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Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea*

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ABSTRACT

The fungal community associated with the terrestrial photosynthetic orchid *Gymnadenia conopsea* was characterized through PCR-amplification directly from root extracted DNA and cloning of the PCR products. Six populations in two geographically distinct regions in Germany were investigated. New ITS-primers amplifying a wide taxonomic range including Basidiomycetes and Ascomycetes revealed a high taxonomic and ecological diversity of fungal associates, including typical orchid mycorrhizas of the Tulasnellaceae and Ceratobasidiaceae as well as several ectomycorrhizal taxa of the Pezizales. The wide spectrum of potential mycorrhizal partners may contribute to this orchid's ability to colonize different habitat types with their characteristic microbial communities. The fungal community of *G. conopsea* showed a clear spatial structure. With 43 % shared taxa the species composition of the two regions showed only little overlap. Regardless of regions, populations were highly variable concerning taxon richness, varying between 5 and 14 taxa per population. The spatial structure and the continuous presence of mycorrhizal taxa on the one hand and the low specificity towards certain fungal taxa on the other hand suggest that the fungal community associated with *G. conopsea* is determined by multiple factors. In this context, germination as well as pronounced morphological and genetic differentiation within *G. conopsea* deserve attention as potential factors affecting the composition of the fungal community.

Introduction

Eucaryotic micro-organisms such as fungi are often regarded as ubiquitously distributed due to their small size and great abundance (Finlay 2002). On the other hand clear distributional patterns have been detected, and are thought to be determined by e.g. large scale soil carbon gradients, land use or small-scale soil textures produced by plant growth (Ettema & Wardle 2002; Kasel *et al.* 2008). The development of plants of the Orchidaceae directly depends on the presence of fungal partners, because orchid seeds lack any nutrient reserves and germination in the wild is only possible upon colonisation by a compatible fungus providing carbohydrates. The developing seedling remains dependent on fungal sugars for several years, a strategy called mycoheterotrophy (Leake 1994; Rasmussen 1995). For most orchid species it is only during further development that the achlorophyllous protocorm becomes autotrophic, although some species remain mycoheterotrophic throughout the adult stage (Abadie *et al.* 2006; Julou *et al.* 2005). As a consequence of this symbiosis the

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degree of specificity between fungus and orchid is an important factor determining chances of successful seedling establishment (Bidartondo & Read 2008). For orchids that require specific fungi, their availability determines the suitability of a given habitat and thus constitutes an environmental factor critical for orchid recruitment. On the contrary for species exhibiting diverse associations this factor may not be limiting (McCormick *et al.* 2004).

Early studies on the specificity between orchids and their mycorrhizal fungi were mainly based on cultivation methods or germination tests under laboratory conditions and found a considerable phylogenetic breadth of associated fungi (Curtis 1939; Knudson 1922). However, physiological compatibility under laboratory conditions may be broader as it does not reflect the complexity of interactions under natural conditions (Masuhara & Katsuya 1994; Perkins *et al.* 1995). Furthermore the general problems of the unculturability of many mycorrhizal fungi or outgrowing contaminants could have additionally biased these early results. Modern PCR-based approaches largely eliminate these biases and allow the direct assessment of the fungal diversity present within an orchid root (Kristiansen *et al.* 2001; Taylor & McCormick 2008).

Indeed, more recent investigations applying molecular methods have shown a more complex picture, pointing to a considerable specificity between some orchid species and their mycorrhizal fungi. Most recorded fungi associated with photosynthetic orchids are *Rhizoctonia*-forming fungi (Roberts 1999) belonging to the Ceratobasidiaceae and Tulasnellaceae (McCormick *et al.* 2004; Otero *et al.* 2002; Rasmussen 2002), whereas mycoheterotrophic and mixotrophic orchids are rather associated with ectomycorrhizal Basidiomycetes like the Thelephoraceae and Russulaceae (Abadie *et al.* 2006; Girlanda *et al.* 2006; Julou *et al.* 2005). However, even some ascomycetous genera have been shown to form true orchid mycorrhizas (Currah *et al.* 1988; Selosse *et al.* 2004).

In the present study we performed a screen for fungal associates of the photosynthetic orchid species *Gymnadenia conopsea*, a still common orchid found in a wide range of different habitat types. We were interested whether a widely distributed species has the ability to associate with multiple fungi, which would likely increase its habitat availability as well as its tolerance to disturbances (McCormick *et al.* 2004). We set out for a comprehensive description of the fungal community of *G. conopsea* by using new ITS-primers that amplify a broad taxonomic spectrum of Basidio- and Ascomycetes. Here we report on the fungal diversity found in the roots of *G. conopsea* and discuss overall geographical differentiation between two study regions in Germany.

Materials and methods

Plant and fungal material

Diversity of fungal root associates was investigated of *Gymnadenia conopsea*, a terrestrial photosynthetic orchid species geographically widely distributed in Eurasia (Tutin *et al.* 1980). Like most other orchids, this species is declining, but is still relatively common in Central Europe and found in various habitat types, ranging from wet to dry grasslands and open woodlands (Gustafsson 2000). We analysed samples from six dry grassland sites located in two geographically distinct regions in Eastern Germany (area of Leipzig; coordinates E1: 11°64′E/51°21′N, E2: 11°65′E/51°30′N, E3: 11°73′E/51°19′N) and Northern Germany (area of Hannover; coordinates N1: 9°51′E/51°87′N, N2: 9°40′E/51°89′N, N3: 9°53′E/51°92′N), approximately 300 km apart. In spring 2006 before flowering, root material of three randomly chosen individuals per site was collected and cleaned several times with sterile water to minimize the detection of soil fungi. Samples were either processed immediately for fungal isolation or lyophilised for molecular analyses.

Fungal isolation and primer design

On the basis of ITS sequences obtained from fungal taxa isolated from the roots of *Gymnadenia conopsea* new ITS -primers were designed. Fungal isolation was performed from three root pieces per individual. 1–2 cm root segments were surface sterilized with 1 % hypochlorite for 2 min and then rinsed three times for 10 min in sterile water before placing onto nutrient agar (Laiho 1970). The plates were kept in the dark at room temperature and growing colonies were separated onto fresh media. DNA was extracted with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). For fungal identification the ITS region of nuclear ribosomal DNA was amplified by polymerase chain reaction (PCR) using the primers ITS1 and ITS4 (White *et al.* 1990). PCR products were purified with MinElute (Qiagen) and sequenced using the BigDye cycle sequencing v.3.1 kit (Applied Biosystems, Darmstadt, Germany), and run on an ABI 3100 genetic analyzer (Applied Biosystems). For taxonomic identification the sequences were compared with known sequences from GenBank using a BLASTN search (Altschul *et al.* 1997). The taxonomic spectrum of identified species was used for the development of new primers. These PCR primers ITS_ufz01: 5′-TGAACCTGCGGARGGATCATTA-3′ and ITS_ufz02: 5′-CCGCTTATTGATATGCTTAAGT-3′ amplify fungal ITS covering a broad taxonomic spectrum of Basidio- and Ascomycetes, but they do not amplify *G. conopsea* ITS. BLAST searches of the primer sequences against orchid sequences available in GenBank showed that only one orchid species perfectly matches the 3′ end of both primers. All other orchid sequences showed mismatch of at least one 3′ terminal nucleotide of each primer.

DNA extraction and PCR amplification

Fungal diversity was assessed directly through PCR-amplification from root extracted DNA of 18 individuals from six sites in total. For each individual DNA was extracted from 6 root pieces separately (equivalent of approximately 6 cm of the root system), using the DNeasy Plant 96 Kit (Qiagen). A separate PCR amplification was conducted for each piece. Fifty microlitre PCR reactions contained 5 μl of 10× HotStart Buffer (Fermentas, St. Leon-Rot, Germany), 2 mM $MgCl_2$, 0.16 mM of each dNTP (Fermentas), 1 μM of each primer (ITS_ufz01 and ITS_ufz02) and 1 U of HotStart *Taq* (Fermentas). The cycling scheme was 95 °C for 5 min, followed by 40 cycles at 95 °C for 40 s, 57 °C for 30 s, 72 °C for 40 s and the final extension step at 72 °C for 10 min.

In order to reduce the number of necessary cloning experiments to two per individual, two pooled reactions, each consisting of products of three individual PCRs were prepared (Renker et al. 2003). We purified 30 μl of this PCR pool with the MinElute Purification Kit (Qiagen) and eluted them with 10 μl EB buffer (Qiagen). Each purified PCR pool was checked on an agarose gel.

Cloning and sequencing

PCR pools were cloned using the pGEM T-Easy vector system (Promega, Mannheim, Germany). Recombinant clones were detected by blue/white screening, colonies picked from plates were used directly as a template in PCR with the standard sequencing primers M13F (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and M13R (5′-TCACACAGGAAACAGCTATGAC-3′), with 20 μl PCR reactions contained 2 μl 10× PCR buffer (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM of each M13 F-and R-primers and 0.5 U of *Taq* (Fermentas). The cycling scheme was 94 °C for 3 min, followed by 30 cycles with 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min and the final extension step with 72 °C for 3 min. PCR products of positive clones were purified with ExoSap-IT (USB, Staufen, Germany) and sequenced as described above.

As several different template sequences were present in PCR pools, formation of amplification chimaeras was possible (Judo et al. 1998; Zylstra et al. 1998). In order to detect and remove chimaeras we compared our sequences to GenBank sequences using BLAST. Those sequences with low bit scores and high *E*-values or with parts obviously corresponding to different species were removed from the dataset as suspected chimaeras.

Data analysis

Sequences were manually trimmed and grouped according to their similarity with Sequencher v.3.1.(Gene Codes, Ann Arbor, MI, USA). To determine taxonomic affiliation sequences were compared with known sequences in the GenBank using BLASTN. Taxonomic affiliation as well as putative ecology was inferred from the closest hits. One representative of each taxon was deposited in GenBank under the accession numbers listed (Accession No. GQ223448-GQ223476).

The fungal taxon composition between the two geographic regions was compared with the Jaccard index based on shared fungal taxa. Diversity of the fungal community was assessed and compared on the spatial levels of regions and populations. Diversity is reported as the fungal taxon richness (*R*, number of fungal taxa in the region/population) and taking abundance of the taxa into account as Shannon diversity (*H*′). The values of the Shannon diversity have to be considered with caution, as abundance after cloning may not exactly reflect abundance in the roots.

A rarefaction analysis (Simberloff 1978) was used to determine whether clone sampling effort saturated the number of taxa, using the analytical approximation algorithm (Hurlbert 1971) embedded in the Analytic Rarefaction freeware program from Steven M. Holland http://www.uga.edu/strata/software/Software.html.

Results and discussion

Diversity of fungi associated with Gymnadenia conopsea

On the basis of BLASTN searches we assigned 330 obtained sequences to 28 different taxa belonging to the Basidiomycetes and Ascomycetes (Table 1). The closest BLAST hits enabled classification to different taxonomic levels, depending on the fungal group. The majority of identified taxa (57 %) has previously been shown to be mycorrhizal, either orchid-mycorrhizas (OM 7 %) or ectomycorrhizas (ECM 50 %). However, a substantial part of taxa (43 %) were unspecific plant endophytes (PE), plant pathogens (PP) or uncultured taxa (UC).

Basidiomycetous mycorrhizas

Most so far identified orchid mycorrhizas are Basidiomycetes of the *Rhizoctonia* group (Rasmussen 1995; Warcup & Talbot 1971; Warcup & Talbot 1967) a polyphyletic assemblage including teleomorphs of the genera *Tulasnella*, *Ceratobasidium*, *Thanatephorus* and *Sebacina* (Moore 1987; Warcup 1981; Warcup & Talbot 1971; Warcup & Talbot 1967). Sequences of all these families were amplified from the roots of *Gymnadenia conopsea*. The Sebacinaceae have also been shown to form ectomycorrhizas on trees (Selosse et al. 2002), like the typical ectomycorrhizal families Russulaceae (Dearnaley 2007) and Thelephoraceae (Abadie et al. 2006), which were also found in the roots of *G. conopsea*.

Ascomycetous mycorrhizas

Ascomycete ectomycorrhizas differ morphologically from their more robust and well established basidiomycetous counterparts. Typically they produce only thin mantles with sparsely growing hyphae. So far they have been less studied and comparatively little is known about the taxonomy and ecology of these fungi. Hence, their importance as mycorrhizas is probably seriously underestimated, a view supported by the fact that Tedersoo et al. (2006) identified several new mycorrhizal taxa within Pezizales. The Pezizales and Helotiales are two ascomycetous orders which have been shown to include taxa interacting as mycorrhizas (Julou et al. 2005). In our study we identified five pezizalean genera *Peziza*, *Terfezia*, *Morchella*, *Geopyxis* and *Wilcoxina*, all previously shown to interact as ectomycorrhizas (Abadie et al. 2006; Buscot 1994; Dahlstrom et al. 2000; Tedersoo et al. 2006). We were not able to characterize sequences assigned as Helotiales in more detail, because taxonomic assignment of Helotiales sequences in the GenBank is poor reflecting difficulties with taxonomy and limited knowledge of this group (Wang et al. 2006b; Wang et al. 2006a). As the Helotiales are an ecologically diverse order including plant pathogens, different types of saprobes, plant endophytes and both ericoid and ectomycorrhizal fungi (Vralstad et al. 2002; Wang et al. 2006b), an ecological function was difficult to assess. Nevertheless, because Helotiales also include ectomycorrhizal species and the only genus identified within the Helotiales was the ectomycorrhizal *Cadophora* (Vralstad et al. 2002), we classified the Helotiales sequences as potentially ectomycorrhizal. Similar problems exist with sequences classified as ‘uncultured’ taxa, because due to the lack of information no ecological characterization

Table 1 – Taxa found within the roots of *Gymnadenia conopsea* and putative ecological roles as inferred from the closest relatives (OM = orchid mycorrhiza; ECM = ectomycorrhiza; PE = plant endophyte; PP = plant pathogen or saprobes) and the number of clones of the respective fungal taxa amplified from the roots of three individuals per site of *G. conopsea* for Eastern German (E) and Northern German (N) region (individual sites)

	Tentative identification[a] and putative ecology		Closest NCBI-Hit (Accession No.)/taxonomic affiliation	ID (%)	Number of Clones	
					E	N
1	Tulasnellaceae (B)	OM	Unc. Tulasnellaceae (DQ925600)/Tulasnellaceae	99	17 (4/13/0)	27 (0/4/23)
2	*Sebacina* sp. (B)	ECM/OM?	Unc. *Sebacina* (EU668266)/Tulasnellaceae	99	1 (1/0/0)	–
3	Ceratobasidiaceae -OM (B)	OM	*Ceratobasidium* sp. (EU668239)/Ceratobasidiaceae	99	34 (0/0/34)	7 (1/6/0)
4	Ceratobasidiaceae –ECM (B)	ECM	Uncultured ectomycorrhiza Ceratobasidiaceae (AY634129)	96	1 (0/1/0)	–
5	*Lactarius* (B)	ECM	*Lactarius pubescens* (AY336958)/Russulaceae	99	1 (1/0/0)	–
6	Russula (B)	ECM	*Russula exalbicans* (DQ974759)/ *R. maculata* (AY061688)/Russulaceae	99 98	3 (0/3/0)	2 (0/2/0)
7	Thelephoraceae (B)	ECM	Unc. *Tomentella* (EU668209)/Thelephoraceae	99	1 (0/1/0)	–
8	*Terfezia* (A)	ECM	*Terfezia* sp. (DQ061109)/Pezizales	86	19 (0/0/19)	4 (0/0/4)
9	*Peziza* (A)	ECM	*Peziza proteana* (DQ491497)/Pezizales	85	–	11 (11/0/0)
10	*Morchella* (A)	ECM	*Morchella spongiola* (AJ539478)/Pezizales	96	1 (0/1/0)	–
11	*Geopyxis* (A)	ECM	*Geopyxis rehmii* (Z96991)/Pezizales	91	1 (1/0/0)	–
12	*Wilcoxina* (A)	ECM	*Wilcoxina rehmii* (AF266708)/Pezizales	98	–	1 (0/1/0)
13	*Cadophora* (A)	ECM	*Cadophora* sp. (DQ317329)/Helotiales	92	–	1 (0/1/0)
14	Helotiales (A)	ECM?	Unc. Helotiales (DQ182424)/Helotiales	100	9 (6/3/0)	17 (12/3/2)
15	*Cenococcum* (A)	ECM	*Cenococcum geophilum* (DQ474346)/Dothideomycetes	99	2 (0/2/0)	1 (0/1/0)
16	*Phialophora* sp. (A)	ECM?	*Phialophora europaea* (EF540756)/Sordariomycetes	91	1 (1/0/0)	–
17	*Tetracladium* (A)	PE	*Tetracladium maxilliforme* (DQ068996)	100	20 (13/7/0)	14 (3/9/2)
18	*Leptodontidium* (A)	PE	*Leptodontidium orchidicola* (AF486133)	98	14 (8/4/2)	40 (13/14/13)
19	*Cryptococcus* (B)	PE	*Cryptococcus carnescens* (AB105438)/Tremellales	99	2 (0/2/0)	–
20	*Verpa* (A)	PE	*Verpa conica* (AJ544206)/Pezizales	97	5 (0/0/5)	1 (0/1/0)
21	*Lecanora* (A)	PE	*Lecanora reuteri* (AF070026)/Lecanorales	95	1 (1/0/0)	–
22	*Exophiala* (A)	PP	*Exophiala salmonis*(AF050274)/Herpotrichiellaceae	95	7 (5/2/0)	11 (6/3/2)
23	*Fusarium* (A)	PE/PP?	*Fusarium oxysporum* (FJ605243)/Hypocreales	100	–	2 (0/0/2)
24	*Neonectria* (A)	PE/PP?	*Neonectria radicicola* (AJ875336)/Hypocreales	100	5 (2/3/0)	12 (0/5/7)
25	Hypocreales (A)	PP	Unc. Hypocreales (FJ552924)	92	–	3 (3/0/0)
26	Herpotrichiellaceae (A)	PP	Uncultured Herpotrichiellaceae (EF619700)	95	6 (0/6/0)	–
27	Pezizomycotina (A)	?	Uncultured Pezizomycotina (DQ182456)	100	2 (0/0/2)	–
28	Uncultured Taxa	?	Taxonomy unknown		12 (7/5/0)	11 (2/6/3)

a A = Ascomycetes; B = Basidiomycetes.

is possible, but a potential role for plant performance cannot be ruled out.

Endophytes

'Endophyte' is a general term referring to organisms that grow inside plant tissues without causing disease symptoms (Carroll 1988; Chanway 1996). Little is known about the role of endophytes for orchid performance, although some endophytes have been shown to confer fitness benefits to host plants, including tolerance to heat, disease and drought (Rodriguez & Redman 2008). Most of the identified endophytes of *Gymnadenia conopsea* are Ascomycetes such as *Exophiala*, *Fusarium*, *Leptodontidium* or *Tetracladium*, some of them possibly also representing surface contaminants. Interestingly, we detected *Tetracladium* in five out of six populations of *G. conopsea* in Germany. Only recently Selosse *et al.* (2008) drew attention to the presence of these aquatic asexual fungi in terrestrial ecosystems. Although they are commonly occurring in running fresh water they were reported as endophytes from healthy looking plant tissue of several species (Abadie *et al.* 2006; Murat *et al.* 2005; Russell & Bulman 2005; Tedersoo *et al.* 2007). Our findings support the hypothesis that some aquatic fungi spend a part of their life in plants and have a planktonic, aquatic and aerial dispersal (Selosse *et al.* 2008).

We found a surprisingly high diversity of fungi associated with *G. conopsea*, indicating that this orchid shows only little specificity to certain fungal clades. The basidiomycetous mycorrhizas are mostly of confirmed mycorrhizal status for orchids (seven of the eight Basidiomycetes), whereas we also identified a variety of ascomycetous taxa which are known to form ectomycorrhizas on other plants. Their detection suggests a potential role as mycorrhizas for *G. conopsea* and emphasizes the need for further investigations of the role of ascomycetous taxa as mycorrhizas in orchids. The wide taxonomic range of mycorrhizal associates found in the roots of *G. conopsea* might contribute to its ability to grow in very different habitat types with their respective fungal communities, including rather disturbed habitats in quarries and mines.

Geographic differentiation of fungal communities

From the total of 28 taxa, seven were widespread and occurred in at least four out of six sites, five taxa occurred at 2–3 sites.

However, the majority of 16 taxa were found at only one site. The total number of fungal taxa differed between regions: 23 taxa (80 %) were detected in the East and 17 (60 %) in the North. Taking into consideration the abundance of the taxa, the Shannon diversity was slightly higher in the East ($H' = 2{,}6$) than in the North ($H' = 2{,}4$). However, for both regions rarefaction analysis showed a clear levelling off after approximately 100 and 65 sequences respectively, with a gain of only three species following additional sampling in both regions (Fig 1). This indicates that our sequence sampling effort, while by no means exhaustive, captured a substantial proportion of the diversity of fungal taxa associated with *G. conopsea*.

Species composition of the two regions showed limited overlap, as only 43% of taxa were shared, including the most abundant ones Tulasnellaceae, Ceratobasidiaceae, *Leptodontidium* and *Tetracladium*. When only more common taxa were considered (present in at least three clones) similarity increased to 64 %, indicating that inclusion of rare species may inflate the sampling error and thus underestimate similarity between regions (Table 1). However, substantial differences in taxon richness were found among populations. Irrespective of region, the number of taxa per population varied between 5 (18 %) and 14 (50 %) (Fig 2), while the mean population taxon richness was similar in both regions (East: $R = 10.3$, SD = 4.7; North $R = 10.0$, SD = 2.6, $p = 0.92$ *t*-test).

Regardless of the observed differences in the community composition of fungal taxa common patterns can be readily recognized. Considering functional groups, in each population at least one basidiomycetous OM of the Tulasnellaceae and/or Ceratobasidiaceae was detected, suggesting that *G. conopsea* utilizes fungi from these known OM families, like most photosynthetic orchids. In addition, in all populations several ascomycetous ectomycorrhizal taxa of the Pezizales and/or Helotiales were also present (Table 1). This pattern holds even when Helotiales are not considered as their ectomycorrhizal status is not confirmed. The presence of ectomycorrhizal taxa in all populations might indicate that *G. conopsea* has the ability to utilize ascomycetous ectomycorrhizal taxa as mycorrhizas.

Selosse *et al.* (2004) suggested that the replacement of the usual *Rhizoctonias* in Neottieae by ectomycorrhizas may be a strategy to secure access to fungal carbohydrates where

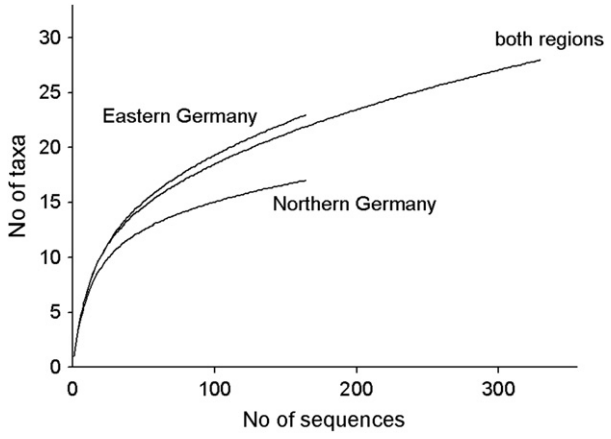

Fig 1 – Rarefaction curve of the number of sequences sampled in Eastern Germany, Northern Germany and in both regions together.

Rhizoctonias are either not available or where photosynthesis rate is limited due insufficient light availability like in forest habitats. The adoption of ectomycorrhizal fungi as mycorrhizas would mean a more stable carbon resource and made light deficient habitats accessible. *G. conopsea* is generally known to colonize a wide variety of different habitat types, typically occurring on open grassland sites, but also found in shaded forest habitats. Hence, the adoption of ectomycorrhizal taxa could have contributed to its ability to grow in such diverse habitats by expanding its potential habitat to shaded conditions. Nevertheless, at the current stage these ectomycorrhizal taxa found in the roots of *G. conopsea* have to be considered as 'potential partners', because the amplification of fungal taxa directly from root extracted DNA does not necessarily imply that these fungi interact as true mycorrhizas. The standard method to test whether a fungus is compatible with an orchid species are germination tests. Unfortunately, ectomycorrhizal taxa are known to be difficult to cultivate. However, the fact, that these fungi are obligatory symbiotic (Erland & Taylor 2002) makes us confident that they are not simply surface contaminants but that they indeed play a role for the performance of *G. conopsea*. Which role exactly, certainly needs further investigation.

In general, the diversity of compatible fungi (degree of specificity) is expected to influence the competition, survival and distribution of an orchid species. For orchids that require specific fungi, availability of appropriate symbionts may determine which habitats allow orchid growth and what environmental factors are critical for orchid recruitment, while diverse associations may be less limiting (McCormick *et al.* 2004). Furthermore, orchids with a broad taxonomic spectrum of potential fungal partners should be expected to be more easily distributed and colonize new habitats as the probability to find a compatible fungus after dispersal should be high. However, Irwin *et al.* (2007) investigated the fungal partners of the common terrestrial orchid *Pterostylis nutans* across its range in eastern Australia. He identified two fungi of the *Ceratobasidium* to be the main fungal partners and showed that specificity occurs in this species, despite its wide distribution. In contrast, Bonnardeaux *et al.* (2007) found that two weed-like orchid species and a widespread native, disturbance-intolerant species in Australia were associated with a diversity of fungal associates and had broad webs of mycorrhizal fungi. Most associated fungi belonged to the *Rhizoctonia* alliance with a worldwide distribution, whereas for *G. conopsea* we additionally identified several ectomycorrhizal taxa as potential fungal partners.

So far only little is known of the factors determining the diversity and composition of fungal communities associated with orchids. On the one hand micro-organisms are hypothesized to be omnipresent, at least in the form of diaspores, forming a basically common species pool. Consequently the same environmental conditions, both biotic and abiotic, should select the same microbial community in different locations (Taylor *et al.* 2006). On the other hand, however, there are also parameters known to influence the fungal community, e.g. extrinsic factors such as habitat type, geography or intrinsic factors like genetic differentiation (Schechter & Bruns 2008; Shefferson *et al.* 2008; Taylor & Bruns 1999b; Taylor *et al.* 2004). Such a complex interaction of different factors was shown to

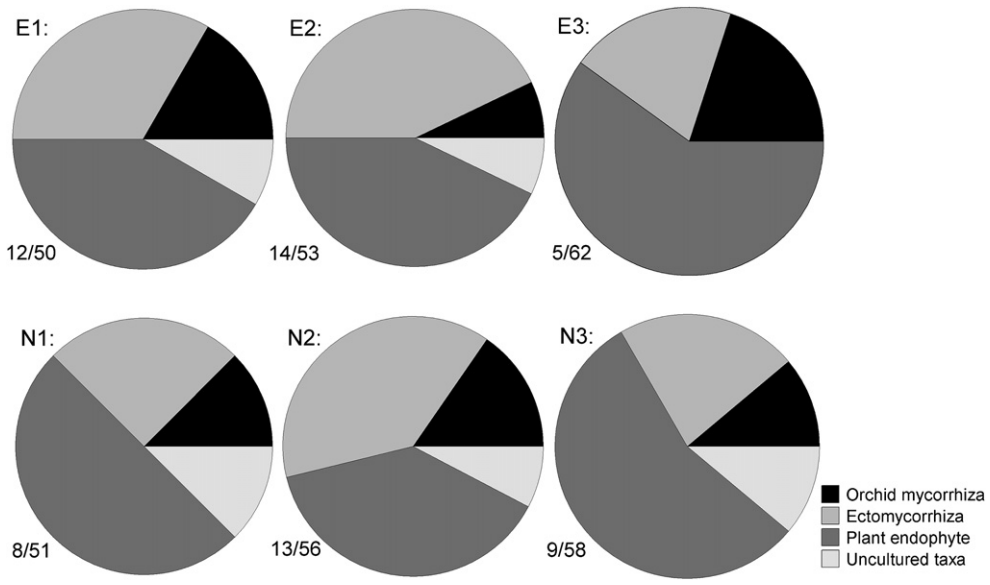

Fig 2 – Distribution of putative ecological roles of the taxa found for each population in Eastern Germany (E1-E3) and Northern Germany (N1-N3). Digits present numbers of taxa found in the respective populations (left) and number of clones checked (right). Endophytes include plant endophytes, pathogens and saprobes.

influence the fungi associated with the fully mycoheterotrophic orchid *Corallorhiza maculata*. Taylor & Bruns (1999a) found that *C. maculata* associated with only one single, never fruiting *Russula* species, whereas there were also six other *Russula* taxa on the same plot. Furthermore, a strong correlation between specificity and plant community was detected as certain *Russula* species were the dominant symbionts of orchids growing in *Quercus* forests, but these ones were never found in samples from nearby coniferous forests (Taylor & Bruns 1999a; Taylor & Bruns 1997). Further studies on this orchid showed that even the genotypes of *C. maculata* individuals played an important role as different genotypes never shared the same *Russula* species, even when growing together (Taylor *et al.* 2004). These investigations on *C. maculata* showed that factors determining the fungi associated with an orchid species can be highly complex and are not solely driven by the absence of alternatives. Such a complex interaction of different factors may also play a role for the determination of the fungi associated with *G. conopsea*. The taxon composition of the fungal partners associated with *G. conopsea* was not homogenous over all localities, but showed a clear spatial structure and only little overlap between regions. This regional differentiation in species composition together with the high variability on the population level suggest that factors at the local scale may strongly affect local species composition and hence diversity at the regional level. *G. conopsea* is known to show a high intraspecific morphological variability (Scacchi & de Angelis 1989; Soliva & Widmer 1999). Currently there are two differentiated subspecies, which can occur sympatrically and genetic differences as well as in the ploidy level have been reported (Gustafsson & Lönn 2003; Marhold *et al.* 2005). Differences in fungal diversity found between the investigated populations of *G. conopsea* might be due genetic differences and indicate an ongoing diversification between populations. In addition environmental factors, like pH or water availability, may differentiate localities. This emphasizes the need for future investigations to integrate multiple factors such as ploidy or habitat type in order to analyse what are the main factors determining the fungal associates of *G. conopsea*. Especially in the light of the high intraspecific variation observed for *G. conopsea* a more detailed analysis of the determining parameters is certainly warranted and might contribute to the general understanding of this very unique relationship between orchids and their mycorrhizal fungi.

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