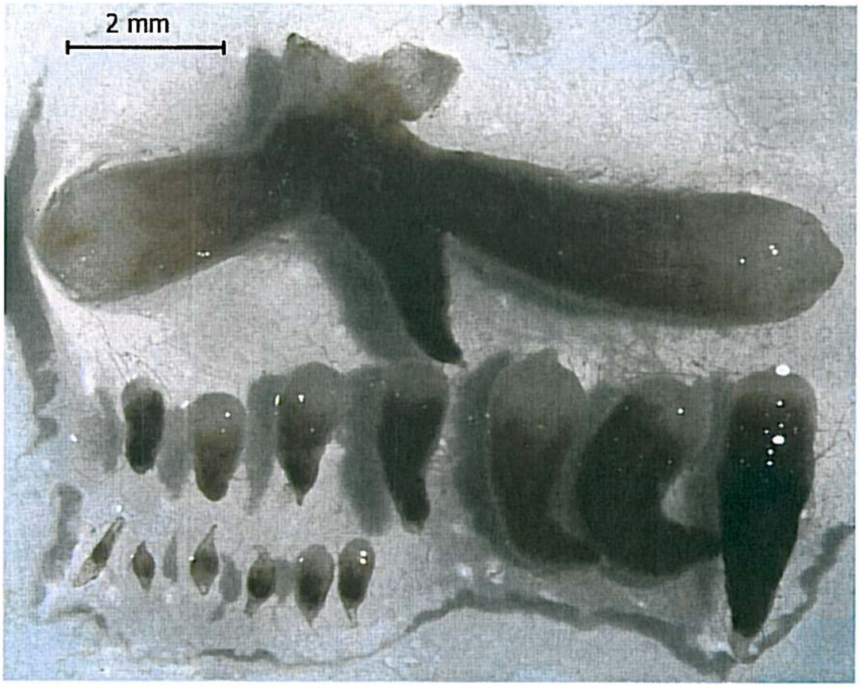
University of South Bohemia
Faculty of Science

Master thesis

Germination ecology in orchids



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České Budějovice 2009

Malinová T. 2009. Germination ecology in orchids [M.Sc. thesis; in English]. Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

Germination ecology of four *Epipactis* species (*E. albensis*, *E. atrorubens*, *E. helleborine*, *E. purpurata*) was studied. Habitat preferences of adult plants were analyzed using phytosociological relevés from the Czech Phytosociological Database. A field experiment was carried out to determine course of germination of *Epipactis* seeds sown in different habitat types. Relationship between ecological preferences and germination ecology, and spatial aspects of seed dispersal and seedling recruitment are discussed.

This study was supported by "Mattoni Awards for Studies of Biodiversity and Conservation Biology" in 2007 (investigator: Tamara Malinová), by grant GA AV IAA600870802, 2008-2011 (investigator: Jana Jersáková), by Socrates-Erasmus exchange program and by grant LC 06073 of the Czech Ministry of Education (investigator: Pavel Kindlmann).

Prohlašuji, že jsem tuto práci vypracovala samostatně, pouze s použitím citované literatury.

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V Českých Budějovicích, dne 6. ledna 2009

Acknowledgement

It is great pleasure for me to thank to all people who helped me in any way to deal with this work. In particular, I would like to thank to Jana Jersáková and Marc-André Selosse for their essential and concerned guidance during my studies. I am grateful to Jakub Těšitel for his devoted support in every moment helping me to overcome severe whiles in French molecular lab or during writing this thesis, and for help with statistical analysis of my data. I thank very much to my family, especially my mother, for great moral and south Moravian food support, as it many times helped to overcome moments of missing inspiration during writing this thesis. I really appreciate the help of Jarda Rydlo with studies on *Epipactis albensis* and I cross fingers for poplar trees in Libický Luh. I thank to Gabriela Říhová for help in molecular lab, and also to Mélanie Roy, Marrie-Pierre Dubois, Chantall, Sofie Veran and other French and Moroccan people who made my stay in Montpellier more pleasant.

Preface

This thesis deals with aspects of seedling recruitment and population establishment in orchids. Regarding the nearly unlimited orchid seed multiplication (producing up to 4 million seeds per a capsule) and the rareness of adult plants, the recruitment is expected to play a crucial role in an orchid life-cycle indicating some constraints during development. However, the germination ecology in orchids used to be difficult to study due to miniature size of the seeds. The long time applied cultivation under laboratory conditions however have not fully resolved this problem as seeds of many orchid species are difficult to germinate *in vitro*, and the observations on the early developmental stages of these species presented rather anecdotal findings in soil. The recent breakthrough method for controllable cultivation of dust-like seeds under natural conditions developed by Hanne Rasmussen and Denis Whigham facilitated not only examination of the germination course, but also evaluation of fine-scale patterns in orchid recruitment. Having seeds with insufficient nutrient reserves, the inevitable dependency upon fungal nutrition is another peculiar feature in the orchid establishment. However, the identification of mycorrhizal partners based on morphology of *in vitro* cultivated isolates or even only on light microscopic observations of fungal structures in roots was often infeasible or insufficient. The wide application of molecular techniques in ecological experiments and advances in methods of microscopic observation (such as electron microscopy) helped substantially to overcome this problem. A combination of these above mentioned techniques enabled significant advancement in our knowledge on principles of population establishment, relationship with mycorrhizal partner or influence of environmental conditions on seed germination.

Despite wide use of seed cultivation methods and large interest in studying orchid recruitment, there are still many unresolved topics such as: to what extent do the abiotic factors or distribution of mycorrhizal partners influence germination success and population establishment? What is the small- and large-scale distribution of orchid mycorrhizal fungi? Is there any influence of ecological factors on mycorrhizal specificity? At what stage of ontogenetic development is the bottleneck of growth expressed? What is the pattern of specificity to a mycorrhizal partner in related species? Do they share fungal partners? The effort to find answers to these questions is challenging not only from evolutionary but also from conservation point of view, as many orchid species belong to highly endangered species and understanding their ecological requirements is crucial for conservation and management of orchid habitats or *ex situ* propagation of threatened orchid species.

In the first part of this thesis, I tried to combine the *in situ* seed cultivation approach and molecular identification of symbiotic fungi to help resolve some of these intriguing topics. I focused on ecology of four *Epipactis* species. I tested their ability to germinate in distinct habitats and identified the fungal symbionts involved. Further, I discuss the possible influence of abiotic factors and landscape-level distribution of mycorrhizal partners on the orchid establishment. In the second part, we discuss the fine-scale aspects of orchid recruitment.

Part I.

Do habitat preferences of adult plants determine germination potential in orchids? A comparative study of four *Epipactis* species.

a manuscript by Tamara Malinová, based on cooperation with Jakub Těšitel, Jana Jersáková, Gabriela Říhová and Marc-André Selosse

Abstract

The orchids with windborne seeds have fast colonization potential, thus the presence or absence of a species might be considered as a manifestation of ecological preferences of species. We included four *Epipactis* species in an extensive seed sowing experiment, which demonstrated broad germination potential even in species with distinct ecological requirements at multiple sites where adult congeners grow. The rate of development in *E. albensis* and *E. purpurata* was very low over 23 months of soil cultivation suggesting a delay in ontogenetic development. Ecologically specialized *E. atrorubens* grew beyond initial germination stage at all study sites, suggesting a potential bottleneck caused by abiotic factors during transition into maturity. As expected, the ecological generalist *E. helleborine* germinated well in all forest types. A detailed study of fungal symbionts in *E. atrorubens* and *E. helleborine* showed that both species associated very similar spectrum of ectomycorrhizal fungal species over all developmental stages, showing clear preference for strains from Pyronemataceae and Tuberaceae families over considerable ecological and geographical range. The distribution of mycorrhizal partners thus does not seem to limit population establishment in various habitats.

Introduction

The orchid family is characterized by mass production of miniature "dust-like" seeds (Rasmussen 1995), which allows easy transportation by wind and decreases dispersal limitation (Shefferson *et al.* 2008). Yet, orchid seeds contain minimal nutrient reserves insufficient for growth, and the successful establishment is fully dependent on external supply of energy by a mycorrhizal fungus (Smith & Read 2008). Thus, all orchids are obligate mycoheterotrophs at least during their initial developmental stages, regardless later photosynthetic dispositions of adults (Leake 1994). The orchid associated fungi represent lineages with diverse trophic strategies, but the two main include saprophytic species from the polyphyletic complex *Rhizoctonia* agg. (including Tulasnellaceae, Ceratobasidiaceae and Sebacinaceae) and ectomycorrhizal (ECM) species from diverse families predominantly of Basidiomycota, but also of Ascomycota (reviewed in Dearnaley 2007). Symbiosis with rhizoctonian strains is typical for fully autotrophic species of open habitats (Rasmussen 2002). Non-green fully mycoheterotrophic and some green mixotrophic (i.e. combining autotrophic and heterotrophic nutrition) forest growing orchids associate with ECM fungi, forming a tripartite symbiosis with surrounding trees via a shared mycorrhizal fungus (e.g. Taylor & Bruns 1997, Selosse *et al.* 2004), with the trees being the ultimate energy source (McKendrick *et al.* 2000).

Numerous terrestrial orchids are recognized for their specialization to particular habitats such as wet meadows, calcareous dune slacks, nutrient poor fens, or distinct forest types (Procházka 1980, Delforge 2006). The ecology and availability of mycorrhizal partners was proposed to strongly influence these habitat preferences and determine the range of environmental factors for successful development (Rasmussen & Whigham 1998, Taylor *et al.* 2002, McCormick *et al.* 2004). Nowadays, little is known about the actual distribution of orchid mycorrhizal fungi within or among sites (Otero & Flanagan 2006). But in general, the composition of saprophytic and ECM fungal community seems to be influenced by multiple abiotic and biotic factors (Erland & Taylor 2002, Etema & Wardle 2002), such as soil litter quality, soil type, nutrient level, climate and also tree species composition in the case of ECM fungi (Ishida *et al.* 2007 and references herein). Consequently, the orchid occurrence might be constrained by distribution of fungi. Thus, the ability to associate with broader range of mycorrhizal fungi increases the probability of finding a suitable mycobiont and colonization of wider range of habitats (Bonnardeaux *et al.* 2007). However, narrow specialization to an ECM partner is supposed to allow the mycoheterotrophic plant highly efficient exploitation of a fungal host, due to higher physiological compatibility (Bruns *et al.* 2002). This ability to effectively exploit surrounding trees for energy is likely to release the orchids from competition for light with other plant species, and allows growth in shady habitats. Indeed, there seems to be a trend of negative correlation between fungal host range and dependency upon fungal nutrition (Taylor & Bruns 1997, Selosse *et al.* 2004, Julou *et al.* 2005, Girlanda *et al.* 2006, Bonnardeaux *et al.* 2007), although there are many exceptions from this pattern (McCormick *et al.* 2004, Shefferson *et al.* 2007). Most of recent studies focused on mycorrhizal associations of adult orchids; however a more detailed insight into mechanisms of symbiosis establishment and its ecological consequences is largely missing.

Considering the mass seed production and relative scarcity of adult specimens, the recruitment is likely to play a crucial role in orchid life-cycle. Several studies investigating the small-scale influence (within populations of orchid species studied) of biotic and abiotic factors on the germination success often revealed the importance of vicinity of adult conspecifics, but also various effects of moisture, organic content, soil acidity or potassium content (Batty *et al.* 2001, McKendrick *et al.* 2002, Diez 2007, Jacquemyn *et al.* 2007). Fungal host range was shown to decrease with the ontogenetic development indicating that multiple related fungal species may trigger initial germination, but only a subset of compatible ones supports advanced growth (Bidartondo 2005, Bonnardeaux *et al.* 2007, Bidartondo & Read 2008). In some cases, fungi supporting successful germination completely differed from those detected in the adults showing a complete switch of fungal partners during ontogeny (Xu & Mu 1990, McCormick *et al.* 2004). Thus, the necessity of certain fungal partner might cause a bottleneck during ontogeny and further reduce suitability of sites for the completion of plant's life-cycle (Bidartondo & Read 2008). However, it remains largely unknown, how species-specific ecological requirements pronounced by adults' distribution correlate with germination potential at landscape level. And to what extent is the germination success influenced by abiotic factors or fungal associations.

The application of cultivation-independent molecular techniques brought much light into determination of fungal symbionts of orchids, as it overcomes problems with cultivability of ECM strains (Taylor *et al.* 2002, Bidartondo *et al.* 2004, McCormick *et al.* 2004), and taxonomic identification of lineages of the anamorphic form-genus *Rhizoctonia* (Rasmussen 1995, Taylor *et al.* 2002). However, molecular identification reveals whole spectra of fungi present without any information on functional status of the fungi detected. Hence, it is advisable to combine molecular assessment with further evidences such as microscopic observations or cultivation assays.

Genus *Epipactis* (Neottieae tribe) comprises numerous species with different ecological requirements, and hence provides great opportunity for a comparative study. It encompasses mostly forest-dwelling rhizomatous species with predominantly Eurasian and North American distribution (Pridgeon *et al.* 2005). Identification of fungi associated with *Epipactis* by molecular techniques (e.g. Bidartondo & Read 2008, Ogura-Tsujita & Yukawa 2008) together with detailed microscopic observations (peloton formation – a typical structure in orchid mycorrhiza – was confirmed by electron microscopy for ECM ascomycetes in Selosse *et al.* 2004; by light microscopy reported for basidiomycetes by Salmia 1988), and isotopic measurements (Gebauer & Meyer 2003, Bidartondo *et al.* 2004) show that *Epipactis* species are mixotrophic at maturity associating mainly ECM fungi (*E. palustris* is the only exception, associating *Rhizoctonia* strains; Rasmussen 1995). The *Epipactis* species were reported to associate with relatively broad range of fungi composed predominantly of ECM ascomycetes, several ECM basidiomycetes, and few *Rhizoctonia* strains (Bidartondo *et al.* 2004, Selosse *et al.* 2004, Ogura-Tsujita & Yukawa 2008). Bidartondo & Read (2008) found *E. atrorubens* to be dependent on ECM fungi also during early developmental stages. Thus, all ECM and *Rhizoctonia* strains can be considered as potentially mycorrhizal in *Epipactis* studied, although the functional status of the symbioses would have to be confirmed by cultivation experiments.

We focused on four *Epipactis* species, of which three grow in distinct forest types with specific soil conditions, while the forth one is an ecological generalist. *E. albensis* is a tiny autogamous species derived from *E. helleborine* agg. This Central European endemic species typically grows in extensive floodplain forests, streamside vegetation or poplar alleys in immediate vicinity of *Populus nigra* or *P.* x *canadensis* (Rydlo 1989). *E. purpurata* is an allogamous species reported to grow in humid shady submontane beech and hornbeam forests on deep clayish neutral soils (Procházka 1980). *E. atrorubens* is an allogamous species confined to relatively dry and open forest types strictly on calcareous bedrock (Procházka 1980, Presser 2002, Delforge 2006). *E. helleborine* is a common allogamous species with wide ecological amplitude, growing in nutrient rich soils in forests, shrubs or partly disturbed vegetation from lowland floodplain forests to mountain spruce forests (Procházka 1980). *E. helleborine* is able to grow in various forest types, including those typical of the other species. The Ellenberg indicator (Ellenberg *et al.* 1992) values for the three studied species (*E. albensis* was not categorized) support these trends and provide further estimate of ecological demands of the species (Table 1).

In this paper, we examined the relationship among ecological preferences, germination pattern and mycorrhizal associations in the four *Epipactis* species. We performed analyses of phytosociological relevés to confirm ecological preferences of the study species related to tree layer composition and abiotic conditions. We used a well-established method for *in situ* orchid seed cultivation developed by Rasmussen & Whigham (1993) to evaluate the species-specific germination rate. Our main goal was to reveal the relationship between specific ecology of adult plants and germination pattern of their seeds at different sites. For each species, we compared the germination potential between its preferred forest type and habitats typical of the other ecologically distinctive species. We also employed PCR-based molecular techniques to reveal mycorrhizal context of observed germination pattern.

Table 1: Ellenberg indicator values for *Epipactis* spp. as indicated in Ellenberg *et al.* (1992) on ordinal scale 1-10.

	Light	Temperature	Continentality	Moisture	Soil reaction	Nutrients
E. atrorubens	6	-	3	3	8	2
E. helleborine	3	5	3	5	7	5
E. purpurata	2	6	4	6	8	6

Materials and methods

Analyses of vegetation relevés

Forest type preferences of four *Epipactis* species were analyzed using phytosociological relevés extracted from the extensive Czech Phytosociological Database (Chytrý & Rafajová 2003). As *Epipactis* plants depend primarily on the ectomycorrhizal associations with trees, we excluded herb and moss species from the analysis and concentrated on tree and shrub cover in all vegetation layers. Plant species occurring only in one relevé were removed from the dataset. In total, we analyzed 181 relevés: *E. albensis* (13), *E. atrorubens* (73), *E. helleborine* (45) and *E. purpurata* (50). We used a linear discriminant analysis (LDA) to delineate the differences in the plant species composition among groups of relevés defined by the presence of individual *Epipactis* species.

The Ellenberg indicator values (Ellenberg *et al.* 1992) deliver a rating of environmental preferences for listed species, and in some studies they were directly used as indices of ecological preferences of mycoheterotrophic species (Gebauer & Meyer 2003, Bidartondo *et al.* 2004). However, to get more accurate insight into species ecology, it is better to use a large dataset, and infer the species ecology from the analysis of Ellenberg indicator values of accompanying plant species. This approach allows not only determination of ecological optima, but also estimation of the range of favorable conditions. The mean Ellenberg indicator values for moisture, light, nutrients, soil reaction, temperature and continentality were calculated for each relevé in the *Epipactis* dataset of the Czech phytosociological database. Except bryophytes and species, which were not categorized by Ellenberg *et al.* (1992), we involved all recorded plant species in the calculations but *Epipactis* spp. to prevent circular reasoning. Relative ecological preferences of the *Epipactis* species studied were consequently assessed by a canonical correspondence analysis (CCA). The Ellenberg indicator values entered the model as predictors and the response consisted of four dummy-variables indicating presence/absence of individual species. The depicted interspecific differences are only relative and do not equal real ecological preferences.

Sowing experiment

We selected up to three sites with a population of each *Epipactis* species throughout the Czech Republic (altogether seven sites) differing in the tree layer composition and soil substrate (for general site description and presence of *Epipactis* species see Table 2, for detailed description of the tree layer composition and soil characteristics see Table 3). We selected three sites of *E. helleborine* which resemble biotic and abiotic conditions at sites of its congeners in order to compare the recruitment success of *Epipactis* species at sites of seed origin (suitable sites, further called as home sites) to that at sites which have similar tree layer composition, but lack conspecific adults (putatively suitable sites). In this sense, both localities Alb and H2 represent a stand typical for growth of *E. albensis*, i.e. a poplar alley in the vicinity of an extensive flood-plain forest, but only the former is colonized by *E. albensis*, the latter hosts *E. helleborine*. Similarly, the sites Atr and H3, or P1, P2 and H1 represent suitable and potentially suitable sites for growth of *E. atrorubens*, or *E. purpurata* respectively. Consequently, we could compare the recruitment success at these (putatively) suitable sites and unsuitable sites (habitats typical of other specialized species). Soil samples from each study site (a mixture of 5 random replicates from 5 to 10cm soil depth) were analysed by standard analytic methods for levels of potassium, calcium, available phosphorus, and soil reaction (distilled water) in the certified laboratory at the Institute of Botany of the Czech Academy of Science in Třeboň.

At each study site, matured seeds from several *Epipactis* specimens were harvested in August and September 2004, and pooled together. The seeds were dried at room temperature and subsequently stored at 4°C until construction of the seed packets and their burial in mid October. Seed quality (i.e. proportion of seeds with well-developed embryo) was examined under a dissecting microscope. For the seed packet construction, we followed the sowing technique developed by Rasmussen & Whigham (1993). Approximately 300 well-developed seeds of *E. helleborine* or *E. purpurata*, 250 seeds of *E. atrorubens*, and 120 seeds of *E. albensis* were placed separately in a 1.5 x 3.5cm pocket (42μm nylon mesh; Silk & Progress Ltd) using a fine scoop, and enclosed into a plastic slide. The slides were marked by a coloured wire and permanent marker, and attached to a nylon line. We sew the seeds in a factorial design: at each locality, we buried 140 seed packets composed of 20 replicates of each *Epipactis* population, 980 seed packets in total. Within the site, the seed packets were placed into the soil in ten groups composed of 14 packets (2 packets per each *Epipactis* population), and attached to a metal peg enabling later recovery using a metal detector. The groups were placed randomly within a study site, but always near an adult *Epipactis* plant. The slides were buried vertically into the top soil layer using a garden knife. According to our preliminary observations (after 6, 9 and 10 months of soil incubation at sites H1, H3 and Atr), germination course in all species proved to be rather slow; therefore, one third of all plastic slides was retrieved after 12 month, and remaining slides after 23 months cultivation in the soil.

Table 2: Site description. At sites H1 and H2, specimens of *Cephalanthera damasonium* are present, at site P1 specimens of *Neottia nidus-avis*.

Study sites (Region)	Site code	Latitude/longitude	Elevation (m a.s.l.)	*Epipactis* sp. present	Plant code	Forest type
Libice n. Cidl. (C Bohemia)	Alb	50°07'N, 15°09'E	190	*albensis*	EAl	poplar alley
Čepice (W Bohemia)	Atr	49°16'N, 13°35'E	488	*atrorubens*	EAt	pine wood on limestone
Milovice (S Moravia)	H1	48°50'N, 16°42'E	218	*helleborine*	EH3	lime wood
Lednice (S Moravia)	H2	48°48'N, 16°49'E	158	*helleborine*	EH1	poplar alley
Kamenný Újezd (S Bohemia)	H3	48°54'N, 14°24'E	461	*helleborine*	EH2	mixed wood on limestone
Brno-Bystrc (S Moravia)	P1	49°13'N, 16°31'E	265	*purpurata*	EP1	hornbeam-lime forest
Milovice (S Moravia)	P2	48°50'N, 16°41'E	271	*purpurata*	EP2	hornbeam-lime forest

Table 3: Overview of soil characteristics and dominant tree species at study sites in context with overall germination rate of four *Epipactis* species. **A.** Proportion of germinated and mycorrhizal seedlings from the total number of sown seeds after 23 months of soil incubation. Values are based on counts of twelve to fourteen seed packets. In case the number exceeded 20%, it is denoted. **B.** The highest achieved developmental stage after 12 and 23 months of soil incubation. Red and green lines depict germination rate in seeds sown at their home sites and sites with similar tree layer composition to the home site, respectively. Abbreviations of *Epipactis* species: EAl = *E. albensis*, EAt = *E. atrorubens*, EH1-3 = *E. helleborine* (seeds from H1-3 sites), EP1-2 = *E. purpurata* (seeds from P1 and P2 sites). Abbreviations of tree species: Ace spp. = *Acer campestre* and *A. platanoides*, Ace cam = *Acer campestre*, Car bet = *Carpinus betulus*, Cor ave = *Corylus avellana*, Fra exc = *Fraxinus excelsior*, Pic abi = *Picea abies*, Pin syl = *Pinus sylvestris*, Pop xcan = *Populus* x *canadensis*, Pop tre = *Populus tremula*, Pru avi = *Prunus avium*, Til cor = *Tilia cordata*.

study site	K g/kg	Ca g/kg	P mg/kg	pH dH_2O	dominant trees	**A** stage	*Epipactis* species EAl	EAt	EH1	EH2	EH3	EP1	EP2	**B** months	*Epipactis* species EAl	EAt	EH1	EH2	EH3	EP1	EP2
Alb	5.0	6.5	35.3	7.2	*Pop xcan, Fra exc, Ace spp.*	germinated		23%		41%	22%			12							
						mycorrhizal								23							
Atr	1.5	288.4	24.9	7.8	*Pin syl, Pop tre*	germinated		54%	57%	66%	72%			12							
						mycorrhizal								23							
H1	5.3	29.8	16.2	7.9	*Til cor*	germinated		78%	78%	81%	85%			12							
						mycorrhizal		35%	21%		22%			23							
H2	5.9	38.2	84.7	7.9	*Pop xcan, Pru avi, Ace cam*	germinated		74%	77%	92%	90%			12							
						mycorrhizal								23							
H3	0.7	78.1	10.1	7.1	*Pic abi, Pin syl, Cor ave*	germinated				24%				12							
						mycorrhizal								23							
P1	1.7	4.7	9.9	5.2	*Til cor, Car bet, Pop tre*	germinated				43%	45%			12							
						mycorrhizal								23							
P2	3.2	2.7	7.7	5.6	*Til cor, Car bet, Pop tre*	germinated		29%	57%	73%	59%			12							
						mycorrhizal								23							

home site

site with similar tree layer composition

A
- 0%
- 0.01-0.99%
- 1-4.99%
- 5-9.99%
- 10-19.99%
- more than 20%

B
- ungerminated
- germinated, nonmycorrhizal
- small mycorrhizal < 0.5mm
- pear-shaped protocorm
- protocorm with primordium
- branched seedling

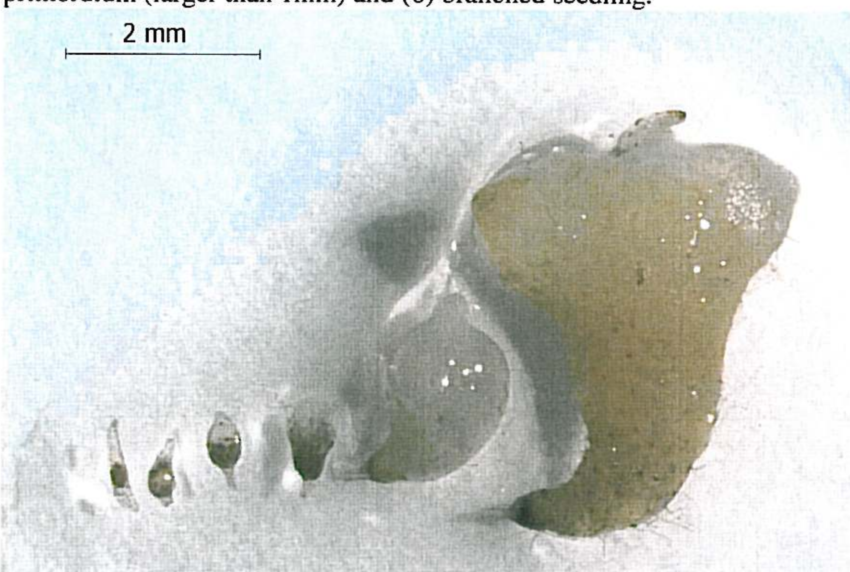
Evaluation of germination and root sampling

The recovered seed packets were kept moist at 4°C until processing within next few days. Prior to the opening the slides were carefully washed by running water to remove soil dirt. Each seed packet was examined under a dissecting microscope (45x magnification) and every seed was than categorized as (1) ungerminated or parasitized, (2) germinated but non-mycorrhizal, (3) small mycorrhizal seedling of oval shape, (4) pear-shaped seedling - protocorm (larger than 0.5mm), (5) seedling with leaf primordium (larger than 1mm), and (6) branched seedling (Fig. 1). All mycorrhizal seedlings were further examined under a high magnification dissecting microscope, and their length was measured using tpsDig software (Rohlf 2006). Seeds and non-mycorrhizal seedlings remaining on the nylon mesh were scanned at 2400dpi (Epson Perfection 1650) for subsequent counting. The germination rate after 23 months of cultivation was used for detailed statistical analysis.

We collected roots of two to four *Epipactis* adults at each study site (except for P2) during August and September 2005 and June and July 2006. In addition, roots of one specimen of *Cephalanthera damasonium* were collected at sites H1 and H2. The roots were carefully rinsed under tap water and surface was carefully cleaned of soil particles using a fine toothbrush. Subsequently, the roots were cut into 1cm long pieces, and thin cross-sections taken at each cutting were checked for mycorrhizal colonization under a dissecting microscope (45 x magnifications). Eight to ten randomly selected infected cross-sections per plant were pooled and analyzed for mycobiont identity together.

Both the mycorrhizal seedlings and root pieces were stored for transportation reasons in 55% ethanol up to 3 weeks, before recovering them for molecular analyses. Then, they were cleaned from external hyphae using fine tweezers, washed in distilled water, and kept at -20°C till DNA extraction.

Fig. 1: Growth categories in seedlings of *E. helleborine*: from left (1) an ungerminated seed, (2) a germinated but non-mycorrhizal seedling, (3) a small mycorrhizal seedling of oval shape (brown pelotons visible), (4) a pear-shaped seedling larger than 0.5mm, (5) a seedling with leaf primordium (larger than 1mm) and (6) branched seedling.



Molecular identification of fungal symbionts

We used standard molecular tools to analyze identity of fungi found in roots of adults *Epipactis* plants and in 23 months old seedlings of *E. helleborine* and *E. atrorubens*. The seedlings of the other two *Epipactis* species were not analyzed, as their numbers were negligible. In order to limit the number of analyses, we created two protocorm pools per *Epipactis* species at each site: (i) up to two small mycorrhizal seedlings (around 0.5mm in length) and (ii) up to two larger seedlings (above 1mm) per packet if possible. In addition, one to six particularly large seedlings per species at each site were analyzed separately. Seedlings sourced from different populations of *E. helleborine* were handled separately. This size approach allows recognizing potential changing, narrowing or switching in fungal endophytic spectrum during plant ontogeny, and it avoids potential bias in dominant mycorrhizal fungi of larger seedlings prevailing over less numerous fungi of small seedlings.

The fungal DNA was extracted from root pieces and seedlings using the DNeasy™ Plant Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's advice and the fungal internal transcribed spacer of ribosomal DNA (ITS) was amplified as in Selosse *et al.* (2002) using primers ITS1F and ITS4. Whenever a unique fragment occurred after amplification of a root pool or a large seedling, it was directly sequenced from both strands using ITS1F and ITS4. PCR products were purified by ExoSAP-IT (USB corporation) according to manufacturer's advice, and sequencing reaction was performed on an ABI3130xl sequencer (Applied Biosystems, Courtaboeuf), 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), according to manufacturer's advice but dividing all amounts of chemicals by five. Ligates were transformed into super-competent cells XL1-Blue (Stratagene, Amsterdam), in order to obtain at least twenty positive clones per PCR. Clones were submitted to PCR using ITS1F and ITS4, as previously, and to restriction fragment length polymorphism (RFLP) analyses using *Hinf*I + *Hae*III and *Hha*I + *Nde*II (Promega). Four to 12µl of PCR product was mixed with 0.5µl of each enzyme, buffer and BSA according to manufacturer's advice, and incubated at 37°C for 1 to 4 h. RFLP patterns were visualized on 3% agarose gels in 0.5x TAE buffer, and up to four clones per unique RFLP pattern were sequenced. Whenever sequences from a given cloning were more than 97% identical, a consensus was built. To check for the presence of Tulasnellaceae, common orchid partners with highly derived ITS sequence, the specific primer pair ITS1 and ITS4-Tul was used as in Selosse *et al.* (2004). Sequences were edited and assembled using ChromasPro, version 1.41 (Technelysium 2007). The presence of chimeric sequences resulting from cloning procedure was examined using BioEdit ver.7.0.4.1 (Hall 1999). In order to identify the putative taxonomical position and ecology of the fungus, the search for similar sequences was conducted using Blast (Altschul *et al.* 1997) at the NCBI page (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). To delimit putative species, we arbitrarily grouped together sequences that were more than 97.0% identical over the whole ITS region. Fungi potentially mycorrhizal in *Epipactis* species (i.e. ECM fungi and rhizoctonias) were then used for statistical analysis. We used genus level of fungal identity in the statistical analyses, as we were not able to create a phylogenetic tree due to highly variable and thus unalignable ITS regions which would allow e.g. phylogenetic independent contrasts (Felsenstein 1985).

Statistical analyses

We performed a mixed model ANOVA in order to evaluate overall germination rate. The identificator of locality entered this analysis as a random effect predictor. The proportions were arcsine transformed prior to the analyses to normalize their distributions. We used *post-hoc* contrasts to compare the germination rate of seeds cultivated at home site vs. the other sites; and seeds cultivated at their home site and a site with similar tree layer composition against the other sites.

Fungal spectra found at the localities and in different *Epipactis* species were analyzed by a canonical correspondence analysis (CCA). Partial CCA (pCCA) with locality identificators as covariates was used to test whether fungal spectra differed between seedlings of *E. atrorubens* and *E. helleborine*, and between adults and seedlings of *E. helleborine* at three home sites.

We used Statistica for Windows, version 8.0 (StatSoft 2008) for ANOVAs and other calculations, SigmaPlot for Windows, version 9.01 (Systat Software 2004) for graphical visualization and Canoco for Windows, version 4.53 (ter Braak & Šmilauer 2002) for the multivariate statistics.

Results

Ecological preferences of adults

The LDA ordination of vegetation relevés (Fig. 2A) and the CCA analysis based on Ellenberg indicator values (Fig. 2B) support the general view on ecology of four *Epipactis* species studied (Table 1). Consistently with presumed ecological preferences, the analyses segregated relevés with *E. albensis* on the basis of presence of *Fraxinus angustifolia*, *Populus nigra*, *P.* x *canadensis* and *Ulmus* sp. which are typical for nutrient rich and moist alluvial forests. Sites of *E. atrorubens* were delimited by the presence of Scotch pines (*Pinus sylvestris*). Their position in the CCA ordination plot is negatively correlated with moisture and nutrients level, but positively associated with light level. In contrast to predicted indicator values for this species (Table 1) most relevés are additionally correlated with higher soil alkalinity compared to the other species. The *E. purpurata* relevés are distinguished by hornbeam (*Carpinus betulus*) and beech (*Fagus sylvatica*) in the LDA analysis, in the CCA ordination space they occupy central part being correlated positively with nutrients, moisture and negatively with light level, continentality and soil reaction. *E. helleborine* relevés occupy the central part of both LDA and CCA ordination diagrams indicating no distinctive ecological preferences relative to other *Epipactis* species. Despite putative differences in tree species composition, some tree species occurred more or less frequently in relevés of all four *Epipactis* species, e.g.

Tilia cordata, *Carpinus betulus*, *Fraxinus excelsior*, *Quercus robur*, *Acer campestre* or *Corylus avellana*.

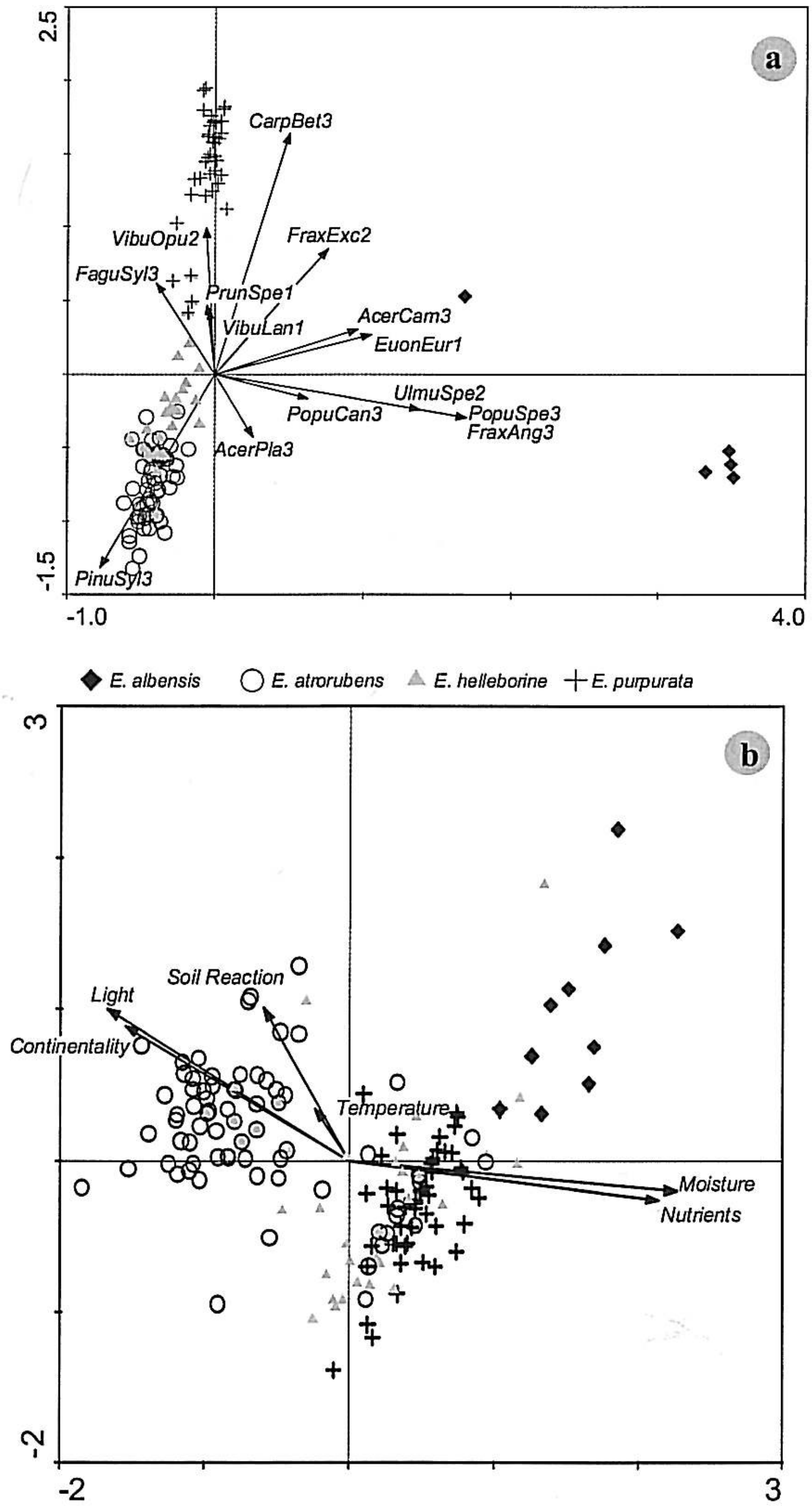


Fig. 2: A. Ordination plot of the linear discriminant analysis (LDA; first two canonical axes are shown) of phytosociological relevés with individual *Epipactis* species. Tree species name abbreviations: AcerCam = *Acer campestre*, AcerPla = *A. platanoides*, CarpBet = *Carpinus betulus*, EuonEur = *Euonymus europeus*, FaguSyl = *Fagus sylvatica*, FraxAng = *Fraxinus angustifolius*, FraxExc = *Fraxinus excelsior*, PinuSyl = *Pinus sylvestris*, PopuCan = *Populus* x *canadensis*, PopuSpe = *Populus* sp. (*P. nigra* or *P.* x *canadensis*), PrunSpe = *Prunus* sp., UlmuSpe = *Ulmus* sp., VibuLan = *Viburnum lantana*, VibuOpu = *Viburnum opulus*. A number behind the tree species abbreviation indicates vegetation layer (3 = tree layer, 2 = shrub layer, 1 = herb layer). The first two canonical axes explain 32.5% and 31.5% of total variability. Result of Monte-Carlo permutation test for all canonical axes: $F = 2.53$, $p < 0.001$ (999 permutations). **B.** Ordination plot of the canonical correspondence analysis (CCA; first two canonical axes are shown) of mean Ellenberg indicator values for phytosociological relevés with individual *Epipactis* species. The first two canonical axes explain 20.9% and 11.3% of total variability. Result of Monte-Carlo permutation test for all canonical axes: $F = 15.83$, $p < 0.001$ (999 permutations).

Two-year germination course of *Epipactis* species

Germination in all four *Epipactis* species studied proved to be rather slow. At harvest after 6 months, we observed no germination at all, but some germinating and small mycorrhizal seedlings were recorded after 9 months. Thus, the germination in *E. helleborine* and *E. atrorubens* started in spring approximately after 7 to 8 months of soil cultivation. After 12 months of soil incubation, numerous seedlings of *E. helleborine* and *E. atrorubens* achieved the stage of mycorrhizal pear-shaped protocorm (Fig. 3, Table 3B), while very few small mycorrhizal seedlings (<0.5mm) and no germination were observed in *E. albensis* and *E. purpurata*, respectively.

After 23 months of growth in soil, the differences in achieved germination stage among species were even more pronounced (Fig. 3, Table 3B). The fastest growth was recorded in *E. atrorubens*, with multiple seeds reaching stage (5) and (6) of seedlings larger than 1mm having leaf primordium or branching, respectively. The largest branched seedling of *E. atrorubens* spanned slightly over 1cm in length. Growth rate of *E. helleborine* was of similar intensity, but seedlings rarely reached size of those of *E. atrorubens*. In *E. albensis*, small mycorrhizal seedlings in stage (3) were observed this time, nevertheless, no protocorms in stage (4) larger than 0.5mm were recorded. In *E. purpurata*, germination onset was recorded this time. We observed small mycorrhizal seedlings of stage (3), and some seedlings growing further into stage (4) of a small pear-shaped protocorm.

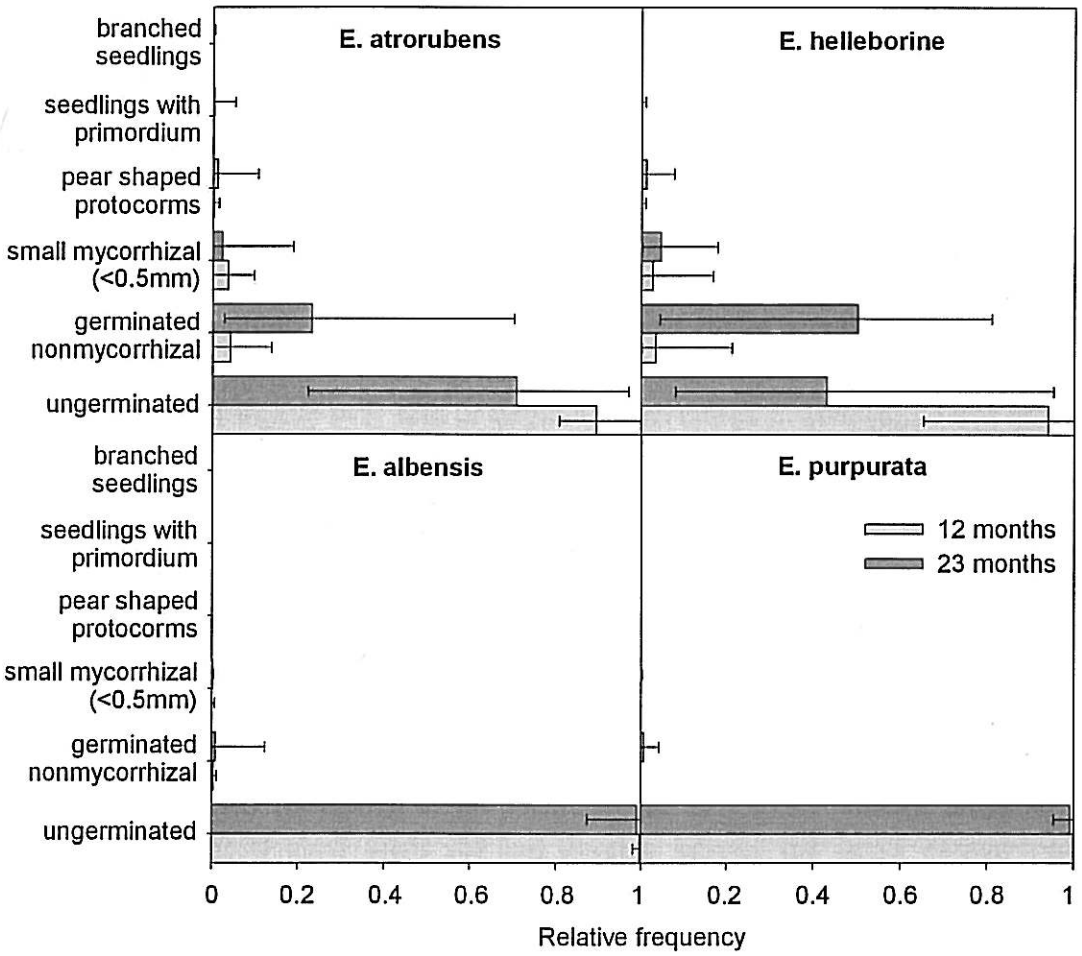
Proportion of germinating seedlings changed markedly between the two years (Fig. 3), as significant part of seeds did not germinate until 12 months. After 23 months the germination rate often exceeded 50% of the total amount of *E. atrorubens* and *E. helleborine* seeds sown per a site (compared to the maximum of 35% of germinated seeds after 12 months). Nevertheless, most seeds did not develop beyond the small non-mycorrhizal seedling stage and less than 20% of all seeds sown became mycorrhizal and grew further (with the exception of 35% of mycorrhizal seedlings of *E. atrorubens* at H1 site; Fig. 3, Table 3A). Germination rates in *E. albensis* and *E. purpurata* were rather low over the whole experimental period.

We also found large spatial heterogeneity in the germination success within a site. In *E. atrorubens* and *E. helleborine*, the difference in germination success sometimes ranged from 1 to 95% among packets of a single species. Nonetheless, at least some germination occurred in cca 90% of *E. atrorubens* and *E. helleborine* packets, and in 50% and 25% of *E. albensis* and *E. purpurata* packets, respectively, over all sites (for more detailed overview of germination rate in four *Epipactis* species and across study sites see Appendix A).

Pronounced differences in germination rate among *Epipactis* species are supported by significant ANOVA tests for overall germination rate, i.e. number of germinating seeds ($F_{3,18} = 17.82$, $P < 10^{-4}$), number of mycorrhizal seedlings ($F_{3,18} = 4.09$, $P < 0.05$) and the highest achieved developmental stage ($F_{3,18} = 38.77$, $P < 10^{-6}$). We also observed intraspecific variability in germination rate of seeds sourced from different populations of *E. helleborine*

($F_{2,12}$ = 12.77, P < 0.001) and *E. purpurata* ($F_{1,6}$ = 6.46, P < 0.05); and in the achieved developmental stage in case of *E. purpurata* ($F_{1,6}$ = 13.29, P < 0.05).

Fig. 3: Distribution of developmental stages in four *Epipactis* species after 12 and 23 months of soil incubation. Median and min-max values for localities are shown.



Germination rate at different sites

After 23 months in soil, we detected seed germination at all study sites and in all *Epipactis* species (Table 3A). Nevertheless, there were marked differences in germination support among study sites; the ANOVA tests were significant for overall germination rate ($F_{6,18}$ = 2.97, P < 0.05), number of mycorrhizal seedlings ($F_{6,18}$ = 2.75, P < 0.05) and the highest achieved developmental stage ($F_{6,18}$ = 4.53, P < 0.05). It is notable that at most localities there was a high level of initial germination, but only at some of them higher levels of mycorrhization and further seedling growth occurred (compare site H1 and site Atr).

We found slightly different germination patterns in the three ecologically specialized species over putatively suitable and unsuitable sites. *E. albensis* germinated at all sites with various forest types, despite adults growing exclusively in floodplain or similar forests, however, small mycorrhizal seedlings occurred only at the sites with poplars (i.e. home site Alb and the putatively suitable site H2) and in lime-hornbeam wood (at H1 and P2). *E. purpurata* successfully germinated at all the study sites but multiple mycorrhizal seedlings were found only at home sites (P1 and P2) where also some protocorms developed; and at site with similar tree layer composition (H1). Two small mycorrhizal seedlings of stage (3) < 0.5mm occurred also at site H3, which is a mixed forest. Calcicolous *E. atrorubens* germinated well and reached stages (5) and (6) of larger mycorrhizal seedlings at all study sites including poplar alleys (Alb and H2) or sites with low pH (P1 and P2). The ecological generalist *E. helleborine* also germinated successfully everywhere. Seedlings of advanced growth stages (5) and (6) were observed at all sites but the site Atr, where mycorrhizal seedlings occurred very rarely and no protocorms developed. A comparison of differences in both the species-specific germination rate and the number of mycorrhizal seedlings between the home sites and the other sites was insignificant ($F_{1,36} = 0.66$, $P < 0.45$; $F_{1,36} = 0.3$, $P < 0.6$); as was the comparison between the home site and site with similar tree layer vs. the other sites ($F_{1,36} = 1.36$, $P < 0.29$; $F_{1,36} = 2.14$, $P < 0.19$).

Fungal diversity in seedlings and adult plants

We succeeded to amplify fungal ITS in all adult plants and in 23 out of 26 seedling pools. Cloning and direct sequencing revealed 148 unique fungal ITS sequences. Beside putatively ECM lineages, the significant portion of ascomycetes and basidiomycetes detected belonged to fungal strains with saprobic or parasitic trophic strategies (Table 4, Appendix B). Sometimes we failed to find any ECM or rhizoctonian lineages in a seedling pool at all. When grouped according to their sequence similarity, we detected 21 and 24 putatively ECM lineages in the seedlings and roots of adult *Epipactis* plants, respectively. Most frequently detected ECM strains in seedlings and adults of all four *Epipactis* species (except *E. purpurata*) at all study sites belonged to ascomycetes in families Tuberaceae and Pyronemataceae, including strains of *Genea* (incl. an unknown strain from *Genea-Humaria* group), *Geopora, Geopyxis-Stephensia* lineage, *Trichophaea* (*T. woolhopeia* group, clade 7, according to Perry *et al.* 2007) and *Wilcoxina.* In addition we found one lineage of *Helvella* (Helvellaceae) in *E. helleborine* and *Hydnotria* (Discinaceae) in *E. purpurata.* Rarely we detected ECM basidiomycetes, which mostly belonged to Thelephoraceae, Hymenogastraceae, Russulaceae (mainly in *E. purpurata* adults) or Tricholomataceae families, one *Ceratobasidium* strain and two Sebacinaceae strains (both clade A and B; Weiss *et al.* 2004). In addition, multiple zygomycetes (*Mortierella* spp.) and two chytridiomycetes were detected. No other fungi were added using the *Tulasnella* specific primer ITS4Tul. Contrary to rather wide spectrum of ECM lineages detected in *Epipactis* species, co-occurring *Cephalanthera damasonium* adults associated exclusively with multiple strains of Thelephoraceae and Hymenogastraceae.

Table 4: Fungi detected in orchid roots and seedlings at seven study sites. Putatively non-mycorrhizal fungi are shown in italics. Mycorrhizal fungi occurring both in seedlings and adult *Epipactis* plants are shown in bold. Species occurring in both adults and seedlings within one site are shown in bold and underlined. Value in parenthesis shows the number of large seedlings in which a fungus was detected if this is higher than one. Grey fields indicate that seedlings of this category were not available. *Epipactis* species abbreviations: EAl = *E. albensis*, EAt = *E. atrorubens*, EH1-3 = *E. helleborine* (seeds sourced from H1-3 site), EP = *E. purpurata*, CD1-2 = *Cephalanthera damasonium* (from sites H1 and H2). Quotation marks indicate less clear genus status of a strain: "Genea" is an unidentified strain from *Genea-Humaria* lineage, "Geopyxis" from *Geopyxis-Stephensia* clade. *Trichophea* belongs to *T. woolhopeia* group, clade 7; according to phylogeny of Pyronemataceae in Perry *et al.* 2007.

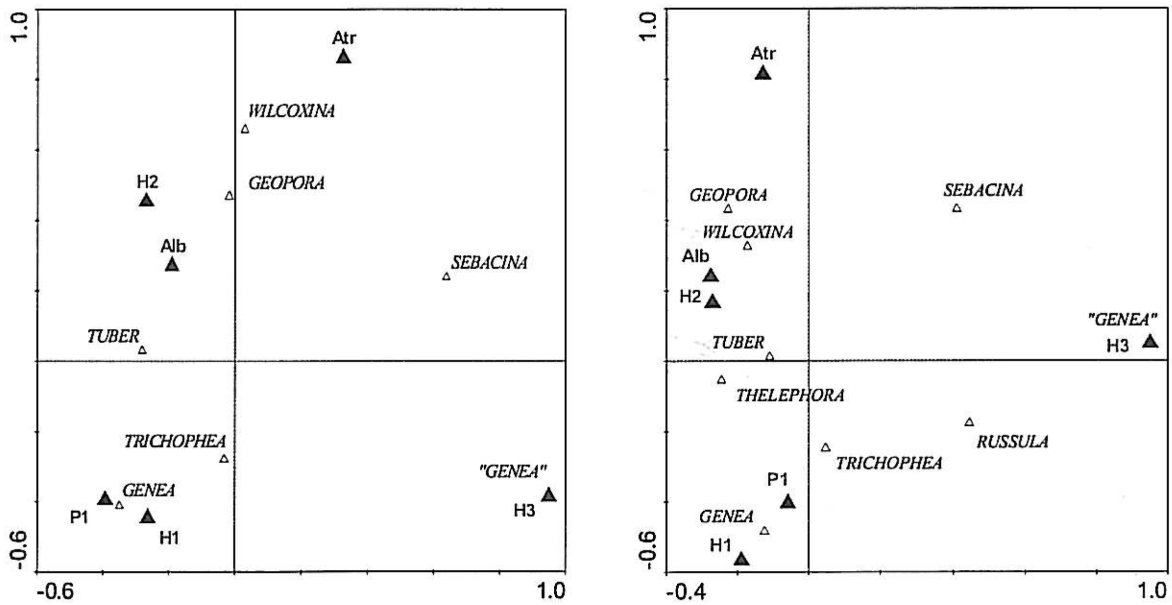
Site	Adults	Fungi in adults	Seedling species	Fungi in different sized seedlings		
				small (~0.5 mm)	medium (~1 mm)	large seedlings
Alb	EAla	**Wilcoxina**, "Geopyxis", Thelephoraceae sp.2, Ceratobasidium, *Penicillium sp.1, Bionectria, Alternaria sp.2, Debaryomyces*	EAt	**Tuber sp.1**, Geopora, *Cladosporium sp.1, Nectriaceae sp.1, Volutella, Arthopyreniaceae*	Trichophaea sp.1, *Cladosporium sp.1, Aspergillus*	
	EAlb	Tuber sp.9, "Geopyxis", Thelephoraceae sp.2 + sp.3, *Penicillium sp.1, Capnodiales, Malasseziales, Malassezia sp.1, Dipodascaceae sp.1, Debaryomyces, Cryptococcus, Candida*	EH2	**Tuber sp.1**, Geopora, *Nectriaceae sp.1, Pleosporaceae, Plectosphaerella, Truncatella, Chaetosphaeriaceae, Mortierella sp.1+sp.2*	**Tuber sp.1 + sp.8**, *Volutella, Plectosphaerella, Leptosphaeria, Peniophora, Clitocybe, Mortierella sp.3, Chytridiomycete sp.2*	
	EAlc	Tuber sp.3 + sp.9, Ceratobasidium, *Diaporthales, Chytridiomycete sp.1*				
Atr	EAta	**Wilcoxina**	EAt	Pyronemataceae, Geopora, *Agaricomycete, Cladosporium sp.1, Nectriaceae sp.3, Eudarluca, Dipodascaceae sp.1*		Sebacina clade B
	EAtb	**Tuber sp.1**				
	EAtc	**Wilcoxina**	EH2	*Cladosporium sp.1, Malasseziales, Malassezia sp.3, Dipodascaceae sp.1, Trichosporon*		
	EAtd	**Wilcoxina, Tuber sp.1**				
H1	EH1a	**Tuber sp.5**	EAt	Trichophaea sp.1 + sp.2, *Tetracladium sp.1*	Trichophaea sp.1 + sp.2	Trichophaea sp.1(3) + sp.2(3)
	EH1b	**Tuber sp.1 + sp.5**, Geopyxis, Hymenogaster sp.1, *Fusarium sp.1+ sp.2*	EH1	Trichophaea sp.1	*Penicillium sp.2, Nectriaceae sp.3, Helotiales sp.3, Exophiala, Malasseziales, Malassezia sp.4, Tremellales, Dipodascaceae sp.1, Agaricostilbomycetidae*	**Genea**, *Mortierella sp.7, Debaryomyces*
	EH1c	**Genea**, 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*		
H2	EH2a	**Tuber sp.5**	EAt	**Tuber sp.5**, *Mortierella sp.1+sp.4+sp.6, Nectriaceae sp.1*	no PCR amplification	**Wilcoxina**, *Tetracladium sp.1, Mortierella sp.1, Malassezia sp.2*
	EH2b	**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
H3	EH3a	**"Genea"**	EAt	**"Genea"**, Trichophaea sp.1, *Entolomataceae, Dipodascaceae sp.1, Itersonilia*	**"Genea"**, Tricholoma, Sebacina clade A, *Cladosporium sp.1+sp.2, Ascochyta, Dipodascaceae sp.1*	
	EH3b	**Tuber sp.5 + sp.7**, Inocybe				
	EH3c	**"Genea"**, Tuber sp.2 + sp.6, Helvella, *Coniosporium*	EH3	**"Genea", Russula sp.1**, *Nectriaceae sp.2*	no PCR amplification	**"Genea"(2)**
	EH3d	**"Genea"**	EH2	no PCR amplification	*Nectriaceae sp.2, Dipodascaceae sp.1*	
P1	EPa	**Wilcoxina, Russula sp.1**, *Malassezia sp.2, Dipodascaceae sp.2, Alternaria sp.1, Trichocladium, Debaryomyces, Filobasidium*	EAt	Trichophaea sp.2, *Nectriaceae sp.4*	Trichophaea sp.2, *Cladosporium sp.1*	**Genea**, Tuber sp.10
	EPb	Hydnotrya, Russula sp.2, *Helotiales sp.1*	EH2	*Tetracladium sp.2, Nectriaceae sp.2, Helotiales sp.2, Leptodontidium*	**Tuber sp.7**, *Nectriaceae sp.2, Leptodontidium, Trichoderma, Candida sp.2*	**Genea, Tuber sp.7(2)**, *Nectriaceae sp.2, Leptodontidium*

Spectra of ECM fungal genera associated with seedlings of *E. atrorubens* and *E. helleborine* were not significantly different within study sites (pCCA – localities as covariates, Monte-Carlo permutation test with 999 permutations: $F = 2.46$, $P < 0.07$). Within the sites, the seedlings of these two species often shared the same fungal strains (Table 4, Appendix B). Nevertheless, the ECM fungal communities (at genus level) associated either with *E. atrorubens* and *E. helleborine* seedlings or both seedlings and adults differed significantly among the sites (Fig. 4). The ordination analyses showed that both seedlings and adults growing at the localities with similar tree layer composition such as lime-hornbeam forests (P1 and H1) or poplar alleys (Alb and H2) associated similar fungal communities.

The number of ECM species was comparable among seedling growth categories, as we always found one to three lineages per cloning and up to two lineages per direct sequencing of mycorrhizal seedlings. We often detected same fungal lineages across all developmental stages (Table 4), and the total number of ECM lineages in seedlings of an *Epipactis* species per site never exceeded four.

ECM spectra in adult plants were sometimes broader and somewhat different from that found in the seedlings (Table 4). While sharing genera like *Genea*, *Tuber*, *Wilcoxina*, *Russula* or Thelephoraceae within and across the sites, other genera (*Geopyxis-Stephensia*, *Hydnotria*, *Helvella*, *Hymenogaster*, *Ceratobasidium* etc.) were never found in the seedlings. Similarly, *Trichophaea*, *Geopora* or *Sebacina* strains were commonly associated with seedlings, but never with the adults. In a more detailed analysis comparing ECM fungal spectra in seedlings and adults of *E. helleborine* at three home sites, we found no difference in fungi between those two developmental stages (pCCA – localities as covariates, Monte-Carlo permutation test with 999 permutations: $F = 2.59$, $P < 0.27$).

Fig. 4: Ordination plot of the canonical correspondence analysis (CCA, first two canonical axes are shown) of fungal genera found within six study sites **A.** in seedlings of *E. atrorubens* and *E. helleborine* and **B.** in seedlings and sympatric adults of different *Epipactis* species. Sites Alb and H2 represent a poplar alley, P1 hornbeam-lime forest, H1 lime forest; Atr is a pine stand on limestone and H3 is a mixed wood on chalk. Monte-Carlo permutation test (999 permutations), rare species downweighted, A: $F = 3.13$, $P < 0.05$; B: $F = 1.71$, $P < 0.05$.



Discussion

Germination pattern

Our study presents better insight into germination ecology of mixotrophic orchids. We did not detect any common rule explaining relationships between ecological requirements of adult plants and recruitment potential in the *Epipactis* species. The germination potential seemed broad in general as we observed initial developmental stages of germination (i.e. small non-mycorrhizal seedlings) in all species in all forest types. There was however striking difference in further development among species. Seedlings *E. helleborine* and *E. atrorubens* developed into advanced growth stages of large seedlings in all forest types (in contrast to low germination rate observed by Bidartondo & Read 2008 at three forest sites), while *E. albensis* and *E. purpurata* reached at most the stage of small protocorms only at some sites. This lower rate of development is unlikely to be caused by the lack of favorable conditions, as at home sites (a site with suitable soil conditions and appropriate fungi), the development was only slightly better (Table 3). Possible reduction of seed quality caused by autogamous self-pollination in *E. albensis* (Mered'a 2002) or by inbreeding depression (Charlesworth & Charlesworth 1987) in populations of *E. purpurata* could offer another explanation. However, initial germination stages observed indicate sufficient seed viability. Hence, the ontogenetic development of both species seems to be just delayed in a comparison with *E. atrorubens* and *E. helleborine*. In *E. albensis*, the stagnation in very first developmental stages might be caused by slower ontogeny, while the observation in *E. purpurata* implies a one-year delay in germination onset. Similar one-year latency was observed in fully mycoheterotrophic *Corallorhiza maculata* (Taylor *et al.* 2002). Further, many seeds of *E. helleborine* and *E. atrorubens* showed similar dormancy pattern, as proportion of all germinating seeds increased markedly between the two years (Fig. 3), suggesting existence of seed bank or intraspecific variability in seed characteristics (van der Kinderen 1995). Due to presumably delayed development, germination course in *E. albensis* and *E. purpurata* could not be fully covered by this study preventing us from conclusions on developmental progress in these two species.

In several studies, high correlation between seed germination rate and seedling recruitment in orchid populations was observed (Diez 2007, Jacquemyn *et al.* 2007) suggesting that the establishment of a population at unsuitable sites is bottlenecked at the early stage of symbiotic protocorm formation. Germination pattern in *E. helleborine* confirmed the broad ecological range of this species, forming protocorms in all forest types. Surprisingly, ecologically specialized *E. atrorubens* grew beyond the early germination stages into large seedlings even in habitats, where the adult plants never occur. Although the fungal genera detected in *Epipactis* over distinct forest types significantly differed (Fig. 4), multiple strain expected to be mycorrhizal in *Epipactis* occurred across several sites (Table 4) with distinct ecological characteristics or tree species composition. Thus, the distribution of mycorrhizal partners does not evidently limit population establishment. Similarly, the suggested developmental bottleneck caused by higher mycorrhizal specificity at the protocorm stage (Bidartondo & Read 2008) does not necessarily occur, because both *E.*

atrorubens and *E. helleborine* associated very similar spectra of fungi over all developmental stages. Hence, there might be a bottleneck based on abiotic factors preventing seedlings from reaching maturity. In case of *E. atrorubens*, this bottleneck can be caused by light conditions at individual stands. Analysis of Ellenberg indicator values of accompanying plant species (Fig. 2B) demonstrated clear preference for higher light levels and dry, nutrient poor soils of higher alkalinity. At these sites, the intensity of plant competition can be expected to be lower, partly due to low levels of soluble and easily exchangeable phosphate (Zohlen & Tyler 2004). The ratio between energy acquisition by means of photosynthesis and mycoheterotrophy varies substantially across mixotrophic species (Gebauer & Meyer 2003, Bidartondo *et al.* 2004). The rate of nutrient acquisition ability from fungal associates is assumed to depend on level of specificity to the mycorrhizal partner allowing higher physiological compatibility (Bruns *et al.* 2002). Consistently with putatively broad spectrum of *E. atrorubens* associated fungi (reported in Bidartondo *et al.* 2004, Bidartondo & Read 2008), the isotopic measurements showed that *E. atrorubens* derives only 15 to 32% of carbon from fungal association (Gebauer & Meyer 2003, Bidartondo *et al.* 2004). Moreover, albinotic individuals (deriving 100% of fungal carbon) have never been reported in this species, although they regularly occur in other *Epipactis* species (including the other three species studied; Procházka 1980, Salmia 1986, Rydlo 1989, Jakubska & Schmidt 2005). Altogether, this indicates that *E. atrorubens* might be unable to utilize fungal nutrition as efficiently as the other species being more dependent on its own photosynthetic activity, and hence available light level. Nevertheless, some other mechanisms affecting efficiency of host exploitation than the level of host specificity might exist, as indicated by e.g. relatively broad range of fungi detected in *E. albensis* (but see discussion bellow).

Fungal associations

The PCR-based cultivation independent methods present a powerful tool for identification of fungal symbionts but they also have several drawbacks that must be taken into account when interpreting the data. High number of analyzed samples together with time-consuming cloning procedure did not allow us for sampling of 50 clones, which is supposed to be sufficient for detection of complete fungal spectrum in environmental soil samples (M.-A. Selosse, personal communication). We sampled a minimum of 20 positive clones, which amount possibly does not fully cover the fungal diversity in a sample (on the other hand we did not expect the fungal diversity to be that high in seedlings cultivated *in situ* as in environmental samples). Nonetheless, this might be the reason for low fungal diversity in EAt seedlings at H1 or the absolute absence of *Trichophea* in adult *Epipactis* specimens, although this might have been caused also by undersampling of adults (Table 4). The concentration of fungal DNA in some samples was very low; hence the cloning had to be repeated several times in order to receive at least 20 positive PCR products. In such cases we often received many non-ECM species, which might have occurred as surface contaminants, or endophytic/parasitic fungi (e.g. EAla, EAlb, EPa; Table 4). This was also the case of multiple seedling pools from which we have not received any ECM or *Rhizoctonia* strains at all. The reason for the absence of ECM strains in seedlings or no PCR amplification at all (although mycorrhizal structures were clearly visible) remains questionable. It could have been probably caused by long storage in ethanol (similarly observed in Zimmer *et al.* 2007), or by too advanced digestion of fungal structures, which decreases the typing success (personal observations). Another explanation could be the presence of fungal species with accelerated evolution of the nuclear ribosomal operon, which are hence not detectable by the standard universal fungal primers we used (this is the case of Tulasnellaceae, which desire specific primers; Taylor & McCormick 2008).

Regarding the fungal spectra detected, we confirmed the predominantly non-rhizoctonian identity of *Epipactis* mycorrhizal fungi, adding symbionts for two *Epipactis* species which have not been previously investigated (*E. albensis* and *E. purpurata*). The most of ECM fungi in all *Epipactis* species in both seedlings and adults belonged to ascomycetes of the Pezizales order. Moreover, most of these fungi belong to genera reported in previous studies on mycorrhizal partners in *Epipactis* spp. In all *Epipactis* species studied (except for *E. purpurata*) and at all the study sites, we detected multiple strains of *Tuber*, which is a taxon frequently detected also by other researchers (Bidartondo *et al.* 2004, Selosse *et al.* 2004, Ogura-Tsujita & Yukawa 2008, Ouanphanivanh *et al.* 2008). Similar pattern was observed in *Wilcoxina* strains, which we found in all forest types in all species but *E. helleborine*. *Wilcoxina* species are known to commonly form ectendomycorrhizae with various pine species (Smith & Read 2008 and references herein), which could explain its frequent detection at Atr site (a Scotch pine stand). Consistently, the *Wilcoxina* strains were the exclusive symbionts in Japanese populations of *E. helleborine* growing in pine plantations (Ogura-Tsujita & Yukawa 2008), yet their sequences were only little similar to ours. We found strains from *Trichophaea woolhopeia* group frequently in seedlings but not adults; however a similar strain was reported from adults of *E. atrorubens* in Estonia (Shefferson *et al.* 2008). It is not without interest, that although the *Trichophaea* strains were present at least at four sites, they were found exclusively in *E. atrorubens* seedlings at three of them. Despite the insignificant result of statistical analysis, it seems that when exposed to same fungal communities *E. atrorubens* shows moderate preferences for Pyronemataceae strains (perceivable also in Bidartondo & Read 2008), *E. helleborine* for Tuberaceae (similar trend observed in Bidartondo *et al.* 2004).

We also detected some pezizalean strains scarcely reported by others, such as *Genea* or *Geopora* (Bidartondo & Read 2008, Ogura-Tsujita & Yukawa 2008, Shefferson *et al.* 2008). Although the strains related to *Genea arenaria* detected at lime-hornbeam forest sites were not similar to any *Epipactis* mycobiont known so far, the *Genea-Humaria* strain (from H3 site) was 98% similar to that detected in seedlings of *E. atrorubens* by Bidartondo & Read (2008). *Geopyxis* species from *E. helleborine* has not been found in *Epipactis* yet, only in adults of *Cephalanthera damasonium* (Julou *et al.* 2005); interestingly, the *Geopyxis-Stephensia* strain (in this study detected in *E. albensis*) was predicted to occur in *E. helleborine* by Ouanphanivanh *et al.* (2008). The additional ascomycetes, *Helvella* (from *E.*

helleborine) and *Hydnotrya* (from *E. purpurata*) were both detected in *E. helleborine* in Japan (Ogura-Tsujita & Yukawa 2008).

The occurrence of ECM basidiomycetes was much sparser, with exceptions of Thelephoraceae (an important mycorrhizal family in *C. damasonium*; Julou *et al.* 2005, our observations) in *E. albensis* adults and in seedlings and adults of *E. helleborine* at sites where they co-occur with *C. damasonium*. The finding of two *Russula* species in both *E. purpurata* specimens analyzed is interesting, as this genus is rarely reported from *Epipactis* (with an exceptional occurrence in roots of *E. helleborine* in Japan; Ogura-Tsujita & Yukawa 2008). The potential affinity of this *Epipactis* species to *Russula* desires a more detailed investigation, as our observation is based on analysis of few roots of two specimens. Nevertheless, the affinity to russuloid fungi may not be so surprising, as it was found in multiple orchid species including *Limodorum*, another genus within the Neottieae tribe (Girlanda *et al.* 2006). We detected also some rhizoctonian *Ceratobasidium* and *Sebacina* strains (distantly related strains from the ECM clade A and the saprophytic clade B; Weiss *et al.* 2004), which were both reported from *Epipactis* spp. by Bidartondo *et al.* (2004), but we do not have further knowledge on their mycorrhizal status, as they may behave as symptomless endophytes (Abadie *et al.* 2006).

Beside the ECM strains, the cloning procedure revealed many fungal strains which are not expected to be functional partners in *Epipactis*, although their nutritional relevance cannot be fully excluded. Several species are likely to grow in *Epipactis* as endophytic fungi, i.e. they grow in living tissues without producing symptoms, such as *Leptodontium* or *Exophiala*, which have been reported as common orchid endophytes (Rasmussen 1995, Ogura-Tsujita & Yukawa 2008). *Tetracladium*, an aquatic hyphomycete, is increasingly known to grow endophytically in plant tissues (reviewed by Selosse *et al.* 2008). Helotiales were reported as root biotrophs or mycorrhizal species (Vrålstad 2004). Some species might even behave as weak parasites (e.g. members of Nectriaceae; Julou *et al.* 2005). The other species are likely to be non-mycorrhizal, behaving as superficial saprobic contaminants (such as *Aspergillus, Fusarium, Mortierella* and others) or soil saprobes, which probably occurred due to insufficient surface cleaning from soil particles. These fungal species were sometimes reported also by other studies focusing on mycorrhizal partners of Neottieae (e.g. Bidartondo *et al.* 2004, Selosse *et al.* 2004, Julou *et al.* 2005, Abadie *et al.* 2006).

Despite *Epipactis* spp. was reported as less specific to mycorrhizal partners (Bidartondo & Read 2008), it seems that the seedlings and adults of both *E. helleborine* and *E. atrorubens* associate predominantly with strains of Tuberaceae and Pyronemataceae over large range of ecological conditions and geographical distances (compare Bidartondo *et al.* 2004, Bidartondo & Read 2008, Ogura-Tsujita & Yukawa 2008 and this study). Occasionally, ECM basidiomycetes were detected, indicating ability to associate more distantly related strains. Similar situation was recorded in *E. microphylla*; Selosse *et al.* (2004) revealed some basidiomycetes by molecular methods, however the peloton formation was confirmed only for ascomycetes. The authors suggested that the basidiomycetes likely colonized only restricted portions of roots or behaved only as rhizoplane colonizers, co-existing along with dominant mycorrhiza-forming ascomycetes. These findings also resemble pattern of mycorrhizal specificity observed over large geographical range in fully mycoheterotrophic, and thus supposedly highly specific, *Epipogium aphyllum* (Roy *et al. in press*). However, our pooling approach in seedling analysis does not allow recognition between prevailing mycorrhizal partners and non-dominant co-occurring species.

The association of *Epipactis* spp. with ECM ascomycetes is unique among orchid genera investigated so far (Dearnaley 2007). Interestingly, many pezizalean ascomycetes have been recently recognized by molecular tools as ectomycorrhizal fungi of various tree species (De Roman *et al.* 2005, Tedersoo *et al.* 2006, Smith & Read 2008). However, the pezizalean ectomycorrhizae seem to form only a minor part of ECM community on tree roots in mature forests, as they were found only on about 5% of root tips with Pyronemataceae colonizing half of them (Tedersoo *et al.* 2006). This relative scarcity of orchid mycorrhizal ascomycetes is in strong contrast to orchid associating basidiomycetes (such as Thelephoraceae or Russulaceae; Dearnaley 2007) which belong to frequent and abundant taxa in North temperate forests (Horton & Bruns 2001). This rareness of mycorrhizal ascomycetes can be hypothesized to have prevented the *Epipactis* spp. from specialization to narrow range of mycobionts within the pezizalean clade, as the probability of finding a highly specific mycorrhizal partner would be very low. Another adaptation to rarely occurring symbionts could be the observed dormancy pattern, which (together with mass seed production) enables effective spatio-temporal screening of the environment for the suitable host (Bruns *et al.* 2002), and enhances the probability for symbiosis establishment.

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Appendix A: Overall germination rate at each study site (A) and in four *Epipactis* species (B) over all sites after 23 months of soil incubation. The values represent arithmetic mean ± SE. *Epipactis* species abbreviations: EAl = *E. albensis*, EAt = *E. atrorubens*, EH = *E. helleborine*, EP = *E. purpurata*.

A)

	Study sites						
	Alb	Atr	H1	H2	H3	P1	P2
% of packets with germinating seeds	66.3 ± 4.8	73.6 ± 4.6	81.3 ± 4.1	73.2 ± 4.5	36.9 ± 5.3	64.4 ± 5.1	70.6 ± 5.6
% of germinating seeds in all packets	14.7 ± 2.0	35.8 ± 3.7	46.2 ± 4.2	49.2 ± 4.3	5.7 ± 1.7	18 ± 2.8	30.6 ± 4.1
% of germinating seeds in packets where germination occurred	22.2 ± 2.5	48.6 ± 3.9	56.8 ± 4.4	67.2 ± 4.2	15.5 ± 4.0	27.9 ± 3.7	43.4 ± 4.7

B)

	Study species			
	EAl	EAt	EH	EP
% of packets with germinating seeds	45.6 ± 5.3	89.8 ± 3.2	93.5 ± 1.5	27 ± 3.3
% of germinating seeds in all packets	2.9 ± 1.1	41 ± 3.4	52.8 ± 2.1	0.6 ± 0.2
% of germinating seeds in packets where germination occurred	6.4 ± 2.3	45.6 ± 3.4	56.4 ± 2.1	2.3 ± 0.5
% of protocorms in packets (packets with no germination excluded)	0 ± 0	3.8 ± 0.8	2.3 ± 0.4	0 ± 0

Appendix B: Fungal strains detected in *Epipactis* spp. adults and seedlings at seven forest sites and *Cephalanthera damasonium* adults at two sites.

Lineage [a]	Putative species [b]	Access number[c]	Isolation source [d]	Closest matches found by BLAST (with BLAST expected value) [e]	Putative ecology[f]
ASCOMYCOTA					
PEZIZOMYCOTINA					
Pezizales					
Tuberaceae	**Tuber sp.1**	1	EAt/Atr(a),EH/H1(a) EAt/Alb(s),EH/Alb(s)	AY634153 Uncultured ECM (Tuber) (0.0)	ECM
				EF362475 Tuber rufum (0.0)	
Tuberaceae	**Tuber sp.2**	2	EH/H3(a)	AY634153 Uncultured ECM (Tuber) (0.0)	ECM
				EF362475 Tuber rufum (0.0)	
Tuberaceae	**Tuber sp.3**	3	EAl/Alb(a)	EF362475 Tuber rufum (0.0)	ECM
Tuberaceae	**Tuber sp.4**	4	EH/H1(s)	EF644166 Uncultured ECM (Tuber) (3.0e-127)	ECM
				EF362473 Tuber rufum (6.0e-124)	
Tuberaceae	**Tuber sp.5**	5	EH/H1(a),EH/H2(a)	EU202708 Uncultured Tuber (0.0)	ECM
				DQ011848 Tuber scruposum (0.0)	
Tuberaceae		6	EAt/H2(s)	AY940165 Uncultured ECM (Tuber) (0.0)	ECM
				DQ011848 Tuber scruposum (0.0)	
Tuberaceae		7	EH/H2(s)	AY940165 Uncultured ECM (Tuber) (0.0)	ECM
				DQ011848 Tuber scruposum (0.0)	
Tuberaceae		8	EH/H3(a)	EF644167 Uncultured ECM (Tuber) (0.0)	ECM
				DQ011848 Tuber scruposum (0.0)	
Tuberaceae	**Tuber sp.6**	9	EH/H3(a)	EU668241 Uncultured Tuber (0.0)	ECM
				DQ011847 Tuber scruposum (0.0)	
Tuberaceae	**Tuber sp.7**	10	EH/H3(a)	EU668243 Uncultured Tuber (0.0)	ECM
				AJ969625 Tuber puberulum (0.0)	
Tuberaceae		11	EH/P1(s)	AJ969625 Tuber puberulum (0.0)	ECM
Tuberaceae	**Tuber sp.8**	12	EH/H2(a)	EU753269 Tuber maculatum (0.0)	ECM
Tuberaceae		13	EH/Alb(s)	AJ893250 Uncultured ECM (Tuber) (0.0)	ECM
				EU753269 Tuber maculatum (0.0)	
Tuberaceae	**Tuber sp.9**	14	EAl/Alb(a)	AJ534706 Tuber sp. (0.0)	ECM
				AJ969627 Tuber maculatum (0.0)	
Tuberaceae	**Tuber sp.10**	15	EAt/P1(s)	FJ013059 Uncultured ECM (Tuber) (0.0)	ECM
				EU753267 Tuber borchii (0.0)	
Pyronemataceae	**Wilcoxina**	16	EAt/Atr(a), EAl/Alb(a)	EU645612 Uncultured ECM (Wilcoxina) (0.0)	ECM
				AF266708 Wilcoxina rehmii (0.0)	
Pyronemataceae		17	EP/P1(a)	EF458013 Wilcoxina sp.(0.0)	ECM
				AF266708 Wilcoxina rehmii (0.0)	
Pyronemataceae		18	EAt/H2(s)	EF458013 Wilcoxina sp. (2.0e-174)	ECM
				AF266708 Wilcoxina rehmii (4.0e-171)	
Pyronemataceae	**Genea**	19	EH/H1(a), EA/P1(s), EH/P1(s)	DQ206858 Genea arenaria (0.0)	ECM
Pyronemataceae		20	EH/H1(s)	DQ206839Genea arenaria(0.0)	ECM

Pyronemataceae	**"Genea"**	21	EH/H3(a,s)	EU668290 Uncultured Genea (0.0)	ECM
				EU819470 Humaria hemisphaerica (6.0e-103)	
Pyronemataceae		22	EH/H3(a)	EU668290 Uncultured Genea (0.0)	ECM
				DQ206851 Genea gardnerii (2.0e-84)	
Pyronemataceae		23	EAt/H3(s)	EU668290 Uncultured Genea (0.0)	ECM
				DQ206851 Genea gardnerii (7.0e-83)	
Pyronemataceae		24	EAt/H3(s)	EU668290 Uncultured Genea (0.0)	ECM
				DQ206851 Genea gardnerii (2.0e-84)	
Pyronemataceae		25	EH/H3(s)	EU668290 Uncultured Genea (0.0)	ECM
				DQ206851 Genea gardnerii (7.0e-83)	
Pyronemataceae		26	EH/H3(s)	EU668290 Uncultured Genea (0.0)	ECM
				DQ206851 Genea gardnerii (2.0e-84)	
Pyronemataceae	**Trichophea sp.1**	27	EAt/H1(s),EH/H1(s) EAt/Alb(s)EAt/H3(s)	AY351623 Uncultured ECM (Pyronemataceae) (0.0)	ECM
				DQ200835 Trichophaea woolhopeia (0.0)	
Pyronemataceae	**Trichophaea sp.2**	28	EAt/H1(s), EH/H1(s)	DQ200835 Trichophaea woolhopeia (0.0)	ECM
Pyronemataceae		29	EAt/H1(s)	DQ200835Trichophaea woolhopeia (0.0)	ECM
Pyronemataceae		30	EAt/P1(s)	DQ200835 Trichophaea woolhopeia (0.0)	ECM
Pyronemataceae	**Geopora**	31	EAt/Alb(s)	FM206417 Geopora cervina (0.0)	ECM
Pyronemataceae		32	EH/Alb(s)	FM206417 Geopora cervina (0.0)	ECM
Pyronemataceae		33	EAt/Atr(s)	FM206417 Geopora cervina (0.0)	ECM
Pyronemataceae	**Geopyxis**	34	EH/H1(a)	Z96991 Geopyxis rehmii (0.0)	ECM
				EU837242 Stephensia bynumii (1.0e-142)	
Pyronemataceae	**"Geopyxis"**	35	EAl/Alb(a)	EU837242 Stephensia bynumii (8.0e-125)	ECM
				Z96990 Geopyxis carbonaria (5.0e-113)	
Pyronemataceae	**Pyronemataceae**	36	EAt/Atr(s)	EU668289 Uncultured Geopora (1.0e-168)	ECM
				EU669387 Pseudaleuria quinaultiana (6.0e-165)	
Discinaceae	**Hydnotrya**	37	EP/P1(a)	AM261522 Hydnotrya tulasnei (0.0)	ECM
Helvellaceae	**Helvella**	38	EH/H3(a)	AF335455 Helvella elastica (0.0)	ECM
Capnodiales					
Davidiellaceae	**Cladosporium sp.1**	39	EAt/H3(s)	EU167574 Cladosporium sp. (0.0)	S/P
Davidiellaceae		40	EAt/Alb(s)	EU759978 Cladosporium sphaerospermum (0.0)	S/P
Davidiellaceae		41	EAt/Alb(s)	EU167574 Cladosporium sp. (0.0)	S/P
Davidiellaceae		42	EAt/Atr(s)	EU167574 Cladosporium sp. (0.0)	S/P
Davidiellaceae		43	EAt/P1(s)	EU167574 Cladosporium sp. (0.0)	S/P
Davidiellaceae		44	EH/Atr(s)	EU167574 Cladosporium sp. (0.0)	S/P
Davidiellaceae	**Cladosporium sp.2**	45	EAt/H3(s)	EU272532 Cladosporium cladosporioides (0.0)	S/P
	Capnodiales	46	EAl/Alb(a)	AY260092 Teratosphaeria bellula (2.0e-170)	S/P
Pleosporales					
Pleosporaceae	**Alternaria sp.1**	47	EP/P1(a)	FJ455502 Alternaria alternata (0.0)	P
Pleosporaceae	**Alternaria sp.2**	48	EAl/Alb(a)	FJ266475 Alternaria conjuncta (4.0e-158)	P
Pleosporaceae	**Pleosporaceae**	49	EH/Alb(s)	EU750693 Pyrenochaeta sp. (0.0)	S
Venturiaceae	**Eudarluca**	50	EAt/Atr(s)	AY607011 Eudarluca caricis (0.0)	M
Leptosphaeriaceae	**Leptosphaeria**	51	EH/Alb(s)	AY336132 Leptosphaeria sp. (0.0)	P

Dothideomycetes					
Arthopyreniaceae	**Arthopyreniaceae**	52	EAt/Alb(s)	DQ682563 Arthopyreniaccae (0.0)	S
Hypocreales					
Bionectriaceae	**Bionectria**	53	EAl/Alb(a)	AB369487 Bionectria ochroleuca (0.0)	P
Nectriaceae	**Nectriaceae sp.1**	54	EAt/Alb(s), EH/Alb(s)	AJ875336 Neonectria radicicola (0.0)	P
Nectriaceae		55	EAt/H2(s)	AB369421 Cylindrocarpon sp. (0.0)	P
Nectriaceae		56	EH/H2(a)	AB369421 Cylindrocarpon sp. (0.0)	P
Nectriaceae	**Nectriaceae sp.2**	57	EH/H3(s)	AJ875330 Neonectria radicicola (0.0)	P
Nectriaceae		58	EH/P1(s), EH/H3(s)	AJ875331 Neonectria radicicola (0.0)	P
Nectriaceae	**Nectriaceae sp.3**	59	EAt/Atr(s)	DQ317342 Nectria sp. (0.0)	P
Nectriaceae		60	EH/H1(s), EH/H2(s)	DQ779785 Nectria sp. (0.0)	P
Nectriaceae		61	EH/H2(s)	DQ779785 Nectria sp. (5.0e-150)	P
Nectriaceae	**Nectriaceae sp.4**	62	EAt/P1(s)	EU754943 Uncultured Nectriaceae (0.0)	P
				AJ608955Cylindrocarpon magnusianum (0.0)	
Nectriaceae	**Volutella**	63	EAt/Alb(s), EH/Alb(s)	AJ301966 Volutella ciliata (0.0)	P
Hypocreaceae	**Trichoderma**	64	EH/P1(s)	EU280074 Trichoderma longipile (0.0)	S
	Fusarium sp.1	65	EH/H1(a)	EF495234 Fusarium redolens (0.0)	P
	Fusarium sp.2	66	EH/H1(a)	FJ037744 Fusarium lateritium (0.0)	P
		67	CD/H1(a), EH/H2(s)	FJ233193 Fusarium oxysporum (0.0)	P
Chaetosphaeriales					
Chaetosphaeriaceae	**Chaetosphaeriaceae**	68	EH/Alb(s)	EF488392 Codinaeopsis sp. (0.0)	P
Phyllachorales					
Phyllachoraceae	**Plectosphaerella**	69	EH/Alb(s)	FJ430715 Plectosphaerella sp. (0.0)	P
Xylariales					
Amphisphaeriaceae	**Truncatella**	70	EH/Alb(s)	AF377300 Truncatella angustata (0.0)	P
Diaporthales					
	Diaporthales	71	EAl/Alb(a)	EU003012 Uncultured ascomycete (0.0)	S/P
				EF110614 Harknessia ipereniae (3.0e-80)	
Leotiomycetes					
	Helotiales sp.1	72	EP/P1(a)	DQ497975 Uncultured ECM (Helotiales) (0.0)	ECM/P
				FM180478 Helotiales sp. (0.0)	
	Helotiales sp.2	73	EH/P1(s)	DQ273336 Uncultured Pezizomycotina (0.0)	ECM/P
				EF029222 Clathrosphaerina zalewskii (0.0)	
	Helotiales sp.3	74	EH/H1(s)	EF644169 Uncultured ECM (Helotiales) (0.0)	ECM/P
				AY706329 Leohumicola minima (0.0)	
	Helotiales sp.4	75	CD/H2(a)	DQ182424 Uncultured Helotiales (0.0)	ECM/P
				U57089 Cistella grevillei (0.0)	
Eurotiales					
Trichocomaceae	**Penicillium sp.1**	76	EAl/Alb(a)	AY373906 Penicillium corylophilum (0.0)	S
Trichocomaceae	**Penicillium sp.2**	77	EH/H1(s)	AY373929 Penicillium roquefortii (0.0)	S
Trichocomaceae	**Aspergillus**	78	EAt/Alb(s)	EF652080 Aspergillus rubrum (0.0)	S
Chaetothyriales					
Herpotrichiellaceae	**Exophiala**	79	EH/H1(s)	AY213652 Exophiala salmonis (0.0)	P

Pezizomycotina					
	Ascochyta	80	EAt/H3(s)	AF520642 Ascochyta sp. (0.0)	P
SACCHAROMYCOTINA					
Saccharomycetales					
Dipodascaceae	**Dipodascaceae sp.1**	81	EAI/Alb(a)EAt/Atr(s) EAt/H3(s),EH/Atr(s), EH/H3(s), EH/H1(s)	DQ286062 Galactomyces sp. (2.0e-175)	P
Dipodascaceae	**Dipodascaceae sp.2**	82	EP/P1(a)	AY787702 Geotrichum sp. (5.0e-143)	P
Dipodascaceae		83	CD/H1(a)	DQ668351 Galactomyces geotrichum (4.0e-171)	P
Saccharomycetaceae	**Debaryomyces**	84	EAI/Alb(a), EP/P1(a), EH/H1(s)	EU569039 Debaryomyces hansenii (0.0)	S
Saccharomycetaceae		85	EAI/Alb(a)	EF643593Debaryomyces hansenii (0.0)	S
	Candida sp.1	86	EAI/Alb(a)	AM117818 Candida diddensiae (0.0)	P
	Candida sp.2	87	EH/P1(s)	DQ269921 Candida sp. (2.0e-161)	P
DEUTEROMYCETES					
	Tetracladium sp.1	88	EAt/H2(s)	EU883431 Tetracladium breve (0.0)	P
		89	EAt/H1(s)	EU883432 Tetracladium furcatum (0.0)	P
	Tetracladium sp.2	EU363517	CD/H2(a)	DQ068996 Tetracladium maxilliforme (0.0)	P
		EU363516	EH/H2(s)	DQ068996 Tetracladium maxilliforme (0.0)	P
		90	EH/P1(s)	FJ000375 Tetracladium furcatum (0.0)	P
	Tetracladium sp.3	91	EH/H2(s)	DQ068996 Tetracladium maxilliforme (0.0)	P
	Coniosporium	92	EH/H3(a)	AJ972792 Coniosporium sp. (0.0)	S
	Leptodontidium	93	EH/P1(s)	AF486133 Leptodontidium orchidicola (0.0)	P
	Trichocladium	94	EP/P1(a)	EU754970 Uncultured Trichocladium (0.0)	S/P
				AM292049 Trichocladium opacum (0.0)	
	Tetracladium sp.1	88	EAt/H2(s)	EU883431 Tetracladium breve (0.0)	P
BASIDIOMYCOTA					
AGARICOMYCOTINA					
Agaricales					
Hymenogastraceae	**Hymenogaster sp.1**	95	EH/H1(a)	AY634136 Uncultured ECM (Hymenogastraceae) (0.0)	ECM
				AF325642 Hymenogaster olivaceus (0.0)	
Hymenogastraceae	**Hymenogaster sp.2**	96	CD/H1(a)	AY634136 Uncultured ECM (Hymenogastraceae) (0.0)	ECM
				AF325642 Hymenogaster olivaceus (0.0)	
Hymenogastraceae	**Hymenogaster sp.3**	97	EH/H1(a)	AF325636 Hymenogaster griseus (0.0)	ECM
Hymenogastraceae	**Hymenogaster sp.4**	98	CD/H2(a)	AY351629 Uncultured ECM (Hymenogastraceae) (0.0)	ECM
				AF325641 Hymenogaster bulliardii (0.0)	
Cortinariaceae	**Inocybe sp.0**	99	EH/H3(a)	AM882888 Inocybe fuscidula (0.0)	ECM
Tricholomataceae	**Tricholoma**	100	EAt/H3(s)	DQ822835 Uncultured ECM (Tricholoma) (0.0)	ECM
Entolomataceae	**Entolomataceae**	102	EAt/H3(s)	DQ974695 Entoloma sp. (0.0)	S
Thelephorales					
Thelephoraceae	**Thelephoraceae sp.1**	103	EH/H1(a)	EF644157 Uncultured ECM (Tomentella) (0.0)	ECM
				EF644116 Tomentella sp.(0.0)	
Thelephoraceae	**Thelephoraceae sp.2**	104	EAI/Alb(a)	EF218826 Uncultured ECM (Tomentella) (0.0)	ECM
				U83482 Tomentella sp. (0.0)	
Thelephoraceae		105	CD/H1(a)	EF218826 Uncultured ECM (Tomentella) (0.0)	ECM

				DQ974780Tomentella sp.(0.0)	
Thelephoraceae	**Thelephoraceae sp.3**	106	EAl/Alb(a)	EU668199 Uncultured Tomentella (0.0)	ECM
				U83482 Tomentella sp. (0.0)	
Thelephoraceae	**Thelephoraceae sp.4**	107	CD/H2(a), EH/H2(s)	EU668199 Uncultured Tomentella (0.0)	ECM
				U83482 Tomentella sp. (0.0)	
Thelephoraceae	**Thelephoraceae sp.5**	108	CD/H2(a)	FJ210768 Uncultured ECM (Tomentella) (0.0)	ECM
				U83482 Tomentella sp. (0.0)	
Thelephoraceae	**Thelephoraceae sp.6**	109	EH/H2(s)	EF655687 Uncultured ECM (Thelephora) (0.0)	ECM
				AJ889980 Thelephora caryophyllea (0.0)	
Thelephoraceae	**Thelephoraceae sp.7**	110	CD/H2(a)	EU563503 Uncultured ECM (Pseudotomentella) (0.0)	ECM
				AF274771 Pseudotomentella tristis (0.0)	
Russulales					
Russulaceae	**Russula sp.1**	111	EP/P1(a)	EF218804 Uncultured ECM (Russula) (0.0)	ECM
				EU819428 Russula nigricans (0.0)	
Russulaceae		112	EH/H3(s)	EF218804 Uncultured ECM (Russula) (0.0)	ECM
				EU819428 Russula nigricans (0.0)	
Russulaceae	**Russula sp.2**	113	EP/P1(a)	AY061660Russula azurea(0.0)	ECM
Cantharellales					
Ceratobasidiaceae	**Ceratobasidium**	114	EAl/Alb(a)	EU002954 Uncultured Ceratobasidium (0.0)	R
				EU273525 Ceratobasidium cornigerum (0.0)	R
Sebacinales					
Sebacinaceae	**Sebacina clade A**	115	EAt/H3(s)	AM161532 Uncultured ECM (Sebacinaceae) (0.0)	R/ECM
				AF490393 Sebacina aff. epigaea (0.0)	
Sebacinaceae	**Sebacina clade B**	116	EAt/Atr(s)	EF127237 Uncultured Sebacinales (0.0)	R
				DQ520096 Sebacina vermifera (2.0e-140)	
Polyporales					
Lachnocladiaceae	**Peniophora**	117	EH/Alb(s)	AF210825 Peniophora aurantiaca (0.0)	S
Agaricomycetes					
	Agaricomycete	118	EAt/Atr(s)	U85799 Athelia pellicularis (0.0)	?
Filobasidiales					
	Cryptococcus	119	EAl/Alb(a)	AF145327 Cryptococcus kuetzingii (0.0)	S
Filobasidiaceae	**Filobasidium**	120	EP/P1(a)	AF190007 Filobasidium floriforme (0.0)	S
Cystofilobasidiales					
	Itersonilia	121	EAt/H3(s)	AB072233 Itersonilia perplexans (0.0)	S
Tremellales					
	Trichosporon	122	EH/Atr(s)	EU559346 Trichosporon asahii (0.0)	S
	Tremellales	123	EH/H1(s)	AF042453 Tremella giraffa (5.0e-127)	S/P/M
PUCCINIOMYCOTINA					
	Agaricostilbomycetidae	124	EH/H1(s)	AF444519 Bensingtonia ingoldii (9.0e-144)	S/P
USTILAGOMYCOTINA					
Malasseziales					
	Malasseziales	125	EAl/Alb(a)	EU915323 Uncultured Malassezia (0.0)	P
				AY743657 Malassezia sympodialis (2.0e-145)	

		126	EH/Atr(s)	EU915323 Uncultured Malassezia (0.0)	P
				AY743657 Malassezia sympodialis (1.0e-148)	
		127	EH/H1(s)	EU915323 Uncultured Malassezia (0.0)	P
				AY743657 Malassezia sympodialis (1.0e-148)	
	Malassezia sp.1	128	EAl/Alb(a)	AY743636 Malassezia restricta (0.0)	P
	Malassezia sp.2	129	EP/P1(a)	AY743636 Malassezia restricta (0.0)	P
		130	EAt/H2(s)	AY743636 Malassezia restricta (0.0)	P
	Malassezia sp.3	131	EH/Atr(s)	EU915456 Malassezia restricta (0.0)	P
		132	EH/H2(s)	EU915456 Malassezia restricta (0.0)	P
	Malassezia sp.4	133	EH/H1(s)	AY743640 Malassezia sympodialis (0.0)	P
ZYGOMYCOTA					
MUCOROMYCOTINA					
Mortierellales					
Mortierellaceae	**Mortierella sp.1**	134	EAt/H2(s)	EU877758 Mortierella sp. (0.0)	S
Mortierellaceae		135	EAt/H2(s)	DQ093723 Mortierella gamsii (0.0)	S
Mortierellaceae		136	EH/Alb(s)	DQ093723 Mortierella gamsii (0.0)	S
Mortierellaceae	**Mortierella sp.2**	137	EH/Alb(s)	AJ890432 Mortierella sp. (0.0)	S
Mortierellaceae	**Mortierella sp.3**	138	EH/Alb(s)	EU877758 Mortierella sp. (0.0)	S
Mortierellaceae	**Mortierella sp.4**	139	EAt/H2(s)	DQ888725 Mortierella sp. (0.0)	S
Mortierellaceae	**Mortierella sp.5**	140	EH/H1(s)	AJ271630 Mortierella alpina (0.0)	S
Mortierellaceae		141	CD/H1(a)	AY310443 Mortierella alpina (0.0)	S
Mortierellaceae	**Mortierella sp.6**	142	EAt/H2(s)	EU918703 Mortierella alpina (0.0)	S
Mortierellaceae	**Mortierella sp.7**	143	EH/H2(s)	EU754996 Uncultured Mortierellaceae (0.0)	S
				EU877758 Mortierella sp. (0.0)	
Mortierellaceae		144	EH/H1(s)	EU754996 Uncultured Mortierellaceae (0.0)	S
				EU877758 Mortierella sp. (0.0)	
CHYTRIDIOMYCOTA					
	chytridiomycete sp.1	145	EAl/Alb(a)	AY997095 Synchytrium macrosporum (7.0e-56)	S/P
	chytridiomycete sp.2	146	EH/Alb(s)	AY997082 Rhizophydium sphaerotheca (8.0e-42)	S/P

[a] Taxonomic classification; order and family level denoted where possible.
[b] Putative species assembling >97% similar sequences.
[c] Sequences will be submitted to NCBI database before the journal submission.
[d] Orchid species / site (developmental stage); EAl = *Epipactis albensis*, EAt = *E. atrorubens*, EH = *E. helleborine*, EP = *E. purpurata*, CD = *Cephalanthera damasonium*; s = seedling, a = adult.
[e] Only the closest BLAST informative for taxonomy is denoted. In case the closest match did not belong to a vouchered specimen, the closest sequence coming from a herbarium specimen or culture is added.
[f] Trophic strategy of the most similar fungal strains (we expect that the startegy is similar for the sequenced species): ECM = ectomycorrhizal; R = rhizoctonian strain; P = plant parasite or endophyte; S = saprophytic; M = mycoparasitic; ? = unknown strategy.

Part II.

Spatial aspects of seed dispersal and seedling recruitment in orchids

Jersáková J & Malinová T. 2007. Spatial aspects of seed dispersal and seedling recruitment in orchids. New Phytologist 176: 237-241.

Spatial aspects of seed dispersal and seedling recruitment in orchids

Growing interest in spatial plant ecology is resulting in new approaches to the study of seed dispersal and seedling recruitment; two important processes determining population dynamics, genetic structure within and among plant populations and the colonization of new areas (Vekemans & Hardy, 2004). In general, seed dispersion patterns are determined by the spatial pattern of reproductive adults, their seed outputs and their seed shadows, while seedling recruitment mainly depends on the probability of seed arrival and the availability of a suitable microsite (Nathan & Muller-Landau, 2000). In the orchid family, successful germination and seedling establishment are crucial life history stages, as orchid seeds are unusual in being among the smallest seeds of all flowering plants, with an undifferentiated embryo that contains minimal reserves. Therefore, at germination, orchids are fully dependent on an interaction with a mycorrhizal fungus, which colonizes the seeds and provides all nutrients essential for seedling development. In the past decade, there have been several attempts to investigate the process of orchid seed germination in a spatial context (Perkins & McGee, 1995; McKendrick *et al.*, 2000; Batty *et al.*, 2001; Feuerherdt *et al.*, 2005; Diez, 2007); however, these studies have told us little about the extent to which seed dispersal and germination are associated with the spatial distribution of recruits. In this issue of *New Phytologist* (pp. 448–459), Jacquemyn *et al.* provide more insights into the within-population spatial genetic structure and recruitment potential of an orchid species, for which little is known regarding seed dispersal patterns and the successful establishment of mycorrhiza-dependent seedlings.

'Such a fine-scale population genetic structure may have serious consequences for the seed quality resulting from pollen transport between neighbouring plants in outcrossing species'

The nature of orchid seed dispersal

Orchid seeds are very small, extremely light and produced in great numbers. The embryo occupies only a very small part of the space inside the seed coat, the remainder of which is filled with air. As a result, orchid seeds can remain airborne for long periods and travel thousands of kilometres. Long-distance dispersal events are well demonstrated by the colonization of volcanic islands, where orchids were among the first plants to grow after island formation (Arditti & Ghani, 2000).

Although orchids have fine dust-like seeds, most studies investigating spatial genetic structure within terrestrial orchid populations have found a significant pattern, which in most cases was explained by limited seed dispersal (Machon *et al.*, 2003). Similarly, the parentage analysis in the study of Jacquemyn *et al.* suggests that seed dispersal with subsequent recruitment within two *Orchis purpurea* populations was limited to median distances of 4 and 7 m. The typical seed rain density in terrestrial orchids thus decreases as a function of distance from the parent plant (Fig. 1), where the long tail represents only a small proportion of seeds, which is, however, sufficient for colonizing new areas.

Such a fine-scale population genetic structure may have serious consequences for the seed quality resulting from pollen transport between neighbouring plants in outcrossing species, as is the case for most orchids. For example, in *Dactylorhiza praetermissa*, a nonrewarding orchid species in terms of nectar production, pollination between plants growing less than 10 m apart yielded seeds with a lower proportion of embryos and decreased germination rates compared with pollination between plants growing more than 20 m apart (Ferdy *et al.*, 2001). This effect may be even more pronounced in orchids producing rewards, as pollinators tend to fly shorter distances in rewarding patches, which leads to increased inbreeding depression in progeny if neighbouring plants are closely related (Vekemans & Hardy, 2004). The orchid family is renowned for an unusually high occurrence of nonrewarding flowers compared with other plant families (Jersáková *et al.*, 2006). The limited seed dispersal thus could indirectly favour the evolution and stability of deceptive pollination systems in orchids, where pollen dispersal distances are greater than in rewarding plants, to compensate for the homogamy that would arise from recruitment in the extreme vicinity (Jersáková *et al.*, 2006).

What is a 'safe site' for orchid recruitment?

The concept of a 'safe site' describes the specific conditions that allow a seedling of a particular species to emerge successfully from the soil and to develop into an adult, reproductive plant (Harper *et al.*, 1965). For orchid seedlings, which are fully

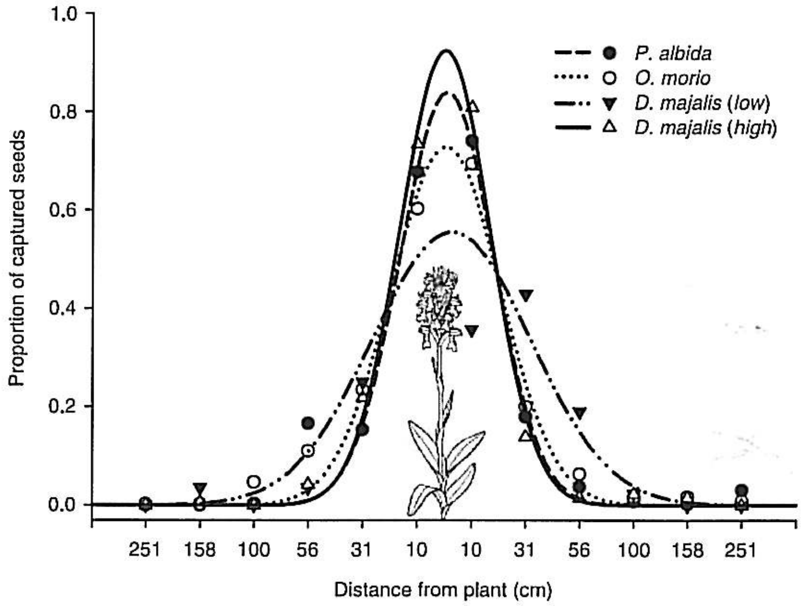

Fig. 1 Proportion of seeds captured by sticky Petri dishes, which were positioned at six distance classes, in six directions, from adult plants of *Pseudorchis albida*, *Anacamptis morio* and *Dactylorhiza majalis* (in low and high vegetation). The value for each distance class is based on the sum for three directions. Data were fitted by Gaussian curves (J. Jersáková, unpublished data).

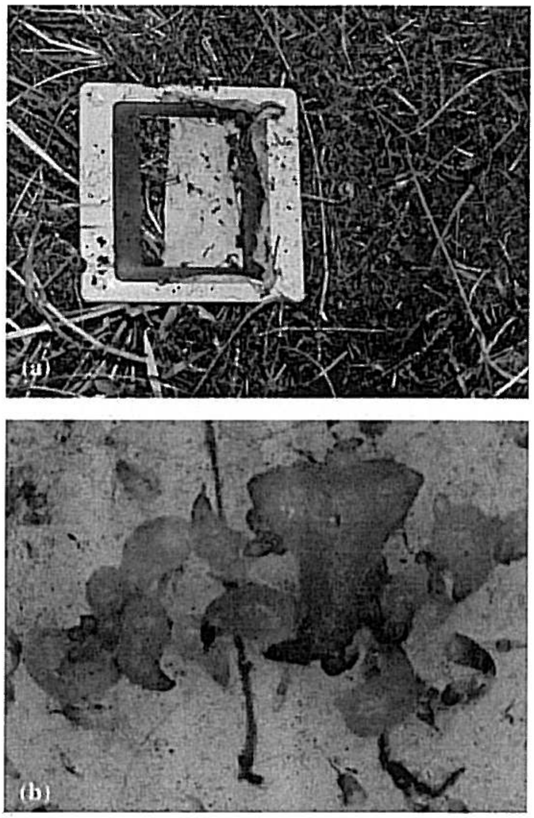

Fig. 2 (a) The *in situ* seed germination technique employs a plastic slide and nylon mesh with enclosed seeds. (b) Developmental stages of *Epipactis helleborine* seedlings after 23 months in the soil.

dependent on nutrients supplied by a mycorrhizal fungus until they reach the autotrophic stage, recruitment success will be strongly influenced by the availability of a suitable fungal strain. Our knowledge of spatial aspects of seed germination was greatly improved when Rasmussen & Whigham (1993) developed an inexpensive and simple method for *in situ* seed germination (Fig. 2a). This method enables seed cultivation under nearly natural conditions and colonization by fungal hyphae from the surrounding soil (Fig. 2b). Not only do the seed packets retrieved allow assessment of germination, but the mycorrhizal seedlings obtained can be used in further molecular analyses and *in vitro* cultivation of fungal symbionts. To date, orchids have been found to associate with several groups of fungi, and differences in the ecology and nutritional demands of these fungi may strongly impact seed germination patterns.

Orchids of open habitats typically associate with saprotrophic basidiomycetes of several lineages, collectively named *Rhizoctonia* after their asexual stage (Rasmussen, 2002). When not in association with orchid roots, these soil-borne fungi are considered to be saprophytic or parasitic on plants, but little is known about their spatial distribution in the environment, their nutrient demands, and their fine-scale propagation. The above-mentioned studies, which focused on the spatial aspects of orchid seed germination, suggest that mycorrhizal fungi have an aggregated distribution within the habitats. Although in ordinary circumstances the mycorrhizal fungi are likely to be distributed independently of the orchids (Feuerherdt *et al.*, 2005), higher abundances of fungal symbionts are typically found close to adult plants (Batty *et al.*, 2001; Diez, 2007). For example, Perkins & McGee (1995) found *Rhizoctonia solani* within 50 cm of adult plants of the orchid *Pterostylis acuminate*. These 'safe sites' probably provide suitable environmental conditions for both fungus and orchid growth, as the seed germination rate was found to be correlated with specific edaphic factors, such as soil organic matter content, potassium content, soil acidity and moisture (Batty *et al.*, 2001; Diez, 2007), which probably play important roles in the growth and density of saprophytic fungi (Ettema & Wardle, 2002).

Conversely, constraints on fungus availability might be more pronounced in the germination of nonphotosynthetic myco-heterotrophic orchids and some green forest orchids, which were found to associate with ectomycorrhizal basidiomycetes and ascomycetes (Julou *et al.*, 2005). In such cases, the fungus belongs to a tripartite symbiosis, in which the orchid indirectly recovers carbohydrates from surrounding trees via a shared mycorrhizal fungus. As the ectomycorrhizal root tips of host trees are usually found in the close vicinity of the root system of mycotrophic plants (Selosse *et al.*, 2002), the successful germination of orchids may not simply depend on the presence of adult plants, but may be largely determined by the occurrence of ectomycorrhizal root tips. This view is supported by a nonsignificant effect of the presence of adult *Corallorhiza trifida* plants on the percentage of germinating seeds (McKendrick *et al.*, 2000).

Making a significant step forward, Jacquemyn *et al.* show that the probability of seed germination and further establishment of protocorms is closely associated with the spatial distribution of recruits. The authors investigated the spatial patterns of seedling recruitment within two populations of *O. purpurea*, an orchid that is likely to associate with Rhizoctonia-like fungi from the Tulasnellales fungal subgroup (GenBank accession number AJ549121). The germination rates were found to differ markedly between the two populations of *O. purpurea*. At the first site, seed germination was confined to particular microsites, where both adults and seedlings were found to be clustered. At the second site, seed germination was not found to be restricted, and hence not all seedlings overlapped with adult clusters. The authors could, however, only speculate on whether the restricted germination at the first site was caused by a lack of appropriate fungus or by a lack of suitable soil substrate. Future studies might focus on establishing the presence or absence of the fungal symbiont in the soil using molecular methods such as those employing specific PCR primers or terminal restriction fragment length polymorphism, which provide fingerprints of whole fungal communities (Dickie *et al.*, 2002).

How many will be lucky enough to grow and reproduce?

Orchids produce an enormous quantity of seeds, but the probability of one seed appearing above the ground as a seedling is extremely low. Seedling recruitment is a fundamental component in population dynamics models, but its value is very difficult to determine for orchids, for which little is known about the time elapsing between the heterotrophic and autotrophic stages and the persistence of the orchid seed bank. Seed baiting techniques can, however, help us to estimate this value more precisely, as seed germination and seedling recruitment rates seem to be highly correlated (Diez, 2007).

Existing studies suggest that a relatively high proportion of orchid seeds start to germinate (ranging from 30 to 89% in suitable microsites; Rasmussen & Whigham, 1993; McKendrick *et al.*, 2000), but only a small proportion of protocorms will reach the advanced stages of plant development (less than 1%; Batty *et al.*, 2001). The insufficient development and subsequent death of seedlings in the later stages of development could stem from the fact that initial mycotrophic germination can be induced by a broader spectrum of mycobionts than is required for the further growth of a mycotroph, as reviewed by Bidartondo (2005) for Ericaceae seeds without food reserves. Convincing evidence, however, is still lacking for orchids. One attempt to calculate seedling recruitment was presented by Batty *et al.* (2001), who used a seed sowing technique with *Caladenia arenicola*. Batty *et al.* found that, of the approximately 34 500 seeds examined, less than 1% reached a stage at which they were able to survive summer dormancy. These data, combined with the mean number of seeds produced per plant per year (1200 seeds), the probability of reaching the seed bank (50%), the probability of finding a 'safe site' (10%), and the duration of the seed bank (< 1 yr), were used to estimate the overall success of *C. arenicola* recruitment as approximately 0.4 seedlings per parent plant per year.

Several relevant long-term studies of orchid population ecology have now been published in which annual seedling recruitment was recorded. However, these studies neglected seed germination and protocorm survival, which are the main steps towards adulthood. Many of these studies are still running; in these and the new studies that are being undertaken, it would be valuable if demographic monitoring of the plants could be combined with seed packet experiments, which can be used as an efficient tool to determine how germination rates translate to population-level recruitment rates.

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Key words: orchids, seed dispersal, seed germination, seed sowing technique, seedling recruitment, spatial analysis.