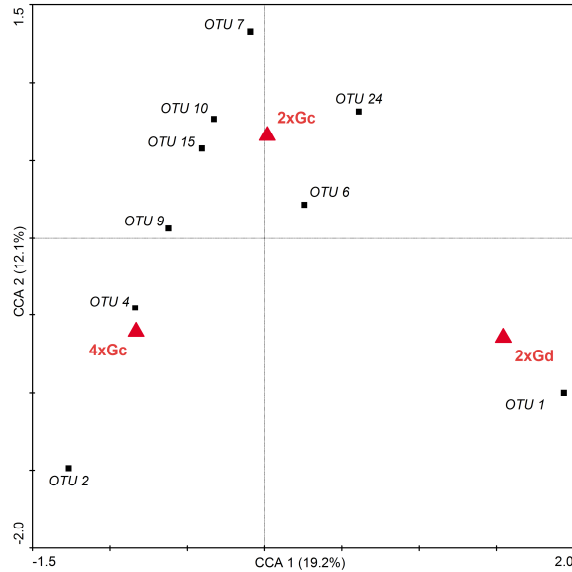

Fig. 3. Canonical correspondence analysis (CCA) ordination plot displaying differences in fungal operational taxonomic units (OTUs) in seedlings and adults of diploid *Gymnadenia densiflora* (2xGd), diploid *G. conopsea* (2xGc), and tetraploid *G. conopsea* (4xGc) sampled at 10 sites. Centroids of each group are denoted by a triangle. Summary of a Monte-Carlo permutation test (999 permutations): F = 3.41, P = 0.001.

Mycorrhizal associations at the microsite scale

To avoid potential effects of uneven spatial distribution (e.g. cytotype grouping) on mycorrhizal associations and verify ploidy-specific differences in the composition of mycorrhizal fungi observed at the regional scale, closely-spaced adults of the four cytotypes/taxa (2xGd, 2xGc, 3x, and 4xGc) were collected at 11 microsites at Zahrady site (see Table 2 for the sampling scheme). In 44 plants, 13 OTUs were identified, seven of which belonged to Tulasnellaceae and occurred in 41 *Gymnadenia* adults (Fig. S3, Table S4). Partial CCA detected significant differences in the mycorrhizal species composition of all majority cytotypes/taxa (pCCA, $F = 8.78$, $P = 0.001$) and also of 2xGc and 4xGc ($F = 10.99$, $P = 0.002$), which were consistent across the microsites. Whereas diploid taxa (2xGc and 2xGd) associated mainly with OTU 1 (and 2xGc also with seven other OTUs), 4xGc associated mainly with OTU 2 and five other OTUs (Fig. 4). No overlap of mycorrhizal symbionts

between 2x and 4x cytotypes was found, except for one individual of 4xGc, in which both OTUs 1 and 2 occurred. Triploids harboured OTUs typical of either 2x or 4x cytotypes (Fig. 4).
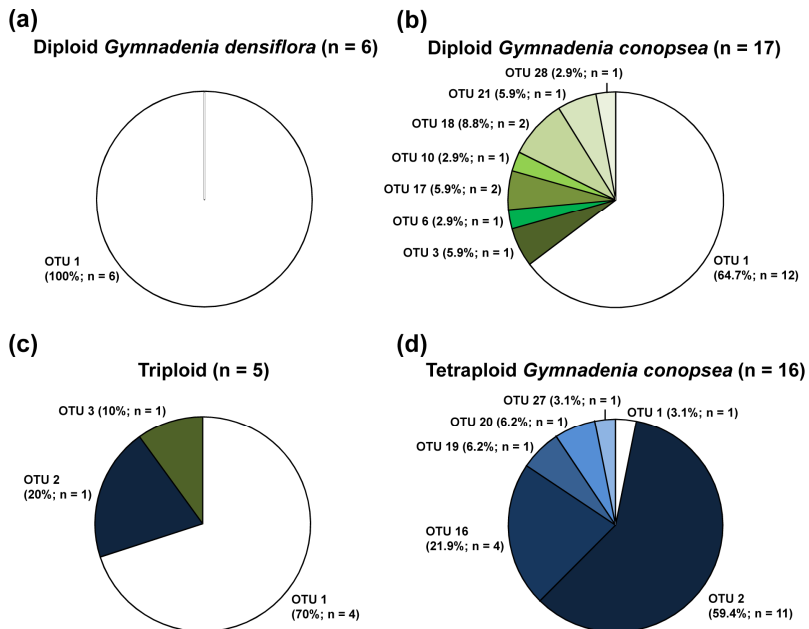
Fig. 4. Relative abundance of fungal operational taxonomic units (OTUs) (weighted by the number of OTUs per plant) and the number of plants associated with each OTU in four *Gymnadenia* cytotypes/taxa collected at 11 microsites at Zahrady pod Hájem site (Czech Republic).

Discussion

Genome duplication can cause instantaneous shifts in ecological behaviour of newly established polyploid lineages, including alterations of interactions between plants and other organisms (Arvanitis et al. 2010; Parisod et al. 2010). In this study, we found distinct mycorrhizal associations in different cytotypes/taxa of the *G. conopsea* group and provide the first evidence for a ploidy-specific divergence of mycorrhizal fungi. The observed patterns were stable (i) across geographically separated single- and mixed-ploidy sites, (ii) within a mixed-ploidy site, and (iii) during plant ontogeny (in seedlings / adults).

Diversity of fungal associations

A wide variety of potentially mycorrhizal fungi from different phylogenetic groups has been detected in *Gymnadenia* (Table 3). The most frequent symbionts in all cytotypes/taxa belonged to Tulasnellaceae, followed by much

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less common Ceratobasidiaceae; this is in agreement with previous reports on fungal diversity in *Gymnadenia* adults (Stark et al. 2009; Jacquemyn et al. 2012b). The occurrence of these two fungal families in seedlings further emphasizes their biological importance for *Gymnadenia*. Tulasnellaceae and Ceratobasidiaceae are very common mycorrhizal symbionts of both terrestrial (e.g. Shefferson et al. 2007; Jacquemyn et al. 2010; Phillips et al. 2011; Martos et al. 2012) and epiphytic orchids (e.g. Suárez et al. 2006; Otero et al. 2007; Martos et al. 2012) worldwide. Tulasnellaceae OTUs associated with any *Gymnadenia* cytotype/taxon did not form a separate lineage but spanned the phylogenetic tree of Tulasnellaceae (Fig. S2). Of interest are the basal OTUs 1, 2, 3, 8, and 16 non-amplifiable with *Tulasnella*-specific primer pair ITS1/ITS4Tul, whose sequences of 5.8S are distinct from any *Tulasnella* reference species stored in GenBank. According to the phylogenies of Suárez et al. (2006) and Shefferson et al. (2007), these OTUs may be related to *Gloeotulasnella cystidiophora*, a saprotrophic wood-decaying fungus. However, this family undoubtedly contains many unknown taxa, and a multigene analysis is required to resolve evolutionary relationships within Tulasnellaceae.

In addition to Tulasnellaceae and Ceratobasidiaceae, various ectomycorrhizal and saprotrophic ascomycetes (especially from Pezizales) and basidiomycetes have been occasionally detected in *Gymnadenia* roots (Stark et al. 2009; Jacquemyn et al. 2012b). Of these, Pezizaceae were the dominant group in our study (recorded in seven plant individuals). Some species from Pezizaceae are known to form ectomycorrhiza with trees (Tedersoo et al. 2006). However, most Pezizaceae OTUs detected in this study were highly similar to saprotrophic species (e.g. *Peziza fimeti*) or to mycorrhizal symbionts of South African orchid genera *Pterygodium* and *Corycium* (Waterman et al. 2011) which belong to saprotrophic lineages (Tedersoo et al. 2013). Although we could have missed some Pezizales because of the predominant use of basidiomycete-specific primers, three Pezizaceae OTUs were detected with ITS1OF/ITS4OF primers, perhaps due to lower annealing temperature during PCR (Taylor and McCormick 2008). Importantly, screening with the ascomycete-specific primer pair revealed only one new Pezizaceae OTU 29 in a single *Gymnadenia* plant (Table S2). The three remaining OTUs of potentially mycorrhizal fungi (OTUs 22, 27, and 28) affiliated to saprotrophic basidiomycetes (*Calocybe, Serpula*, and *Fuscoporia*) and were detected only once in our dataset; there are no previous records from orchids. Together with their absence in mycorrhizal seedlings, which are obligately dependent on

fungal nutrition, it seems that these fungal taxa are only occasional associates of *Gymnadenia* roots with uncertain physiological function.

The other ascomycetes detected by ascomycete-specific primers may rather act as root endophytes or parasites, since they have been rarely isolated directly from mycorrhizal structures (Herrera et al. 2010; Kohout et al. 2013), and their biological function requires further investigation. In contrast to low diversity of mycorrhizal fungi per *Gymnadenia* individual, these ascomycetes were relatively diverse and averaged 3.6 OTUs per plant (Table S2). Our observation corresponds to high diversity of endophytes reported for *Gymnadenia* (Stark et al. 2009), other orchid species (e.g. Kohout et al. 2013), and also other types of mycorrhiza (Tedersoo et al. 2009).

Ploidy-specific shifts in mycorrhizal fungi

The main finding of our study was a distinct segregation of mycorrhizal symbionts according to the ploidy level of the plant. The most pronounced segregation was observed between diploids and their autotetraploid derivatives that grew closely intermingled at a mixed-ploidy site (Fig. 4, Table 2). Obligate dependence on fungal nutrient supply during germination (Rasmussen and Rasmussen 2009) and at least partial dependence at maturity (Cameron et al. 2006) can result in strong plant competition for fungal resources. Thus, ploidy-specific shifts in mycorrhizal symbionts can contribute to niche partitioning and facilitate the establishment and co-existence of different cytotypes. In our particular case, the below-ground niche divergence can be further supported by the low richness of mycorrhizal OTUs associated with *Gymnadenia* plants. Although no comparable data for other polyploid species are available, a distinct segregation of mycorrhizal fungi has been previously observed at species level among co-occurring orchids (Waterman et al. 2011; Jacquemyn et al. 2012a,b) and also among plants forming other types of mycorrhiza (e.g. Vandenkoornhuyse et al. 2003; Sýkorová et al. 2007; Morris et al. 2008). Manifestation at different levels of biological organization (species/cytotypes) suggests that the divergence in mycorrhizal symbionts among co-existing individuals with similar ecological requirements could be a more widespread adaptation that limits resource competition.

The divergence in mycorrhizal fungi associated with *G. conopsea* cytotypes was also obvious when geographically distant single- and mixed-ploidy populations were investigated (Fig. 2); however, the selectivity was lower, and certain overlap was observed also among co-existing but distantly-spaced individuals. Individual *Gymnadenia* cytotypes/taxa showed strong preferences for particular OTUs (Fig. 3), but the association was not obligatory;

for instance, the exclusive symbiont of 4xGc, OTU2, occurred at all 4xGc sites, but not in all 4xGc individuals (Tables S3, S4). Several OTUs were detected in only one plant individual while others (e.g. Tulasnellaceae OTUs 4 and 15) had rather low ploidy specificity. Similarly, Ceratobasidiaceae associated mostly indifferently of the plant ploidy level (Fig. 3, Tables S1, S3). A comparison of the two *Gymnadenia* taxa with the same ploidy level (2xGc vs. 2xGd) revealed a slightly different pattern. Whereas there was no segregation in mycorrhizal symbionts at the microsite scale (both species commonly associated with OTU 1), at the regional scale 2xGd and 2xGc clearly preferred OTU 1 and OTU 7, respectively. The lack of the latter fungal OTU at Zahrady site could explain this discrepancy; OTU 7 was not detected there in any of 66 investigated adults and was also absent in protocorms.

A deeper insight into the mechanisms underlying significant mycorrhizal selectivity of different cytotypes is not possible at this stage of investigation. We can speculate that inter-ploidy differences in nutrient requirements (polyploids have larger cells and nuclei but generally slower metabolic processes; Levin 2002), changes in plant anatomy (thicker cell walls in polyploids) or levels of secondary metabolites may result in the selection of mycorrhizal symbionts that best meet plant´s physiological demands. The identity of mycorrhizal fungi in triploid individuals, however, suggests that a change in ploidy level does not necessarily lead to an immediate switch of mycorrhizal symbionts. Triploids of *Gymnadenia* always occur as a minority either in exclusively diploid or mixed 2x+4x populations (Trávníček et al. 2011, 2012). Their association with fungi typical for both diploid (predominantly) and tetraploid (less commonly) parent taxa (Figs 2, 4) may be related to different pathways of triploid´s origin (i.e. syngamy of reduced and unreduced gametes of diploids or hybridization between di- and tetraploids). Similarly to other traits of newly established autopolyploid lineages (Parisod et al. 2010), mycorrhizal selectivity may be shaped by a genetic component of the plant (e.g. genic redundancy, higher heterozygosity in polyploids), leading to adaptive changes in the selectivity over a longer time frame.

Evolutionary consequences of ploidy-specific symbiotic interactions
Newly arising polyploids are likely to face difficulties to become established due to mating with sympatric parents (Levin 1975); hence, to overcome minority cytotype exclusion they must evolve efficient breeding barriers (Levin 2002; Husband and Sabara 2004; Parisod et al. 2010; Husband et al. 2013). Despite mixed-ploidy populations of the *G. conopsea* group are relatively common in some parts of Central Europe (Trávníček et al. 2011, 2012), the

conditions underlying the co-existence of multiple cytotypes have not been sufficiently understood. Jersáková et al. (2010) investigated potential pre-mating barriers among co-occurring 2xGd, 2xGc, and 4xGc. The authors did not find any assortative pollinator behaviour and concluded that phenological segregation likely acts as an important pre-zygotic barrier between early (2xGc) and late (2xGd, 4xGc) flowering cytotypes. On the contrary, this barrier is unlikely to exist between 2xGd and 4xGc whose flowering times significantly overlap, and heteroploid hybridization yields viable seeds (J. Jersáková, unpublished).

Although segregation in mycorrhizal symbionts cannot directly prevent the detrimental loss of gametes due to heteroploid mating, it may significantly influence intrapopulation spatial structure of obligately mycorrhizal plants and thus indirectly alter the frequency of different mating interactions. Despite their dust-like nature with a potential for long-distance dispersal, it has been shown that most orchid seeds actually fall in the vicinity of a mother plant (Jersáková and Malinová 2007). Since the mycorrhizal symbionts seem to be stable during the ontogeny of *Gymnadenia*, germinating seedlings have higher chance to find an appropriate symbiont just around the mother plant. The relationship between fungal distribution and successful seedling establishment is likely to lead to clumping of individuals with similar mycorrhizal fungi, as was indeed confirmed for different *Gymnadenia* cytotypes (Trávníček et al. 2011) and also observed in co-occurring species of other orchids (Jacquemyn et al. 2012a,b). Because pollinators tend to forage over short distances and between neighbouring plants (Husband and Sabara 2004), cytotype clumping can limit inter-ploidy pollen flow.

To conclude, explaining the origin and maintenance of biological diversity are central questions of ecological research, and niche differentiation among co-existing organisms is widely acknowledged to play a key role in this respect (Coyne and Orr, 2004). In mycorrhizal plants, niche differentiation can be achieved via the segregation of associated fungi (van der Heijden et al. 2003; Sýkorová et al. 2007). Our study is the first to provide evidence for divergence of mycorrhizal fungi according to the ploidy level of the same plant species. Genome duplication of one organism can thus greatly affect other components of the ecosystem, indicating that the consequences of genome duplication are much more far-reaching than generally assumed.

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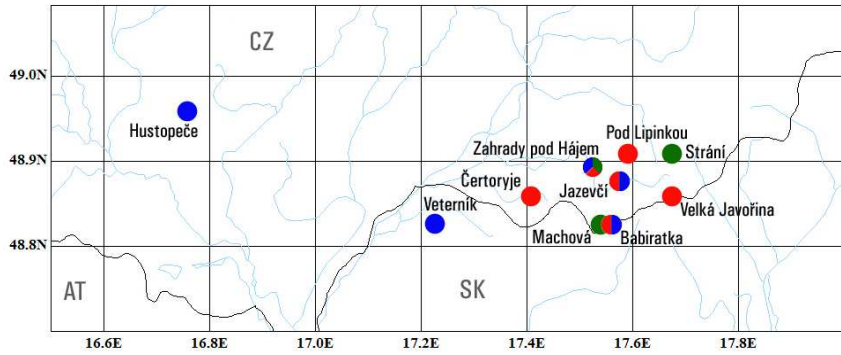
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Fig. S1. Location of experimental sites in the broader area of the White Carpathians Mts. in the Czech Republic and the Slovak Republic. Green, diploid *Gymnadenia densiflora*; red, diploid, and blue, tetraploid *G. conopsea*.

Fig. S2. Bayesian majority-rule consensus tree (based on 5.8S and flanking region of 28S) showing phylogenetic relationships among representatives of Tulasnellaceae OTUs associated with the four *Gymnadenia* cytotypes/taxa, their closest matches in GenBank (Table 3; source organism and geographic origin of the sequences is provided where available), and *Tulasnella* vouchers. Letters in brackets indicate primer pairs (a = ITS1F/ITS4, b = ITS1OF/ITS4OF, c = ITS1/ITS4Tul) that amplified particular OTUs. The values above the branches denote Bayesian posterior probabilities. The tree was rooted with three Cantharellales taxa (*Ceratobasidium cornigerum*, *Multiclavula vernalis*, and *Hydnum albidum*).

Fig. S3. Rarefaction curves of OTUs (based on 97% sequence similarity) of potentially mycorrhizal fungi in individuals of four cytotypes/taxa of the *Gymnadenia conopsea* group (i.e. diploid *G. densiflora*, diploid and tetraploid *G. conopsea*, and triploids), based on adults from the regional (blue) and the microsite (red) samplings, and mycorrhizal seedlings (green).
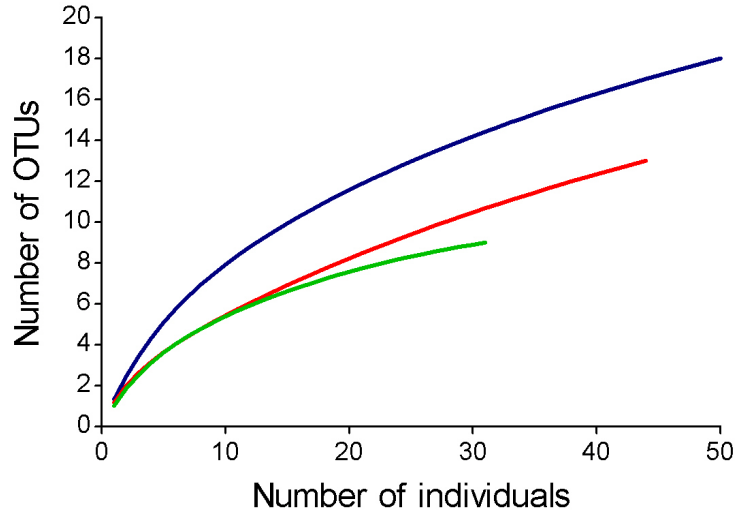
Table S1. Fungal OTUs associated with 2-year-old protocorms from different microsites at Zahrady pod Hájem and Jazevčí sites.

Site	Year of seed sowing	Microsite code[a]	Dominant cytotype within a 1-m radius[b]	Protocorm ploidy (n) (Mother-Father)[c]	Tulasnellaceae OTU 1	OTU 2	OTU 4	OTU 6	OTU 8	OTU 15	OTU 24	OTU 25	Ce. OTU 10
Zahrady pod Hájem	2006	M1A	2xGd	2xGd	1								
		M3A	4xGc	2xGc							1		
				2xGc									1
				2xGc							1		1
		M3B	4xGc	2xGc (2)									2
		M4B	4xGc	2xGc						1			
		M5A	2xGc	4xGc		1							
		M6trial	2xGc	2xGd (3)	3								
				4xGc (2)		2							
		M7B	mix of all	2xGc					1				
		M8A	mix of all	2xGd							1		
				2xGc									1
				4xGc (2)									2
		M8B	mix of all	2xGc							1		1
				2xGc									1
				4xGc									1
				4xGc									1
	2007	1	2xGd	2xGd (2xGd-2xGd)								1	
		2	mix of all	3x (2xGc-4xGc)					1				
		3	2xGc	4xGc (4xGc-4xGc)		1							
Jazevčí	2007	1	4xGc	3x (2xGc-4xGc)		1							
				3x (2xGc-4xGc)		1							
				3x (2xGc-4xGc)		1							
				3x (4xGc-2xGc)		1							
		2	2xGc	3x (4xGc-2xGc)			1						
		3	4xGc	2xGc (2xGc-2xGc)				1					

[a] Microsites used for sowing in 2006 were divided into two 0.5 m distant subplots (A and B).
[b] Dominant flowering adult cytotype(s) near sowing microsites; note that vegetative and dormant individuals of other ploidies could possibly also be present.
[c] Seeds sown in 2006 and 2007 originated from spontaneous and hand pollination, respectively (ploidy of parental individuals in brackets); *n* provided if > 1.
Each row represents a separate seed packet.
Ce. Ceratobasidiaceae.
2xGd, 2x *G. densiflora*; 2xGc, 2x *G. conopsea*; 3x, triploid; 4xGc, 4x *G. conopsea*.

Table S2. Fungal OTUs obtained by cloning with ascomycete specific primer pair ITS1F/ITS4Asco and their occurrences in nine individuals of the *Gymnadenia conopsea* group from Zahrady pod Hájem site.

OTU	GenBank Accession number	Sequence length (bp)	Taxonomic position	Closest taxonomically informative match in GenBank	Max. identity (%)	E-value	2xGd (n=2)	2xGc (n=2)	3x (n=3)	4xGc (n=2)
OTU 29	KC243960	601	Perizaceae	*Peziza pseudoviolacea* voucher 16504 (JF908564)	97	0		1		
OTU 30	KC243961	580	Cordycipitaceae	*Cordyceps* sp. 97005 (AB044636)	99	0	2	1	2	2
OTU 31	KC243962	575	Cordycipitaceae	*Beauveria bassiana* strain G61 (GU566276)	100	0	2	1	1	
OTU 32	KC243963	550	Nectriaceae	*Ilyonectria robusta* strain CBS 117814 (JF735265)	100	0		1	1	1
OTU 33	KC243964	593	Pleosporales	Uncultured Pleosporales isolate 1 (DQ182451)	97	0	1			
OTU 34	KC243965	560	Davidiellaceae	*Davidiella macrospora* strain CBS 138.40 (EU167591)	99	0	1		1	
OTU 35	KC243966	594	Saccharomycetales	*Candida* sp. DBVPG 5510 (JN688670)	92	$2e^{-33}$		1		
OTU 36	KC243967	710	Pezizomycotina	Uncultured leotiomyceta clone 7_a18 (HQ211920)	90	0		1		1
OTU 37	KC243968	635	Chaetothyriales	Uncultured Chaetothyriales clone 9J6S58N (HQ389454)	85	0		1		
OTU 38	KC243969	617	Chaetothyriales	Uncultured Chaetothyriales clone 9J6S58N (HQ389454)	87	0			1	
OTU 39	KC243970	609	Herpotrichiellaceae	*Cladophialophora modesta* strain CBS 985.96 (GU225939)	87	0	1			
OTU 40	KC243971	634	Herpotrichiellaceae	Uncultured Herpotrichiellaceae (HE605255)	99	0			1	
OTU 41	KC243972	640	Herpotrichiellaceae	*Exophiala* sp. Ppf18 (GQ302685)	98	0				1
OTU 42	KC243973	636	Herpotrichiellaceae	Uncultured *Exophiala* clone NG_T_D10 (GU055730)	99	0				1
OTU 43	KC243974	544	Sclerotiniaceae	*Botryotinia fuckeliana* isolate Bot. 1283 (EF207415)	99	0			1	
OTU 44	KC243975	557	mitosporic Ascomycota	*Tetracladium furcatum* strain CCM F-11883 (EU883432)	89	0	1			
OTU 45	KC243976	560	mitosporic Ascomycota	Uncultured *Tetracladium* clone NG_R_H03 (GU055705)	99	0				1
OTU 46	KC243977	637	mitosporic Ascomycota	*Leptodontidium* sp. Papocht 07 (HQ731635)	99	0		1	1	1

Taxonomic position refers to GenBank taxonomy.
2xGd, 2x *G. densiflora*; 2xGc, 2x *G. conopsea*; 3x, triploid; 4xGc, 4x *G. conopsea*.

Table S3. Fungal OTUs associated with adults of four cytotypes/taxa collected at ten sites in the White Carpathians Mts. Both single- and mixed-ploidy populations were studied.

Site	Ploidy (*n*)	Tulasnellaceae												Ceratobasidiaceae				Ly.	Pe.
		OTU 1	OTU 2	OTU 3	OTU 4	OTU 5	OTU 6	OTU 7	OTU 11	OTU 13	OTU 15	OTU 24	OTU 26	OTU 9	OTU 10	OTU 12	OTU 14	OTU 22	OTU 23
Zahrady pod Hájem	2xGd (3)	3																	
	2xGc (3)	2				1													
	3x (7)	5	1	1															1
	4xGc (3)		1	2	1														1
Jazevčí	2xGc (3)						2							2	1				
	4xGc (3)		1				2							2		1			
Babiratka	2xGc (3)							3											
	4xGc (3)		2							1									
Machová	2xGd (3)	3																	
Stráni	2xGd (3)	1					2												
Čertoryje	2xGc (3)				1		1	1	1								1		
Pod Lipinkou	2xGc (3)							3				1	1		1				
Velká Javořina	2xGc (3)							3				1						1	
	3x (2)							2											
Hustopeče	4xGc (2)		1					1			1								
Veterník	4xGc (3)		3	1															

Numbers indicate counts of plants in which a particular OTU was detected.
Ly., Lyophyllaceae; Pe., Pezizaceae.
2xGd, 2x *G. densiflora*; 2xGc, 2x *G. conopsea*; 3x, triploid; 4xGc, 4x *G. conopsea*.

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Table S4. Fungal OTUs associated with adults of four cytotypes/taxa collected at 11 microsites at Zahrady pod Hájem.

Microsite code	Ploidy (*n*)	Tulasnellaceae							Ce.	Pezizaceae			Se.	Hy.
		OTU 1	OTU 2	OTU 3	OTU 6	OTU 16	OTU 17	OTU 19	OTU 10	OTU 18	OTU 20	OTU 21	OTU 27	OTU 28
4	2xGd (1)	1												
	2xGc (2)	2		1										
	3x (1)	1												
	4xGc (2)		1					1						
5	2xGc (1)	1												
	3x (2)	1	1											
	4xGc (2)		2											
6	2xGc (2)	2												1
	3x (1)	1												
	4xGc (1)	1	1											
9	2xGd (1)	1												
	2xGc (2)	2												
	4xGc (2)		2		1									
14	2xGd (1)	1												
	2xGc (1)	1												
	4xGc (1)		1											
15	2xGc (1)	1												
	4xGc (1)		1										1	
16	2xGc (3)						2		1	1		1		
	4xGc (1)		1											
17	2xGd (1)	1												
	2xGc (1)	1												
	4xGc (1)										1			
18	2xGc (1)			1										
	3x (1)	1		1										
19	2xGd (1)	1												
	2xGc (2)	1									1			
	4xGc (2)		2											
20	2xGd (1)	1												
	2xGc (1)	1												
	4xGc (3)					3								

Numbers indicate counts of plants in which a particular OTU was detected.
Ce., Ceratobasidiaceae; Se., Serpulaceae; Hy., Hymenochaetaceae.
2xGd, 2x *G. densiflora*; 2xGc, 2x *G. conopsea*; 3x, triploid; 4xGc, 4x *G. conopsea*.

**Evolutionary shift from autotrophy to mycoheterotrophy in
the orchid genus *Neottia* is associated with a change
of *Sebacina* mycobionts**

Těšitelová T, Kotilínek M, Jersáková J, Joly F-X, Košnar J, Tatarenko I,
Selosse M-A (submitted manuscript)

Těšitelová T, **Kotilínek M, Jersáková J, Joly F-X, Košnar J, Tatarenko I, Selosse M-A: Evolutionary shift from autotrophy to mycoheterotrophy in the orchid genus *Neottia* is associated with a change of *Sebacina* mycobionts.**

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Summary

- Plant dependence on fungal carbon (mycoheterotrophy) evolved repeatedly. The evolutionary pathways to chlorophyll loss are blurred by frequent absence of green species within genera with full mycoheterotrophs.

- We focused on the genus *Neottia* (Orchidaceae) which comprises both green and non-green species. We analyzed mycorrhizal associations in green *N. ovata* and *N. cordata* from 41 sites covering a broad geographic scale, different habitats, and two developmental stages and also investigated their carbon and nitrogen nutrition using stable isotopes. Additionally, we identified mycorrhizal symbionts of the greenish but leafless *N. camtschatea* and analyzed phylogenetic relationships among five available *Neottia* species, including a non-green *N. nidus-avis* and green *N. smallii*.

- The green *N. ovata* and *N. cordata* predominantly associated with non-ectomycorrhizal Sebacinales Clade B (35 operational taxonomic units; OTUs), regardless of the developmental stages, geographical localization or habitat type, although the Sebacinales symbionts of both species did not overlap. 23 OTUs of other rhizoctonias and ectomycorrhizal fungi were found. Stable isotope abundances indicated dominant carbon gain via photosynthesis, even in shaded forest environments. *Neottia camtschatea* associated with ectomycorrhizal Sebacinales Clade A and *Geopora* and formed a monophyletic group with non-green *N. nidus-avis*.

- In the genus *Neottia*, association with non-ectomycorrhizal Sebacinales Clade B and prevailing autotrophy seem ancestral, while secondary evolution to mycoheterotrophy is linked to a shift to ectomycorrhizal Sebacinales Clade A. Such a shift in the ecology of mycorrhizal associates within the same fungal taxon is for the first time documented in orchids and may represent one of the pathways towards mycoheterotrophy.

Tamara Těšitelová participated in planning and conducting of the field sampling and was responsible for organizing the molecular analyses, for analyzing the data, and writing the manuscript.

CHAPTER VI

Summary of the results

SUMMARY OF THE RESULTS

The main objective of this thesis was to contribute to better knowledge of mycorrhizal and other root-inhabiting fungi of several orchid species. In the thesis, I tried to uncover how these cryptic associations may translate into above-ground behaviour of the orchids, including ecological preferences, distribution, or evolution.

As expected, in case of the meadow orchids studied, *Pseudorchis albida* (*Chapter II*, *IV*) and *Gymnadenia conopsea* group (*Chapter V*), we found mycorrhizal symbionts belonging to rhizoctonia, specifically to Tulasnellaceae. In case of the forest *Epipactis* species, we have further broadened the knowledge of mycorrhizal symbionts in this genus, which (very rarely among orchids) belong to ectomycorrhizal Ascomycota even in presumably little photosynthetic *E. purpurata* (*Chapter III*). The mycorrhizal symbionts of forest *Neottia cordata* and forest and meadow species *N. ovata* could be regarded as the most interesting finding (*Chapter VI*) – despite their inclusion in the Neottieae tribe which comprises mainly forest species associated with ectomycorrhizal fungi and close relatedness to non-green orchids, we found that these *Neottia* species associate (similarly to meadow species) with saprotrophic or endophytic rhizoctonia, specifically Sebacinales Clade B. Sebacinales Clade B are common endophytes of many plant species as well as mycorrhizal fungi in ericoid plants worldwide. Nevertheless, they were never found as dominant mycorrhizal symbionts of European orchids.

Naturally-grown seedlings are the least studied stages in orchid life-cycle owing to difficulties with obtaining them. Mycorhizal symbionts of seedlings may, however, sometimes differ from those in adult individuals. In all studied orchids, we found that seedlings associated with identical fungi as adult plants (*Chapter II*, *III*, *V*, *VI*).

Besides dominant mycorrhizal symbionts, we detected a variety of other fungi – these are interesting from (at least) three points of view described in this thesis: (i) the endophyte diversity which is usually much higher than in case of mycorrhizal fungi (*Chapter IV*) and may indicate orchid roots as a specific environment suitable for endophyte presence; however, this group is largely aside the main research interest and many such data are not presented in the research papers. Thus, it is difficult to judge, whether orchids (or particular orchid species) are exceptional in their endophyte diversity or roots of other plant species are similarly colonized. (ii) repeated occurrence of putative endophytes over broader geographic scale – in roots of several *Gymnadenia* adults we found different Pezizaceae species (Pezizales, Ascomycota) (*Chapter V*). Pezizaceae were found also in *Gymnadenia* adults in Germany opening the

chance that this unexpected fungal family may have some (even mycorrhizal) function in rhizoctonia-associated *Gymnadenia*. (iii) irregular and rare occurrence of presumably mycorrhizal fungi, including rhizoctonia, ectomycorrhizal, or ericoid mycorrhizal species along with dominant mycobionts (*Chapter II*, *III*, *V*, *VI*) – their function, either for mycorrhiza formation in orchids or presence as symptomless endophytes, or potential for formation of common mycelial network stays fully unknown.

Mycorrhizal fungi were shown to influence spatial distribution of different orchid species – i.e. divergent mycorrhizal communities lead to clustered distribution of adults. This was found also for different cytotypes of the *Gymnadenia conopsea* group (*Chapter V*). Different cytotypes often hybridize together and their co-existence in contact zones is often insufficiently understood: it is usually attributed to change of ecological preferences, pollinator community, phenology, or reproduction mode. The influence of different mycorrhizal fungi was little studied. Although mycorrhizal fungi cannot influence hybridization, in case of *Gymnadenia*, the clustered distribution of adults sustained by divergence in mycorrhizal symbionts may act in concert with a shift in phenology. Altogether this may decrease the mating frequency between different cytotypes and lead to cytotype establishment. The associations of fully mycoheterotrophic species in the genus *Neottia* seem to be another example of influence of mycorrhizal fungi orchid evolution (*Chapter VI*). Basal *Neottia* species are likely fully autotrophic in adulthood and associate with Sebacinales Clade B. Sebacinales can be separated into two clades, Clade B comprising mainly endophytic and ericoid mycorrhizal fungi and Clade A mainly with ectomycorrhizal fungi. Clade A was found in likely derived non-green (*N. nidus-avis*) and greenish, leafless (*N. camtschatea*) species. The association with Sebacinales Clade B in the basal *Neottia* species could serve as a predisposition to evolution of full mycoheterotrophy, because a shift to closely related fungi only with changed ecology could be more feasible. Similar shift in symbiont ecology but within the same fungal taxon was observed also in green and non-green Aneuraceae (liverworts).

The role of mycorrhizal fungi in determination of ecological preferences of orchids is even less understood, and virtually nothing is known about ecological preferences at the seedling stage. In case of calcicolous *Epipactis atrorubens* we found that the ecological niche may be broader at the seedling stage compared to adults, and that the distribution of mycorrhizal fungi was not limited in diverse forest types with different soil conditions (*Chapter III*). In this case, (ecto)mycorrhizal fungi identity little influences

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orchid ecology which may be shaped by other factors affecting later ontogenetic stages.

This work revealed several novel aspects of interactions between orchids and mycorrhizal fungi and raises a number of questions worth of future investigation, such as what is the function of the endophytes in the roots? What underlies the ploidy-specific interaction? Is the symbiosis with an unsuitable fungus followed by impaired growth or is it totally avoided? Is the mycorrhizal fungi distribution unlimited in different habitat types? What are the reasons for orchid ecological specializations? Do the findings in four *Neottia* species apply to the whole genus? Was the predecessor of Neottieae autotrophic, and mycoheterotrophy arose repeatedly or was it partially mycoheterotrophic, and several genera reversed back to autotrophy?

The thesis represents rather a set of pilot studies in little investigated directions, and further research should evaluate its general value. With the prompt development of new high-throughput sequencing technologies, many of these questions, especially those regarding fungal distribution in soil or fungal communities in roots, become accessible to answer for reasonable time and financial costs. Detailed sequencing, however, does not help to understand the function of species-rich fungal communities often detected in orchid roots, and more studies using *in vitro* germination with cultivated fungi would certainly bring valuable additions, such as on the function of endophytes or reaction of different ploidies on different mycorrhizal fungi. Unfortunately, many orchid species and fungi are hard to germinate *in vitro* and many experiments may be impossible to achieve in such controlled conditions. In this respect, the seed-baiting techniques allow monitoring of the orchid growth of such 'difficult' species under nearly natural conditions. Unfortunately, the nylon mesh usually used for inserting the seeds in soil limits advanced orchid growth and the seeds start to decay after some time. Thus, the monitoring is possible only for a limited time (up to *ca* four years) while it may take even a decade before the orchid appears above ground. Our views on orchid establishment are thus based only on very early developmental stages, and the aspect of a transition to adulthood accompanied by complete physiological change is entirely unexplored, although it may be of crucial importance for population establishment and potentially explain reasons for habitat specialization. This problem is certainly difficult to solve, but the use of slowly degradable materials for seed insertion allowing germination monitoring in early stages but not constraining advanced growth or long-term monitoring of experimental plots with freely sown seeds could bring further advancement. Certainly, we

can await many exciting discoveries on these aspects of orchid biology in near future.