Molecular ecological analyses of specific interactions between symbionts in the arbuscular mycorrhizal symbiosis

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Summary

Arbuscular mycorrhiza is an ancient symbiosis between the majority of land plants and fungi from the phylum Glomeromycota. Arbuscular mycorrhizal fungi (AMF) colonize plant roots and contribute to the mineral nutrient uptake of the hosts in exchange for carbohydrates. AMF species diversity and identity was reported to have a decisive influence on the composition and productivity of natural plant communities. Only around 200 glomeromycotan species described so far were thought to colonize the majority of higher plant species and thus, their host specificity was thought to be very low.

In this thesis, molecular methods were used to investigate ecological aspects of root colonizing AMF. The community composition of these fungi was analyzed in two plant species-rich grasslands facing different environmental conditions and harboring different plant communities. One site consisting of two meadows located close to each other was situated in the upper montane zone of the Swiss Alps. The other was located in the lowland in France on the edge of the Jura mountains. The roots were analyzed using AMF-specific nested PCR, RFLP screening and sequencing of rDNA small subunit and internal transcribed spacer regions. AMF sequences were analyzed phylogenetically and used to define monophyletic sequence types.

Overall, 27 different AMF sequence types were detected in the root samples from both field sites. The overlap between the AMF communities in the alpine and lowland site was relatively small - they shared just six sequence types. These results indicate strong geographical differences in the AMF community composition, reflecting different environmental conditions and plant species communities in each site.

The question was adressed, whether different host plant species co-occurring in the same area host distinct or similar AMF communities. *Gentiana verna*, *G. acaulis* and *Trifolium* spp. growing in two alpine species-rich meadows harbored significantly different AMF communities, whereas the differences between the two sites were negligible. These results indicate that within a relatively small area with similar soil and climatic conditions, the host plant species can have a major influence on the AMF communities within the roots. In these alpine sites, there was also a focus on green plants from the family Gentianaceae. In contrast to their mycoheterotrophic relatives, the green gentians did not show a high level of specificity towards AMF. The plants sampled harbored AMF communities comprising multiple phylotypes from different fungal lineages.

In the lowland site – a calcareous grassland – different culturing methods and their influence on the AMF community composition in the roots were adressed. Four plant species were sampled i) directly in the field, ii) in a bait plant bioassay conducted directly in that

grassland and iii) in a greenhouse trap experiment using soil and a transplanted whole plant from that grassland as inoculum. The community composition in their roots was strongly influenced by the experimental approach, with additional influence of cultivation duration, substrate and host plant species in some experiments.

Some fungal phylotypes, e.g. *Glomus mosseae* and several members of *Glomus* group B, appeared predominantly in the greenhouse experiment or in bait plants. These phylotypes can be considered r strategists, rapidly colonizing uncolonized ruderal habitats in early successional stages of the fungal community. Other phylotypes as *Glomus badium* and GLOM-A16 were detected almost exclusively in roots sampled from plants naturally growing in the grassland or from bait plants exposed in the field, indicating that they preferentially occur in late successional stages of fungal communities and thus represent the K strategy. The only phylotype found with high frequency in all three experimental approaches as well as in the alpine site was GLOM A-1 (*Glomus intraradices*), which is assumed to be a generalist.

These ecological strategies of different AMF species or phylotypes should be considered in AMF experimental work. In greenhouse trap experiments it is difficult to establish a root-colonizing AMF community reflecting the diversity of these fungi in the field roots, because fungal succession in such artificial systems may bias the results. However, the field bait plant approach might be a convenient way to study the influence of different environmental factors on AMF community composition directly under the field conditions.

Finally, the co-existence of the Glomeromycota in the root samples with fungi from the basidiomycotan order Sebacinales was addressed. These fungi are widely distributed and known to form various types of presumable mycorrhizal associations of different morphology (ecto-, ectendo-, orchid, ericoid, jungermannoid) with a broad range of host plant species. However, their presence in plants forming arbuscular mycorrhiza has not been reported yet. Therefore, the root samples originating from the two species-rich grasslands mentioned above were analyzed with Sebacinales-specific primers for the D1/D2 region of the nuclear 28S rDNA subunit. Fungi from the order Sebacinales were present in the majority of the root samples from both sites, where they co-existed with the Glomeromycota. In agreement with studies targeting the Sebacinales in ericoid plants, the phylogenetic analysis of sebacinoid sequences from our samples did not reveal any patterns according to their host plant species or geographical origin.

Chapter 1

General introduction

1.1. Mycorrhiza – general definition and its main types

Mycorrhiza can be defined as a mutualistic symbiotic association between roots and some groups of soil fungi. Mycorrhizal associations have been found between fungal hyphae and the thalli of many bryophytes, as well as the roots of vascular plants (Smith and Read 1997). In the mycorrhizal relationship, the fungus colonizes the roots during periods of the active growth, and a new organ, the "mycorrhiza", is formed. The host plant receives mineral nutrients from the fungus while the fungus obtains from its plant partner photosynthetically derived carbon compounds. Indeed, mycorrhizas, not roots, are the main organs of nutrient uptake by most terrestrial plants. Mycorrhiza can exist in many forms, its morphology is determined by the characteristics of each partner involved and by the specific plant-fungus combination.

Mycorrhizas are classified morphologically according to the fungal growth and structures in the roots. The ectomycorrhizal symbiosis in which fungal hyphae grow in the intercellular spaces but do not enter the plant cells is mainly formed between trees (e.g. members of Pinaceae, Fagaceae, Myrtaceae, Salicaceae) and fungi belonging to the phyla Basidiomycota and Ascomycota. The plant roots get shorter, thicker and highly branched. The fungus forms a hyphal sheath on the root surface and an extraradical mycelium to acquire water and mineral nutrients in the soil. The nutrient exchange between the plant root and the fungus takes place in a hyphal network, the Hartig net, in the intercellular spaces of the outer root cortex layers (Smith and Read 1997).

Arbuscular mycorrhiza (AM) is characterized by the inter- and intracellular growth of the fungus. It is formed by fungi from the phylum Glomeromycota (Schüssler et al. 2001) and mostly herbaceous plant species and tropical trees. The nutrient exchange unit is localized intracellularly, often in the form of a tree-like structure called arbuscule (for details see Chapters 1.2.1. and 1.2.3.). Ectendomycorrhiza is a morphologically intermediate type between the ecto- and arbuscular mycorrhiza. Further, several special types of mycorrhiza were described and named after the plant taxa they occur in: orchid, ericoid, monotropoid and arbutoid mycorrhizae (Smith and Read 1997).

Interestingly, several predominantly nonmycorrhizal taxa developed during evolution (members of e.g. Brassicaceae, Chenopodiaceae, Caryophyllaceae), but this is nevertheless a relatively rare phenomenon (Harley and Harley 1987).

1.2. Arbuscular mycorrhiza - its morphology and life cycle

1.2.1. General features of arbuscular mycorrhiza

Arbuscular mycorrhiza is an ancient symbiosis thought to date back to the Ordovician, 460 million years ago, when the first plants were colonizing the land (Redecker et al. 2000). It is estimated to occur in about 80% of plant species (Bonfante and Perotto 1995). Arbuscular mycorrhizal fungi (AMF) colonize plant roots, produce an extensive network of extraradical mycelium in the soil and as mentioned above, contribute to the mineral nutrient uptake of the hosts in exchange for carbohydrates (Smith and Read 1997).

Arbuscular mycorrhizal fungi are present in most terrestrial ecosystems and crop production systems and mediate plant root and soil interactions. The roots are regularly colonized by AMF and in most habitats it is almost impossible to find non-mycorrhizal individuals. AMF are asexual obligate symbionts, i.e. they cannot grow without their plant hosts. They form a coenocytic mycelium - i.e. it lacks hyphal septa.

1.2.2. The AMF life cycle

AMF spores (diameter 40-800 μm) are commonly present in soils. They possess layered walls and contain several hundreds to thousands of nuclei (Becard and Pfeffer 1993). Under favorable environmental conditions, the spores germinate and the hyphae colonize susceptible roots. AMF hyphal growth and branching in the rhizosphere of host plants can be increased by strigolactones produced as branching factors in root exudates, whereas non-host plants do not have such effects on the hyphae (Smith et al. 2006). Similar to the branching factors, which are produced by plants as a compatibility signal for the fungus, the branched fungal hyphae secrete a diffusible signal to the roots (Kosuta et al. 2003). Thus, a crosstalk between the plant and the fungus is established.

After the recognition of the host plant root, the hyphae form appressoria on the root epidermis (the root hair or other epidermal cell) and excrete small amounts of cell wall degrading enzymes such as pectinase and cellulase. Nevertheless, the low rate of production of these enzymes suggests that AMF penetrate the root surface mostly by mechanical force (Bonfante and Perotto 1995). The entry of the fungus is assisted by the plant by the establishment of a special cytoskeletal arrangement, the prepenetration apparatus (Genre et al. 2005). The appressorium formation is followed by hyphal penetration of the cell lumen and proliferation of the intraradical hyphae in the upper cell layers of the root cortex.

Furthermore, the hyphae enter the middle and inner cortex of the root. However, the

fungus never enters meristems or vascular cylinders, perhaps because of its inability to degrade suberin and lignin in the endodermal cell walls (Bonfante and Perotto 1995). Although the fungal hyphae may grow inside the host cells, they always stay surrounded by

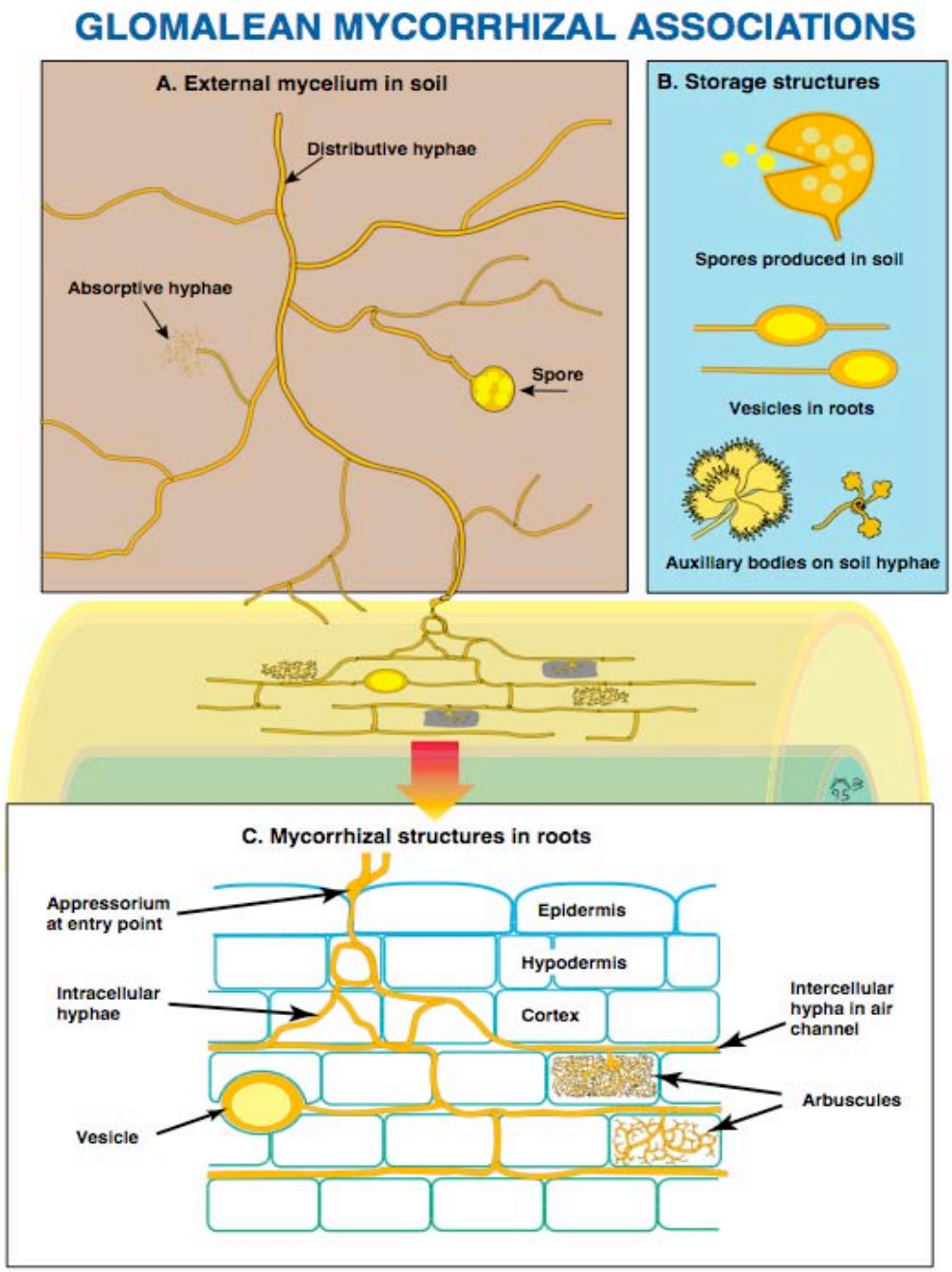

Fig. 1 Structures of arbuscular mycorrhiza. From http://www.ffp.csiro.au/research/mycorrhiza/method.html

the intact plasma membrane of the plant host. In the deeper cortical layers, the fungal hyphae may form arbuscules - a highly branched tree-like structures - in the plant cells. A plant periarbuscular membrane surrounds the thin fungal branches of the arbuscules forming an

extremely large contact surface between the two symbionts. Here, the nutrients are transferred between the plant and the fungus, mediated through a series of specific proteins (Ferrol et al. 2002). For further details see Chapter 1.4.1.

Some AMF taxa (families Glomeraceae and Acaulosporaceae according to http://invam.caf.wvu.edu/fungi/taxonomy/classification.htm) form intra- or intercellular vesicles in the host plant roots. Vesicles are lipid-filled storage structures with an ovoid shape.

Once the fungal colonization is established in the root, extensive growth of the extraradical mycelium (ERM) begins. Its finely branched fans (2 µm according to Friese and Allen (1991) are well adapted to the exploration of soil pores, mineral nutrient uptake and association with soil particles and thus stabilization of soil aggregates. The ERM forms a complex network, which can link roots of plants of the same or different species (Smith and Read 1997). The life cycle of the AMF is completed by the formation of spores or sporocarps on the ERM or rarely inside the roots.

1.2.3. *Paris* and *Arum* type of arbuscular mycorrhiza

Two types of AM symbiosis were defined according to the morphology of the intraradical colonization (Gallaud 1905). They were named after the plants in which they were first described: the *Arum* and the *Paris* type.

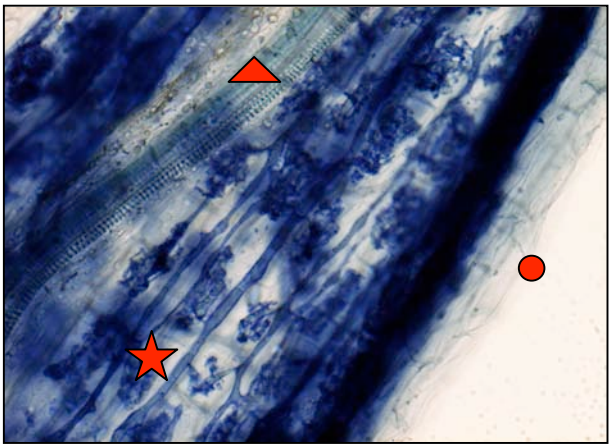

Fig. 2 The *Arum* type of AM. Root of *Inula salicina* stained with trypan blue, 200x magnified. The star shows the intercellular hyphae with intracellular branches forming arbuscules, the triangle indicates the central cylinder and the circle shows the rhizodermis.

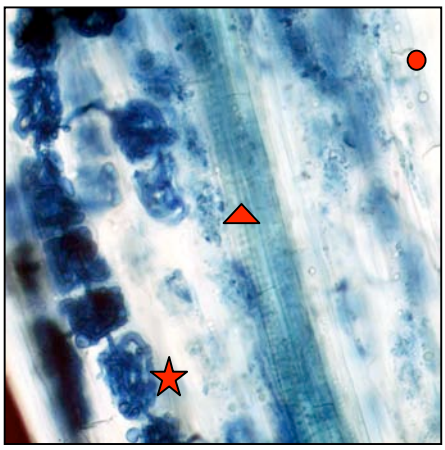

Fig. 3 The *Paris* type of AM. Root of *Gentiana acaulis* stained with trypan blue, 200x magnified. The star shows the intracellular hyphal coils, the triangle indicates the central cylinder and the circle shows the rhizodermis.

In the *Arum* type (Fig. 2), the AMF produce long intercellular hyphae whose branches form typical arbuscules inside the cortical cells. Despite their central role in the nutrient exchange, arbuscules have a relatively short life. They collapse and degrade already after a few days or weeks, leaving the host plant cell intact (Dickson and Smith 2001). The *Paris* type (Fig. 3) is characterized by predominantly intracellular hyphal growth and formation of extensive intracellular hyphal swellings, coils and/or arbusculate coils in the root cortex (Smith and Smith 1997). In addition to arbuscules, coils are also believed to participate in the nutrient transfer between the symbionts. Both arbuscules and coils increase the contact area between the fungus and the plant cell (Smith and Read 1997).

The two AM types differ not only in their morphology, but also in the kinetics of root colonization. The development of *Paris* type colonization within the root is much slower than in the *Arum* type (Azcon- Aguilar et al. 1994). The type of AMF colonization seems to be largely determined by the host plant genotype, as the same AMF species known to form the *Arum* type in some host plant species produces the *Paris* type in other hosts (Demuth and Weber 1990; Sýkorová et al. 2003; Ahulu et al. 2006).

1.3. Genetics of AMF

The glomeromycotan fungi are thought to reproduce clonally (asexually) by spore formation or by formation of coenocytic (multinucleate) hyphal networks. Studies using molecular marker genes have detected no genetic recombination or only low levels (Kuhn et al. 2001).

Sanders et al. (2003) reported that there is an unusual level of within-individual genetic diversity. Different variants of rDNA (with up to 24.1% variation among ITS sequences) as well as protein-coding genes may co-exist within a single AMF spore (Sanders et al. 1995; Kuhn et al. 2001; Jansa et al. 2002b). There are conflicting reports on the possible cause of this high diversity and on the question whether the nuclei in the mycelium and spores of one AMF organism are genetically identical or different. Pawlowska and Taylor (2004) proposed a homokaryotic system with intranuclear rDNA polymorphism, whereas Hijri and Sanders (2005) presented evidence for a heterokaryotic system with genetically different nuclei.

Hijri and Sanders (2005) also addressed the question of polyploidy as a source of genetic and nuclear DNA content variation in AMF as proposed by Pawlowska and Taylor (2004). They provided evidence that AMF even with large nuclear DNA contents are haploid. Further, these authors proposed that the nuclei are genetically different and that probably not all of them are inherited by every spore. However, the frequent anastomoses among hyphae of the same species or isolate (de la Providencia et al. 2005)

were suggested to mediate the re-establishment of the genome diversity of the fungus. At the moment, the question of genetic structure of AMF is a topic of intensive discussions. Further investigations are necessary to understand the mechanisms how these genetic features contribute to the ecology, evolution and symbiotic efficiency of the Glomeromycota.

1.4. Beneficial features of arbuscular mycorrhiza

1.4.1. Nutrient exchange between the symbionts

The symbiosis between AMF and autotrophic plants is generally regarded as mutualistic. With the exception of few achlorophyllous species, AM host plants are autotrophic and, although normally colonized by mycorrhizal fungi in the field, they are usually capable of satisfactory growth in the absence of mycorrhizal colonization, provided that mineral nutrient supplies are adequate. In contrast, the glomeromycotan fungi are obligate symbionts, as their extraradical hyphae are unable to take up carbohydrates (Pfeffer et al. 1999). Thus, they depend on photosynthates supplied by the plant and utilize a considerable proportion of its assimilated C (probably 4-20% of net photosynthate; Jakobsen and Rosendahl 1990).

It is predicted that phosphate and carbon transfer occur at the arbuscule/cortical cell interface, although direct evidence for carbon transfer at this location is lacking (Javot et al. 2007). The assimilate transfer includes sucrose breakdown into glucose and fructose, their export across the plant plasma membrane and active uptake by hexose transporters across the fungal plasma membrane, driven by an increased H^+ATPase activity at the arbuscular membrane (Gianinazzi-Pearson et al. 2000). H^+-ATPases are assumed to drive the transmembrane proton gradient that is required for some of the transmembrane transporter activities and are responsible for an acidic pH in the periarbuscular space. The only monosaccharide transporter of the Glomeromycota described so far has been found in *Geosiphon pyriforme* (Schüssler et al. 2006). This fungus is known to form bladders but not arbuscules (see Chapter 1.5.1.). In the fungal cytosol, the hexoses are then converted into triacylglycerides, amino acids or incorporated into glycogen pools. The major storage forms of carbon in spores and hyphae are lipids, trehalose and glycogen (Pfeffer et al. 1999).

Hyphae from colonized roots extend into the soil and aid root hairs in absorbing water and mineral nutrients. Phosphorus is a major limiting nutrient in many ecosystems and enhanced phosphorus supply by AMF plays a key role in ecosystem functioning (e.g. Klironomos et al. 2000). Phosphate is taken up from the soil by AMF through fungal phosphate transporters. Within the extraradical fungal hyphae, phosphate is condensed to polyphosphate, and transported into the intraradical hyphae. In the arbuscules, polyphosphate

is hydrolysed by enhanced phosphatase activity and released into the periarbuscular space (Ohtomo and Saito 2005). Phosphate is then taken up by the plant through a transmembrane transporter, such as the *Medicago truncatula* MtPt4, located in the periarbuscular membrane (Harrison et al. 2002; Javot et al. 2007). Similarly to carbon, the uptake of phosphate and other nutrients by arbuscular plant cells may be linked to the high plant and fungal H^+-ATPase activity observed at the periarbuscular membrane (Gianinazzi-Pearson et al. 2000).

Although most of the nutrient transfer between the two symbiotic partners takes place in the arbuscules, they do not seem to be the sole place of the nutrient exchange. It is almost certain that the nutrient transfer may additionally occur at the intracellular hyphal coils and swellings, as many plant species form the *Paris* type of mycorrhiza, which may lack the arbuscules (see Chapter 1.2.3.; Smith and Smith 1997).

1.4.2. Other beneficial features of arbuscular mycorrhiza

AM associations have been shown to benefit the plants by increasing mineral nutrients (mainly phosphorus) and water absorption; root health and longevity (resistance to pathogens and insect herbivores); tolerance to drought, high soil temperature, toxic heavy metals, extremes in pH and transplant shock (Smith and Read 1997). Many studies have shown that mycorrhizal colonization is followed by a considerable stimulation of growth (Smith and Read 1997). However, it was demonstrated that the impacts of mycorrhizal association differ according to the plant species and fungus involved; there is a range of plant growth responses from strongly positive, through neutral to negative (Francis and Read 1995).

Hyphae of mycorrhizal fungi are important stabilizing agents in the soil. In many soils, the roots and hyphae together with other organic components play a major role in stabilisation of soil aggregates (Schreiner and Bethlenfalvay 1995), erosion control and water management (Piotrowski et al. 2004). Moreover, the plants in their communities are functionally interconnected with the mycelial network of AMF. The potential importance of these hyphal links is discussed in the Chapter 1.6.2. A healthy AM community with intact soil structure is of high importance for the agriculture, as symbiotic plants achieve higher biomass and crop yields particularly in nutrient poor soils (Klironomos et al. 2000). The AMF colonization may also lead to an increased overall plant fitness and resistance against nematodes or pathogens, which can, to a certain extent, alleviate the necessity of a high input of agrochemicals in the agricultural practice.

1.5. Identification, phylogeny, systematics and diversity of the Glomeromycota

1.5.1. Systematics and phylogeny of the Glomeromycota

Previously, AMF were placed into the order Glomales and belonged to the phylum Zygomycota (Morton and Benny 1990). However, based on their obligate symbiotic habit, the apparent lack of zygospores (i.e. spore produced through the conjugation of specialised hyphae during the sexual reproduction) and the rDNA phylogeny, Schüssler et al. (2001) defined the phylum Glomeromycota as a sister clade of Basidio- and Ascomycota.

Glomeromycota currently comprise approximately 200 described species distributed among ten genera, most of which were defined primarily based on the morphology of their spores or spore-bearing structures (http://www.lrz-muenchen.de/~schuessler/amphylo/). The spores can be analyzed under the microscope, certain spore wall structures can be stained. The way the spore is formed on the hypha ("mode of spore formation") has been important to define genera and families; and the number of the walls, their layer structure as well as ornamentation to distinguish species (Morton 1988). Although these characteristics are specific, they can differ according to the environmental conditions and on the stage of the spore; aged, dry or decaying spores may look different, even if they belong to the same species. Moreover, it is quite difficult and requires expert knowledge; to distinguish and classify the spores by their morphology.

Recently, DNA sequences have also been used to describe AMF taxa and their phylogenetic relationships (e.g. Schwarzott et al. 2001; Redecker and Raab 2007), for details see Chapter 1.5.2.). Molecular methods allow the identification of spores as well as the AMF community currently colonizing plant roots or growing in the soil at any given time. However, the possible sequence variation within individuals (see Chapter 1.3.) makes it difficult to interpret molecular community data when sequences are obtained from colonized plant roots. Hence, a conservative approach in the evaluation of phylogenetic analyses is advisable; e.g. by defining a monophyletic well supported sequence cluster as an AMF phylotype (i.e. sequence type).

At the present time, the phylogeny of all genera of AMF is based entirely on analyses of the nuclear small subunit RNA gene. Although additional genes have begun to be sequenced from some taxa (e.g. Helgason et al. 2003; Corradi et al. 2004; Redecker and Raab 2007), phylogenetic hypotheses based on multilocus DNA sequence data have yet to be incorporated into the AMF classification.

Four orders, containing eight families and ten genera have been delimited within the Glomeromycota (Figs. 4,5). The largest genus within this phylum, *Glomus*, as originally defined by the spore morphology and formation was revealed to be polyphyletic according to molecular data (Schwarzott et al. 2001). Therefore, this genus was divided into the following five genera: *Glomus*, *Paraglomus*, *Archaeospora* (Morton and Redecker 2001), *Pacispora* (Oehl and Sieverding 2004) and *Diversispora* (Walker et al. 2004). The remaining species of the genus *Glomus* are further divided into three clades A, B and C (Schwarzott et al. 2001). Further glomeromycotan genera are *Scutellospora* and *Gigaspora*, which form the family Gigasporaceae; and *Acaulospora* and *Entrophospora*, belonging previously to the family Acaulosporaceae (Fig. 5). However, just recently, the

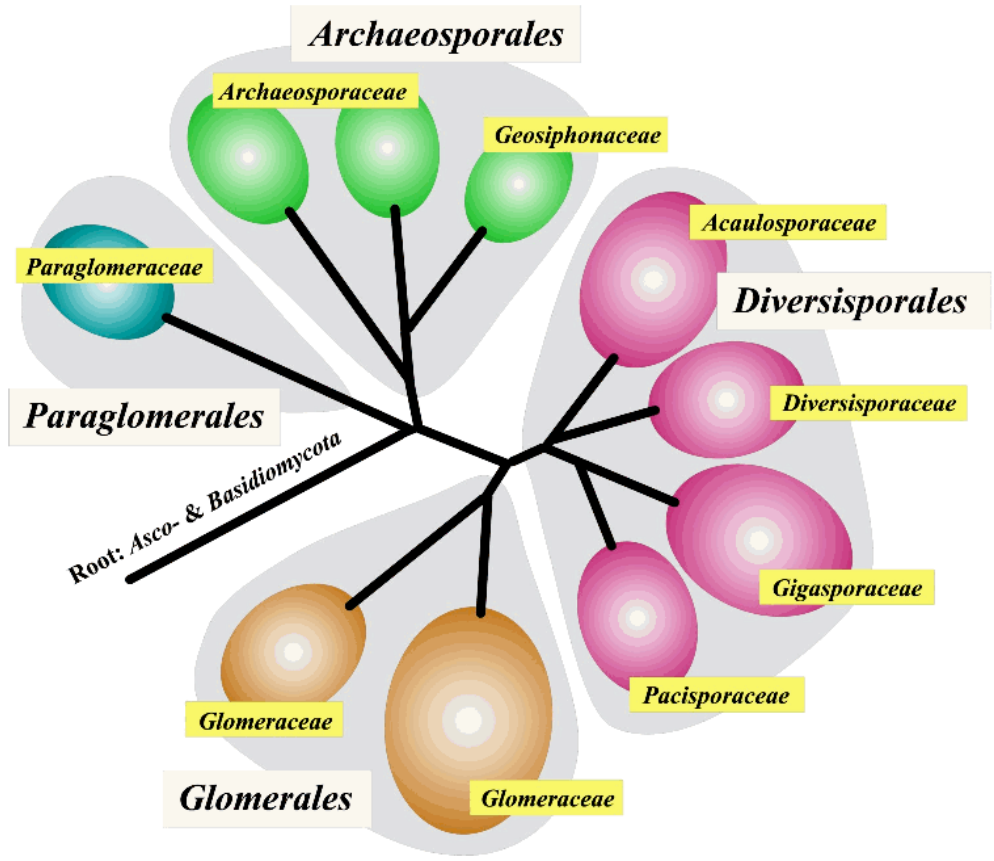

Fig. 4 Phylogenetic tree implementing recent changes in the taxonomy of the *Glomeromycota*. The tree shows the glomeromycotan taxa before the establishment of the families Appendicisporaceae and Entrophosporaceae. From http://www.lrz-muenchen.de/~schuessler/amphylo/.

genera *Archaeospora* and *Entrophospora* have been revised by several groups of authors (Sieverding and Oehl 2006; Spain et al. 2006; Walker et al. 2007). Based on the mode of spore formation, spore wall structures, trypan blue staining intensity of the fungal intraradical structures as well as DNA analyses, two new families have been established: Appendicisporaceae and Entrophosporaceae. However, in the following chapters, old

nomenclature will be retained, because these changes happened only recently.

Glomus groups A and B form a monophyletic clade (Fig. 5). Their spores are formed by budding from a hyphal tip and typically have a layered wall structure. The sporogenic hyphae often remain attached to the mature spore. This glomoid mode of spore formation occurs in all other glomeromycotean families with exception of the Acaulosporaceae, Entrophosporaceae and Gigasporaceae (http://invam.caf.wvu.edu/fungi/taxonomy/classification.htm). Members of the *Glomus* group A are the dominant and most diverse AMF in many field sites (e.g. Öpik et al. 2006). This group also includes the ubiquitous species *Glomus intraradices*. *Glomus* group B contains several AMF species like *Glomus etunicatum, Glomus claroideum* and *Glomus lamellosum,* which are difficult to distinguish (Rodriguez et al. 2005). *Glomus* group C (recently defined as a new family Diversisporaceae; Walker et al. (2004) is more closely related to the Acaulosporaceae than to *Glomus* groups A and B based on phylogenies of nuclear-encoded ribosomal genes (Schwarzott et al. 2001) and also contains tropical species forming large sporocarps, e.g. *Glomus fulvum* (Redecker et al. 2007).

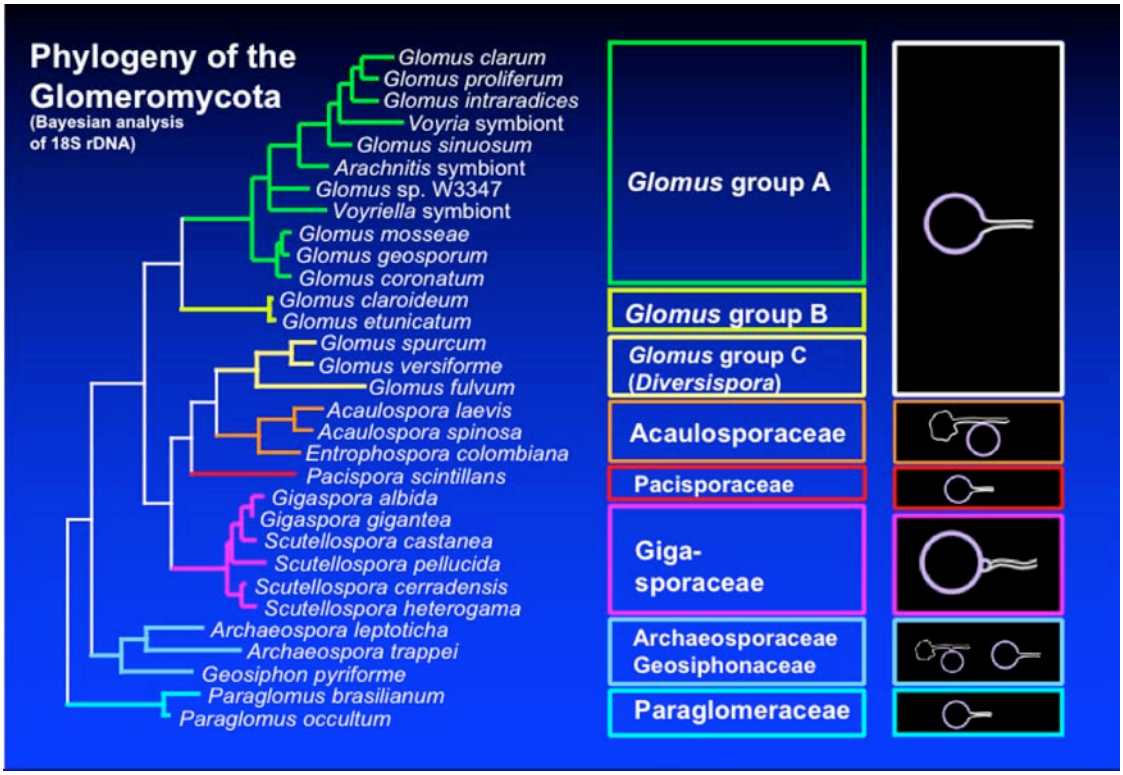

Fig. 5 Phylogenetic tree based on analysis of ribosomal small subunit sequences. *Glomus* subgroups as defined by Schwarzott et al. (2001). The tree shows the glomeromycotan taxa before the establishment of the families Appendicisporaceae and Entrophosporaceae. The boxes on the right hand side show the delimitation of glomeromycotan families or subgroups and the mode of the spore formation in each group. Tree by D. Redecker, unpublished.

The Acaulosporaceae form spores laterally next to “sporiferous saccules”, (Fig. 5) which collapse or completely disappear after the spore maturation. A germ tube of the Acaulosporaceae emerges from a spherical "germination orb".

The Gigasporaceae do not form vesicles within the roots but form so-called “auxiliary cells” on the extraradical mycelium (Fig. 1). Their spores are are generally larger than 200 µm in diameter at maturity and are formed from a morphologically specialized bulbous sporogenous cell formed terminally on a fertile hypha (http://invam.caf.wvu.edu/fungi/taxonomy/classification.htm). The genus *Scutellospora* possesses a “germination shield”, a membraneous structure that is used during the spore germination to penetrate the spore wall. In contrast, the genus *Gigaspora* lacks this germination shield and as well as the flexible inner spore wall.

Pacispora species form spores terminally on the hyphae like members of the genus *Glomus* but have flexible inner walls and germinate by means of a germination shield (Walker et al. 2004). The genus *Pacispora* was recently established by Oehl and Sieverding (2004).

The Archaeosporaceae and Paraglomeraceae are thought to be the basal members of the Glomeromycota. This conclusion is based on phylogenetic studies of the nuclear rDNA by Morton and Redecker (2001) and the possession of unique fatty acids which could not be found in other glomeromycotan fungi (Graham et al. 1995). Their intraradical structures stain very faintly and they do not seem to form vesicles. Some members of the Archaeosporaceae form dimorphic spores – the acaulosporoid type formed similarly to the *Acaulospora* on the neck of a sporiferous saccule that is formed terminally on a fertile hypha, and the glomoid type (Spain et al. 2006). Paraglomeraceae form their spores like members of the genus *Glomus*.

The new family Appendicisporaceae contains e.g. *Appendicispora gerdemanni*, *Ap. appendicula*, *Ap. fennica*, which were tranferred from the family Archaeosporaceae. *Appendicispora* is a dimorphic genus, forming both acaulosporoid and glomoid spores. This genus may form vesicles in the roots and the fungal intraradical structures stain pale with trypan blue (Sieverding and Oehl 2006; Walker et al. 2007).

Geosiphon pyriformis is a glomeromycotan species belonging to the order Archaeosporales. It is the only fungus in the Glomeromycota currently known to form a symbiosis with a cyanobacterium: it produces bladders that harbor symbiotic *Nostoc punctiforme* (Schüssler et al. 1994). Despite its different morphology and life strategy, molecular phylogenetic analysis has shown that *Geosiphon* is a member of the Glomeromycota (Schwarzott et al. 2001).

1.5.2. Molecular identification of AMF using PCR-based techniques

Molecular methods enable the identification of AMF in the host plant roots or directly in the soil. However, as the Glomeromycota are obligate symbionts, it is difficult or even impossible to obtain their pure biomass, which is necessary for the development of molecular markers. Only some AMF can be cultivated in sterile conditions using transformed plant roots (Fortin et al. 2002), others in non-sterile greenhouse pot cultures (e.g. Oehl et al. 2005b) and others are probably non-cultivable or non-sporulating at all. AMF spores or sporocarps, which are the biggest separable and distinguishable units of the fungal tissue, usually host numerous non-glomeromycotan organisms like other fungi or bacteria (Hijri et al. 2002) if they originate from pot cultures or the field. PCR-based methods using AMF-specific primers enable the amplification even of very small amounts of template DNA from the fungi growing in the soil or roots. The specificity of the primers is essential, as a single root or soil sample may be simultaneously colonized by glomeromycotan fungi as well as by numerous fungal pathogens and saprophytes.

During the past ten years, nuclear-encoded ribosomal DNA (rDNA) has been well established for molecular identification and phylogeny of AMF. These genes are present in multiple copies and contain conserved coding (small subunit - SSU and large subunit - LSU) as well as variable non-coding parts (internal transcribed spacers - ITS). Thus, they are useful to distinguish taxa at many different levels (Redecker 2006). The ITS region evolves faster than the conserved regions and therefore provides more information about close relationships, whereas the conserved regions enable primer construction and taxa resolution on the genus and family level. A comprehensive molecular phylogeny has been based largely on sequences of the small subunit (18S) rDNA, as these sequences were available first (Simon et al. 1993).

Different authors have tried to develop AMF-specific primers (Fig. 6) targeting different regions of the small subunit of the rDNA (18S). The first primer designed to be AMF-specific, the VANS1 primer for the 5’ end of the SSU (Simon et al. 1992), does not amplify all glomeromycotan lineages (Clapp et al. 1995). Moreover, its targeted region is not variable enough to distinguish taxa and amplification problems appeared when it was used for field root samples (Clapp et al. 1999). Helgason et al. (1998) designed the AM1 primer, which in combination with the universal primer NS31 amplifies the variable central region of the SSU. This primer combination is also widely used in field studies (Öpik et al. 2006). However, it does not amplify Archaeosporaceae and Paraglomeraceae and neither *Glomus* group B. Other authors constructed primers targeting the LSU of rDNA (Kjoller and Rosendahl 2000; Gollotte et al. 2004). Similarly to the AM1/NS31 primer pair, these primers also amplify only a subset of the glomeromycotan taxa. Redecker (2000) designed a primer set for the 3’ end of the SSU and ITS regions allowing to detect seven genera of the

Glomeromycota including Archaeosporaceae and Paraglomeraceae, which is the largest possible portion of taxon diversity recognized so far. An interesting approach was developed by Renker et al. (2003), who selected for glomeromycotan PCR products by a restriction digest with *Alu*I. As most AMF lacked the target site for this enzyme in the IST region, they were amplified preferentially.

Usually, the PCR products obtained by the primer combinations mentioned above have to be cloned and sequenced, as many different AMF taxa may be present in a single root sample and many different variants of the same gene can be present in a single spore. For screening of the clones, different techniques may be used: restriction fragment length polymorphism (RFLP; Helgason et al. 1999), single strand conformation polymorphism (SSCP; Kjoller and Rosendahl 2000) and denaturing gradient gel electrophoresis (DGGE; de Souza et al. 2004). A useful approach to fingerprint the AMF community in large numbers of samples without the necessity of cloning and sequencing seems to be t-RFLP (terminal restriction fragment length polymorphism; Mummey and Rillig 2006; Lekberg et al. 2007). However, this approach may be biased by the impossibility of detection of the non-specific (non-AMF) PCR amplicons of used primers.

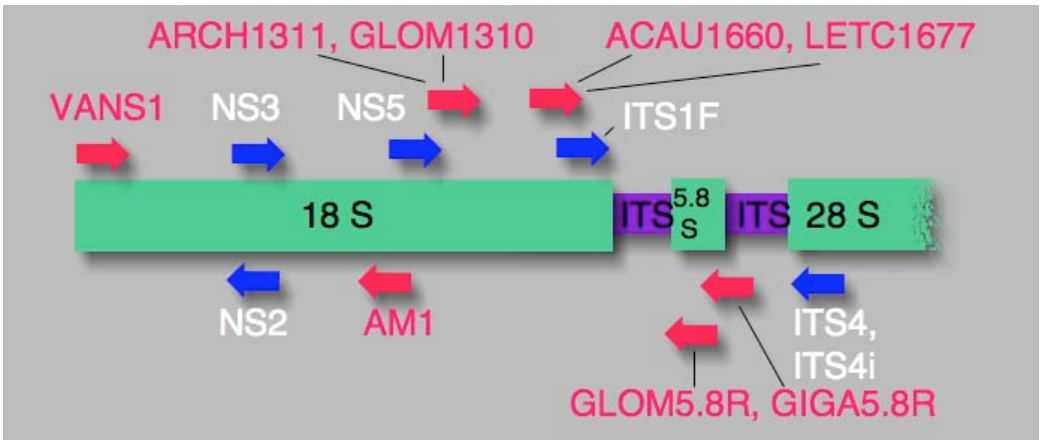

Fig. 6 Ribosomal DNA structure and annealing sites of different primers used in AMF community studies. In red AMF-specific primers, in white universal primers

Some authors use the relative numbers of clones corresponding to different sequence types (definition see Chapter 1.5.1.) as a measure of the relative abundance of these sequence types in the roots (Helgason et al. 1999). This method is controversial, as it assumes equally efficient amplification and proportional cloning of all taxa.

1.5.3. Methods to assess AMF diversity – their pros-and-cons

The diversity of arbuscular mycorrhizal fungi at a given field site can be evaluated using either i) fungal spores originating directly from the field, ii) fungal spores produced in

greenhouse trap cultures with field soil and „universal“ host plant species, iii) fungal mycelium growing in the soil or iv) intraradical fungal mycelium.

The trap culture approach is commonly used to cultivate and harvest healthy spores from a field using “universal host plants” (e.g. *Plantago lanceolata*, *Trifolium pratense*, *Zea mays*, *Allium porrum*) inoculated with field soil in greenhouse pot cultures. It is known that this approach does not necessarily reveal the same community of AMF species as the direct analysis of spores in the field (Jansa et al. 2002a; Oehl et al. 2003). Similarly, a strong difference between the AMF community composition in the roots from an agricultural field site and from corresponding trap plant roots was found by Hijri et al. (2006) using molecular methods. This phenomenon was attributed to selective effects of the trap plant species (Jansa et al. 2002a; Ahulu et al. 2006), different growth conditions in the greenhouse or by influence of time and spatial succession in the culture pots (Oehl et al. 2003).

Molecular studies of AMF field communities from the last decade (e.g. Husband et al. 2002a; Wubet et al. 2004; Börstler et al. 2006) revealed numerous previously unknown phylotypes, which do not correspond to any morphologically- defined and sequenced species. Thus, the diversity of the Glomeromycota may be strongly underestimated. However, not all of the 200 morphologically described species have been characterized by molecular methods so far, which makes it difficult to compare the results of the morphological surveys with the molecular ones. It is also problematic to evaluate and compare results of different molecular studies focused on the diversity of AMF in the field among each other, as different research teams target different parts of DNA and define the sequence types inconsistently (see e.g. Öpik et al. 2006).

As mentioned in the Chapter 1.5.2., molecular methods allow the identification of the symbiotic community currently colonizing the roots of an individual plant at any given time. Interestingly, strong discrepancies have been reported between the taxa present as spores in the field or produced in the trap cultures using field soil, and the fungal community currently detected in the roots (e.g. Clapp et al. 1995; Kowalchuk et al. 2002; Wubet et al. 2003; Renker et al. 2005; Ahulu et al. 2006; Börstler et al. 2006). Actually, these two approaches - AMF spores and AMF in the roots - target different ecological parameters: the studies using spores characterize the inoculum potential of a particular soil sample, whereas the molecular studies analyzing AMF colonizing plant roots can reveal the fungi actively and currently involved in the symbiosis.

Moreover, the results of field AMF community studies using the approaches mentioned above may be influenced by several other methodical factors. Sampling effort and the way of pooling of the analyzed samples may influence the assessment of the species richness and composition of the local community (Renker et al. 2006; Whitcomb and Stutz 2007). Further,

the seasonality of AMF community composition in a single field was reported using spore morphology (Bever et al. 2001) and molecular methods targeting plant roots (Daniell et al. 2001), respectively.

1.6. Ecology of AMF

1.6.1. AMF in different ecosystems: their diversity and species richness

A global survey of root-colonizing AMF communities ranked different ecosystems according to their AMF species richness (Öpik et al. 2006). The species-richest habitats were the tropical forests with eighteen AMF taxa/host plant species; followed by the plant-species-rich non-disturbed grasslands. Approximately eight AMF taxa were associated with a single host plant species here. In the temperate forests and habitats under strong antropogenic influence like arable fields and polluted sites, approximately five AMF taxa associated with a single host plant species. In European grasslands and woodlands, using both molecular and morphological approaches, AMF species richness was also reported to be higher in diverse natural plant communities compared to arable fields (Helgason et al. 1998; Daniell et al. 2001; Oehl et al. 2003; Oehl et al. 2004; Öpik et al. 2006). However, evidence for a relatively high AMF diversity in arable sites with low-input agriculture was presented by Hijri et al. (2006). Ten to 24 AMF phylotypes were detected in the roots of two to 18 plant species growing in different temperate grasslands (Vandenkoornhuyse et al. 2002; Gollotte et al. 2004; Scheublin et al. 2004; Börstler et al. 2006).

Using molecular and morphological methods, some evidence for ecological specialization of different AMF species was found. For instance, *Acaulospora alpina* was described in plant species-rich grasslands of the Swiss Alps at altitudes between 1800 and 2700 m above sea level (Oehl et al. 2006) and *Glomus badium* was found in grasslands, grass-intercropped vineyards or olive fields, or non-tilled arable lands in Central Europe (Oehl et al. 2005a). In contrast, *G. intraradices* has been reported as a ubiquitous AMF species with global distribution (Öpik et al. 2006) occurring in many different native (e.g. Appoloni 2006) and human-influenced ecosystems (e.g. Hijri et al. 2006). *Glomus mosseae, G. caledonium, G. claroideum, G. intraradices* and *G. etunicatum* were frequently found in intensively managed tilled arable soils from different sites in Europe (Helgason et al. 1998; Daniell et al. 2001; Jansa et al. 2002a; Hijri et al. 2006). These species were also found in trap cultures from several agroecosystems already after two to four months, in some cases after six months, as the first sporulating ones (Oehl et al. 2003; Oehl et al. 2004; Oehl et al. 2005b). However, further molecular and morphological investigations are necessary to elucidate the ecological

preferences of AMF.

1.6.2. The ecological importance of AMF diversity and richness

Interactions among AMF, host plants and environmental factors (particularly the availability of nutrients in the soil and other soil organisms) are complex. Mycorrhizal associations of plant roots with fungi are ubiquitous in nature and therefore, this symbiosis is of enormous ecological importance.

AMF were reported to have a decisive influence on the composition of natural plant communities. Plant diversity and community productivity increased with AMF diversity (van der Heijden et al. 1998; Klironomos et al. 2000; Landis et al. 2004) and also depended on the AMF species identity (Stampe and Daehler 2003; Vogelsang et al. 2006). An increased diversity of AMF may also cause an increase in microbial diversity (Johansson et al. 2004). Furthermore, evidence for functional specificity has been reported: Helgason et al. (2002); Burleigh et al. (2002); Klironomos (2003) and van der Heijden et al. (2003) observed that in certain combinations of AMF and plant hosts stronger beneficial effects for the plant were detected than in other combinations. Differences in functionality were observed even among isolates of a single AMF species (Munkvold et al. 2004). Stampe and Daehler (2003) reported a relation between AMF species identity and the invasion success of an invasive plant in a microcosm experiment. From the plant point of view, a different host plant community may also select for a different symbiotically-active AMF community, as has been shown in microcosm experiments by (Bever et al. 1996; Bever 2002; Johnson et al. 2003).

Because more than one plant can be colonized by the same fungus and each plant may be host for many fungal species, a large plant-fungal network may be established. This lack of absolute host specificity (for details see Chapter 1.6.3.) and therefore the assemblage of a mycorrhizal network connecting different plants of the same (Malcová et al. 2001) or different species (Newman et al. 1994) potentially enables the establishment of colonization in seedlings (Mcgee 1990; Malcová et al. 2001), the distribution of nutrients (Koide and Dickie 2002) and the redistribution of nutrients from senescent roots to other plants (Newman and Eason 1989). Non-photosynthetic plants may parasitize their neighboring plants via "cheating" on the shared fungal network – they take up the carbohydrates originating from green neighboring plants (Bidartondo et al. 2002). However, the possible influence of hyphal links on the species coexistence, possibly even dependency, and maintenance of the plant diversity remains to be elucidated.

1.6.3. Host specificity or preferences and other factors shaping the AMF community in the roots in the field

The small number of AMF species described so far was thought to colonize the majority of higher plant species and their host specificity was thought to be very low (Smith and Read 1997). Moreover, most plant species have been successfully inoculated with many different AMF species under greenhouse conditions (e.g. Klironomos 2003). The progress in molecular methods enables the study of AMF communities colonizing plant roots in the field and thus the study of their host preferences as well. Using rDNA as molecular marker, Bidartondo et al. (2002) reported high specificity of non-photosynthetic mycoheterotrophic plants towards a few highly restricted clades of AMF. Host specificity in green plants appears to be lower, but in several cases statistically significant differences were detected among different host plant species growing in the same habitat, mainly in grasslands (Helgason et al. 2002; Vandenkoornhuyse et al. 2002; Vandenkoornhuyse et al. 2003; Gollotte et al. 2004; Scheublin et al. 2004).

In other studies, environmental factors other than host preference appeared to be dominant in shaping the AMF community in plant roots. Husband et al. (2002a) observed a very complex structure of AMF communities in the roots of two host plant species in a tropical forest in Panama: AMF community composition differed depending on sampling time, host plant species and field site. Interestingly, significantly different fungal populations colonized two age classes of seedlings on the same field site sampled at the same time point (Husband et al. 2002b). Öpik et al. (2003) observed site-dependent differences in AMF community composition in two *Pulsatilla* species, but no host preferences. However, these authors perfomed a pot experiment with a mixture of natural soil and sterile sand, which is a relatively artificial system.

Nielsen et al. (2004) did not observe host specificity in their study analyzing *Littorella uniflora* and *Lobelia dortmanna* in Swedish lakes, whereas they showed evidence for site-specific differences in AMF community composition. Santos et al. (2006) investigated the roots of *Festuca pratensis* and *Achillea millefolium* from a Swedish pasture along a fertilization gradient. They did not observe host specificity, whereas the influence of sampling season and soil nitrogen content on the AMF community composition was clearly significant. In some investigations, species composition in plant roots changed over time (Helgason et al. 1999; Daniell et al. 2001; Vandenkoornhuyse et al. 2002; Heinemeyer et al. 2004), but other authors reported the absence of seasonal changes (Rosendahl and Stukenbrock 2004).

Another factor, which may influence the AMF community composition in the roots of a single plant species, is the identity of the neighboring plants. Helgason et al. (1999) and Clapp et al. (1995) observed that the target plant *Hyacinthoides non-scripta* harbored distinct

AMF species under different canopies but there was also a seasonal shift of the frequencies of some of their AMF symbionts. Mummey et al. (2005) observed strong AMF community shifts in grass roots growing in the close proximity of the invasive herb *Centaurea maculosa* in Montana, USA.

1.7. Aims of the thesis

The aims of this thesis were i) to characterize the communities of arbuscular mycorrhizal fungi in the roots of several selected plant species originating from plant species-rich grasslands in different parts of Switzerland using molecular methods and ii) to address the question of the preferences of these root-colonizing AMF for specific host plant species and environmental conditions.

In order to implement this aim, a set of PCR primers was used that enables to detect the largest possible proportion of taxa of the Glomeromycota (Redecker 2000). I wanted to compare the communities of AMF in the roots of different host plant species from a single site and to evaluate whether the differences, if any, can be explained by host preferences or even specificity. Furthermore, the influence of the site as well as local climatic and soil conditions was addressed by comparing the AMF communities of the two different field sites: two species-rich grasslands facing different climate and environmental conditions and harboring different plant species communities.

The lowland site, a calcareous grassland, was located on the edge of the Jura mountains and the AMF diversity was already investigated there using the trap culture approach (Oehl et al. 2003). Therefore, the root-colonizing AMF and the AMF spore community in this site could be compared. In this site, the question of the influence of different experimental approaches to study the AMF community was addressed. I wanted to test whether plant roots directly sampled in the field or grown in different culture systems using the same inoculum would harbor similar AMF communities. These data were expected to provide useful insights into possible ecological preferences and life history strategies of the AMF phylotypes and would also be of practical value for planning experiments with AMF species consortia.

The alpine site was located at approximately 2000 m above sea level, therefore facing much longer winters and a colder climate than the lowland site. This site was also chosen because of the high abundance of *Gentiana verna* and *G. acaulis*. Based on my own previous research (Sykorova et al. 2003) and the work on mycoheterotrophic members of the family Gentianaceae (Bidartondo et al. 2002), it was intriguing to elucidate whether these green gentians would show similar specificity towards AMF as their achlorophyllous,

mycoheterotrophic relatives. These data would contribute to a better understanding of the evolution of mycoheterotrophy in the Gentianacae and the role of mycorrhizal specificity.

Finally, the possible coexistence of AMF with basidiomycetes from the order Sebacinales in the roots was addressed. The Sebacinales have been shown to form symbiotic associations with a broad spectrum of plants (Weiß et al. 2004) and therefore, it was interesting to test whether they also colonize arbuscular mycorrhizal host plants. If so, possible specificity or preferences towards their host plants and as well as geographical distribution patterns could provide important clues to better understand this interaction.

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Chapter 2

Cooccurring *Gentiana verna* and *Gentiana acaulis* and their neighboring plants in two Swiss upper montane meadows harbor distinct arbuscular mycorrhizal fungal communities

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Fig. 1 Site 2A, Ramosch, Engadin, Switzerland. Photo by D. Redecker

Fig. 2 Site 11, Ramosch, Engadin, Switzerland. Photo by D. Redecker

Fig. 3 *Gentiana verna* in the site 2A. Photo by D. Redecker

Fig. 4 *Gentiana acaulis* in the site 2A. Photo by D. Redecker

2.1. Abstract

The community composition of arbuscular mycorrhizal fungi (AMF) was analyzed in roots of *Gentiana verna*, *Gentiana acaulis* and accompanying plant species from two species-rich Swiss alpine meadows located in the same area. The aim of the study was to elucidate the impact of host preference or host specificity on the AMF community in the roots. The roots were analyzed by nested PCR, RFLP screening and sequencing of rDNA small subunit and ITS regions. The AMF sequences were analyzed phylogenetically and used to define monophyletic sequence types.

The AMF community composition was strongly influenced by the host plant species whereas it did not significantly differ between the two sites. Detailed analyses of the two cooccurring gentian species *G. verna* and *G acaulis*, as well as neighboring *Trifolium* spp. revealed that their AMF communities differed significantly. All three host plant taxa harbored AMF communities comprising multiple phylotypes from different fungal lineages. A frequent fungal phylotype from *Glomus* group B was almost exclusively found in *Trifolium* spp., suggesting some degree of host preference for this fungus in this habitat.

In conclusion, the results indicate that within a relatively small area with similar soil and climatic conditions, the host plant species can have a major influence on the AMF communities within the roots. No evidence was found for a narrowing of the mycosymbiont spectrum in the two green gentians in contrast to previous findings with their achlorophyllous relatives.

2.2. Introduction

Arbuscular mycorrhiza is an ancient symbiosis (Redecker et al. 2000) between the majority of land plants and fungi from the phylum Glomeromycota (Schüssler et al. 2001). Arbuscular mycorrhizal fungi (AMF) colonize plant roots and contribute to the mineral nutrient uptake of the hosts in exchange for carbohydrates (Smith and Read 1997).

The diversity of AMF can be evaluated using microscopic analysis of spore morphology or molecular methods. The production of spores is highly dependent on environmental conditions, the physiological status and life strategy of the particular mycorrhizal fungus. Molecular methods allow the identification of the symbiotic community currently colonizing the roots of an individual plant at any given time. The majority of recent molecular studies have used AMF-specific primers for nuclear-encoded ribosomal RNA

genes (rDNA). However, identification of AMF on the species or even isolate level is complicated by the heterogeneity of rDNA within glomeromycotan spores and isolates. Several authors have shown that different variants of rDNA genes coexist within single AMF spores (Sanders et al. 1995; Jansa et al. 2002). Hence, a conservative approach in the evaluation of phylogenetic analyses is advisable by, e.g., defining a well-supported monophyletic sequence cluster as an AMF phylotype.

On the basis of the morphological features of their spores, only about 200 species of AMF have been described so far (http://www.lrz-muenchen.de/~schuessler/amphylo/). This small number of species was originally thought to colonize the majority of higher plant species, and as a consequence, their host specificity or preference was thought to be very low (Smith and Read 1997). However, molecular studies of AMF field communities from the last decade (e.g. Husband et al. 2002a; Wubet et al. 2004; Börstler et al. 2006) revealed numerous previously unknown phylotypes. In several cases, significant differences were observed between the AMF communities inhabiting roots of different host plant species in the same habitat, mainly in grasslands (Helgason et al. 2002; Vandenkoornhuyse et al. 2002; Vandenkoornhuyse et al. 2003; Gollotte et al. 2004; Scheublin et al. 2004). In contrast, an apparent lack of host specificity was reported by other authors (Öpik et al. 2003; Santos et al. 2006).

Achlorophyllous mycoheterotrophic members of the Gentianaceae were among the plants showing the strongest host specificity known so far in arbuscular mycorrhiza (Bidartondo et al. 2002). Therefore, it was intriguing to see whether their green relatives show a similar restriction to a narrow clade of fungal symbionts. Previous studies provided evidence suggesting the possibility of a partly non-mutualistic interaction with AMF in gentians, based on the observation of a strong similarity of the mycorrhizal morphology between *Gentiana* spp. and mycoheterotrophic plants (Imhof 1999). Such a morphology, a *Paris*-type mycorrhiza with hyphal coils and pronounced swellings but without apparent arbuscules, was also observed in the *G. acaulis* and *G. verna* roots used in our study (not shown). Moreover, we previously found that *G. verna* could be colonized only with AMF from a living host plant of another species acting as the AMF donor (Sýkorová et al. 2003). Therefore, data about the mycorrhizal specificity of gentians may provide insight into the presumed transition from a mutualistic arbuscular mycorrhizal symbiosis to mycoheterotrophy by identifying evolutionary trends in the mycorrhizal interactions.

In the present study, we used the set of primers designed by Redecker (2000), allowing us to detect seven genera of Glomeromycota, which is the largest possible portion of taxon diversity recognized so far. The aims of this study were i) to analyze and compare the community of AMF in roots of two green gentian species and some of their surrounding

plants sampled in the Swiss Alps and ii) to evaluate whether green gentians would show specificity towards AMF similar to that of their achlorophyllous mycoheterotrophic relatives (Bidartondo et al. 2002). The present study is also the first one to use molecular methods to analyze AMF communities in the European upper montane zone.

2.3. Materials and methods

2.3.1. Field sites

The study sites were two species-rich meadows with approximately 80 plant species in two 25 m^2 plots per site (K. Maurer, pers. comm.). They were situated close to the village Ramosch in the Engadin region of Switzerland in the upper montane forest zone (definition according to Körner, 2003): site 2A (Fig.1) is at 10°23'30"E/ 46°51'40"N and 1820 m ASL (above sea level) and site 11 (Fig. 2) is at 10°23' 00"E/ 46°51'30"N and 2010 m ASL. The timberline in this part of the Alps is around 2200 m ASL. Both meadows are mown regularly but neither grazed by cattle nor fertilized (Maurer et al. 2006). They are situated about 600 m apart, separated by a forest and exposed to the southeast. The altitude difference between the two sites is ca. 200 m. Site 11 is relatively steep and situated close to the forest line, with scattered ericaceous shrubs and *Juniperus communis*, whereas site 2A is a relatively flat meadow without interspersed shrubs. The dominant grass in both sites was *Nardus stricta*; they shared approximately further 50 plant species, the remaining 30 were unique to each site (K. Maurer, pers. comm.). At site 2A the soil pH (H_2O) was 6.6, the level of sodium acetate-extractable phosphorus was relatively low at 9 ng/g and the total carbon content was 4.2% (w/w; laboratory F.M. Balzer, Wetter-Amönau, Germany). Both sites were subjected to plant diversity research project (Maurer 2006).

2.3.2. Sampling

In May 2003, sixteen soil cores with a diameter of ca. 20 cm and a depth of 15 cm were sampled in each meadow. The cores were randomly removed from areas of approximately 40 and 30 m in diameter. Each core contained one *Gentiana verna* (Fig. 3) or *Gentiana acaulis* (Fig. 4) plant, and up to eight taxa of the surrounding plants. The plants were separated from each other and identified to species level if possible, and their roots were washed carefully and blotted dry using paper tissue. Aliquots of 50 mg consisting of root pieces assembled from a single root system were frozen in liquid nitrogen and stored at -80°C until use. Roots of the following plant species were used for further analyzes: *Crocus albiflorus* Kit., *Hieracium hoppeanum* Schult., *Leontodon hispidus.*, an unidentified grass ("*Poaceae* sp."),

Polygala vulgaris, *Ranunculus montanus*, *Thymus pulegioides*, and *Trifolium* spp. Plants belonging to the genus *Trifolium* turned out to be difficult to identify to the species level, as they were not flowering; therefore we decided to pool all *Trifolium* root samples into one category: "*Trifolium* spp.". Plant records performed by Katrin Maurer (personal communication) suggest that this form taxon may comprise *T. pratense*, *T. montanum*, *T. repens* and *T. badium*, as these were the predominant *Trifolium* species at both field sites.

2.3.3. DNA extraction and polymerase chain reaction

Roots were ground in liquid nitrogen using a pellet pestle within a 1.5 ml tube. DNA was extracted from roots using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in two steps (each step used 50 µl of elution buffer). DNA extracts were diluted 1:10 or 1:100 in TE buffer and used as template for the first PCR reaction. PCR was performed in a nested procedure as described by Redecker (2000) using Taq polymerase from Amersham (Basel, Switzerland) or New England Biolabs (BioConcept, Allschwil, Switzerland), 2 mM $MgCl_2$, 0.5 µM primers and 0.13 mM of each desoxynucleoside triphosphate. The first round of amplification was performed using the universal eukaryote primers NS5 and ITS4 (White et al. 1990). The cycling parameters were: 3 min at 94°C, followed by 30 cycles of 45 sec at 94°C, 50 sec at 51°C and 1 min 30 sec at 72°C. The program was concluded by a final extension phase of 10 min at 72°C.

The PCR products were diluted 1:100 in TE buffer and used as a template in the second round. Five separate PCR reactions were performed using the primer pairs GLOM1310/ITS4i, LETC1677/ITS4i, ACAU1661/ITS4i, ARCH1311AB/ITS4i, NS5/GIGA5.8R (Redecker 2000; Redecker et al. 2003). The PCR parameters for the second round differed from the first one only in the annealing temperature (61°C). Moreover, a "hot start" at 61°C was performed manually to prevent non-specific amplification. In order to check the success of amplification, PCR products were run on agarose gels (2% NuSieve - 1% SeaKem, Cambrex Bio Science, Rockland, ME, USA) in Tris-acetate buffer at 120V for 30 min.

2.3.4. Cloning, restriction fragment length polymorphism analyses and sequencing

PCR products were purified using the High Pure Kit from Hoffman LaRoche (Basel, Switzerland) and cloned into a pGEM-t vector (Promega/Catalys, Wallisellen, Switzerland). Inserts were re-amplified, preferably ten positive clones of each PCR product were digested

with *Hinf*I and *Mbo*I and run on agarose gels as described above. Restriction fragment patterns were compared to a database modified from the spreadsheet developed by Dickie et al. (2003). Representative clones of new restriction types were then re-amplified, purified using the High Pure Kit and sequenced in both directions. The BigDye Terminator Cycle Sequencing Kit (ABI, Foster City, CA, USA) was used for labeling. Samples were run on an ABI 310 capillary sequencer. Sequences were deposited in the EMBL database under the accession numbers AM384904-AM384984 shown in the phylogenetic trees.

2.3.5. Sequence analyses

Sequences were aligned to previously published sequences in PAUP*4b10 (Swofford 2001). The glomeromycotan origin of the sequences was initially tested by BLAST (Altschul et al. 1997). Separate ITS alignments were prepared for each of the target groups of the specific primers LETC1677, GLOM1310, ACAU1661, and ARCH1311AB. In addition, an alignment of the partial 3' end of 18S rDNA small subunit was compiled for the sequences amplified with GLOM1310 and ARCH1311AB (Bidartondo et al. 2002).

Phylogenetic trees were obtained primarily by distance analysis (the neighbor-joining algorithm) in PAUP*4b10 using the Kimura two-parameter model and a gamma shape parameter of 0.5. Results were verified by performing maximum likelihood analyses based on parameters estimated in Modeltest 3.5 (Posada 2004).

2.3.6. Definition of sequence phylotypes

Sequence phylotypes were defined in a conservative manner as consistently separated monophyletic groups in the phylogenetic trees. Only those clades that were supported by neighbor joining bootstrap analysis and also present in the respective maximum likelihood tree were used. In case of GLOM A and ARCH phylotypes, the clades had to be supported by both 18S partial subunit and ITS trees. We avoided splitting the lineages unless there was a positive evidence for doing so. The sequence phylotypes were designated after the major clade they belonged to, followed by a numerical index (x in the following examples) identifying the type (Hijri et al. 2006): GLOM A-x (*Glomus* group A), GLOM B-x (*Glomus* group B), ACAU-x (Acaulosporaceae), ARCH-x (Archaeosporaceae). Representative sequences of each sequence type were checked manually for possible chimaeras, which were excluded from further analyses.

2.3.7. Statistical analyses

The presence or absence of AMF phylotypes in each root sample was used to construct the sampling effort curves (with 95% confidence intervals, using the analytical formulas of Colwell et al. (2004) in the program EstimateS 7.5 (Colwell 2005), and calculate Shannon diversity indices [$H=-\Sigma p_i * \ln(p_i)$] for each plant species. *G. verna, G. acaulis* and *Trifolium* spp. were represented by 9-13 root samples each and thus these plant taxa were analyzed in detail. The data for the remaining host plant species were pooled, as only 1-3 root samples per plant species were analyzed due to technical limitations.

The influence of host plant species and field site on the number of sequence types found in the root samples was analyzed using the program NCSS (NCSS, Kaysville, UT, USA). In order to investigate the influence of environmental factors (host plant species and field sites) on the distribution of the AMF phylotypes in the root samples, ordination analyzes were conducted in Canoco for Windows v. 4.5 (ter Braak and Smilauer 2004) using the presence/absence data for each root sample. Initial Detrended Correspondence Analysis (DCA) suggested a unimodal character of the data response to the sample origin (the lengths of gradients were >4), therefore the Canonical Correspondence Analysis (CCA) was used. The variance-partitioning method with permutations in blocks defined by the co-variables was used to compare the influence of host plants with that of field sites. Host plants were considered covariables when the influence of field sites was tested as a variable, and vice versa. Monte Carlo Permutation Tests were conducted using 499 random permutations. The subsequent forward-selection procedure ranked the environmental variables according to their importance and significance for the distribution of the sequence types.

2.4. Results

2.4.1. PCR yields and sequence types detected in the root samples

Using our PCR approach with four nested primer sets, 45 of the 67 extracted root samples (67%) yielded 119 PCR products (693 clones after the cloning) of which 71 (60%) could be ascribed to AMF phylotypes. Whereas all DNA extracts of *Trifolium* spp. yielded PCR amplicons of AMF origin, only 75% of *G. verna* and 42% of *G. acaulis* extracts did. No PCR products or only non-AMF amplicons were obtained from the remaining samples.

After RFLP screening, 166 clones obtained from 10 root samples of *G. verna*, 9 samples of *G. acaulis*, 13 samples of *Trifolium* spp. and 13 samples of other surrounding plants were sequenced and analyzed phylogenetically. Altogether, 17 different sequence types were found, ten of which belonged to *Glomus* group A (group definitions are according to

Schwarzott et al. (2001), three to *Glomus* group B, two to the Acaulosporaceae and two to the Archaeosporaceae (Figs. 5, 6, 7, 8, 9; Tab. 1). The sampling effort curves (Fig. 10) showed that for *G. verna*, *G. acaulis* and *Trifolium* spp., the number of analyzed root samples was sufficient to detect the majority of sequence types present in their roots, as the curves approach saturation. On the contrary, the curve for the pooled data of the remaining host plant species did not level off.

By far the most abundant sequence type (found in 29 root samples) was GLOM A-1 (Fig. 5), which corresponds to the morphologically defined species *Glomus intraradices*. The second- and third-most-frequent sequence types were GLOM B-4 (Fig. 6) (which could not be assigned to any morphologically described species) and GLOM A-25 (Fig. 5) (which corresponds to *Glomus proliferum*). Interestingly, no sequence types belonging to the genera *Paraglomus*, *Scutellospora*, or *Gigaspora* were found.

Fig. 5 (on the next page) Phylogenetic tree of Glomeromycota obtained by neighbor-joining analysis of 310 characters of the 18S rDNA subunit. Numbers above branches denote neighbor-joining bootstrap values from 1000 replications. The tree was rooted by *Paraglomus occultum* and *P. brasilianum*. Sequences obtained in the present study are shown in boldface. They are labeled with the database accession number, (e.g. AM384944), internal identification number (e.g. ZS359_360), the host plant species they were obtained from (e.g. *Leontodon*), the field site code (2A or 11) and the soil core code (1-8 or A-C). Except with *Gentiana verna* and *G. acaulis*, the last letter (a or v) indicates whether the host plant was collected in a soil core with *G. verna* (v) or with *G. acaulis* (a). The brackets show the delimitation of the sequence types.

Fig. 5 (legend see on the previous page)

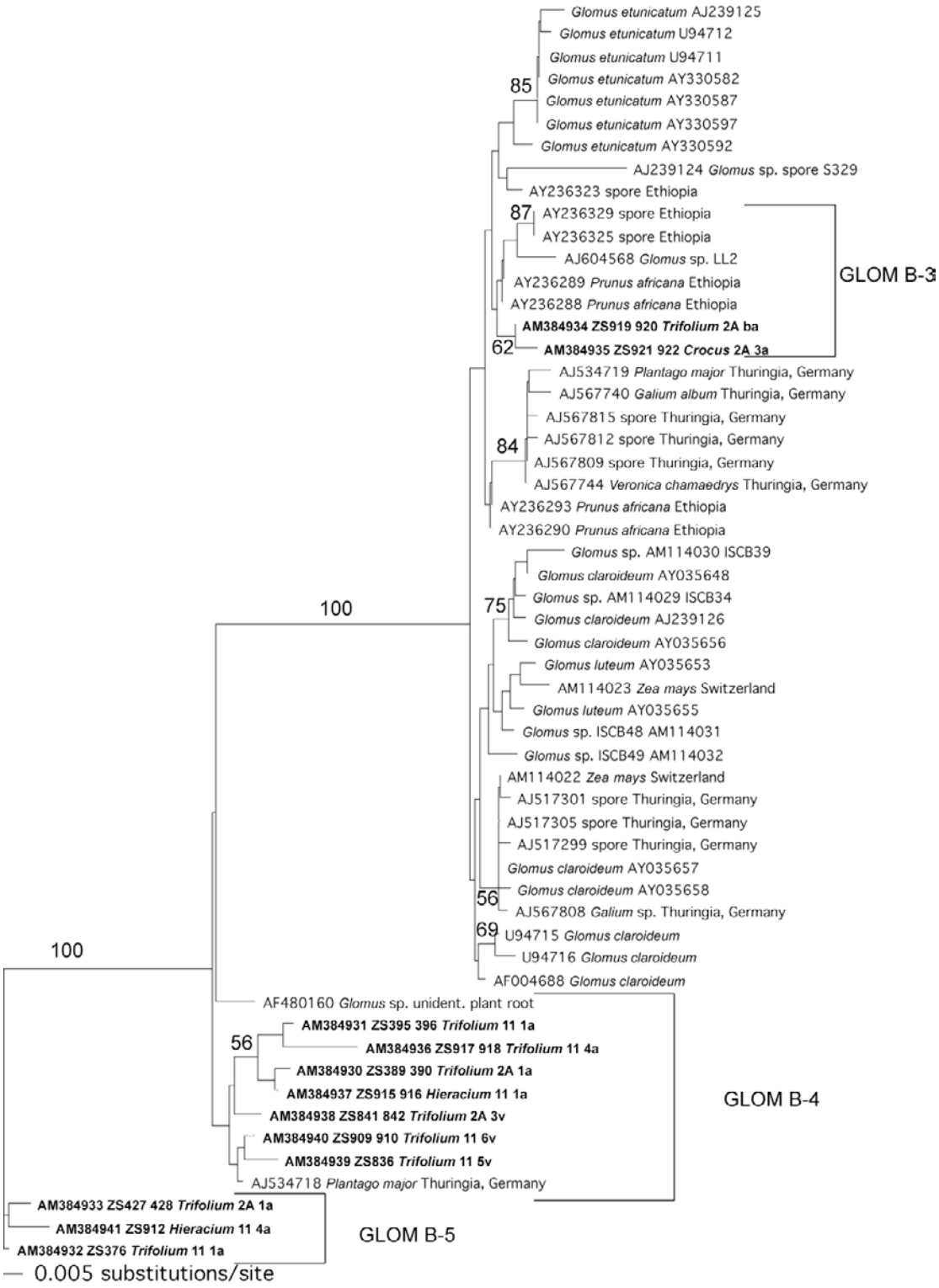

Fig. 6 Phylogenetic tree of *Glomus* group B based on neighbor-joining analysis of 375 characters of ITS2 and 5.8S rDNA sequences. Numbers above branches denote neighbor-joining bootstrap values from 1000 replications. The tree was rooted using the sequence type GLOM B-5. Sequences obtained in the present study are shown in boldface and are labeled as described in the legend of Fig. 5. The brackets show the delimitation of the sequence types.

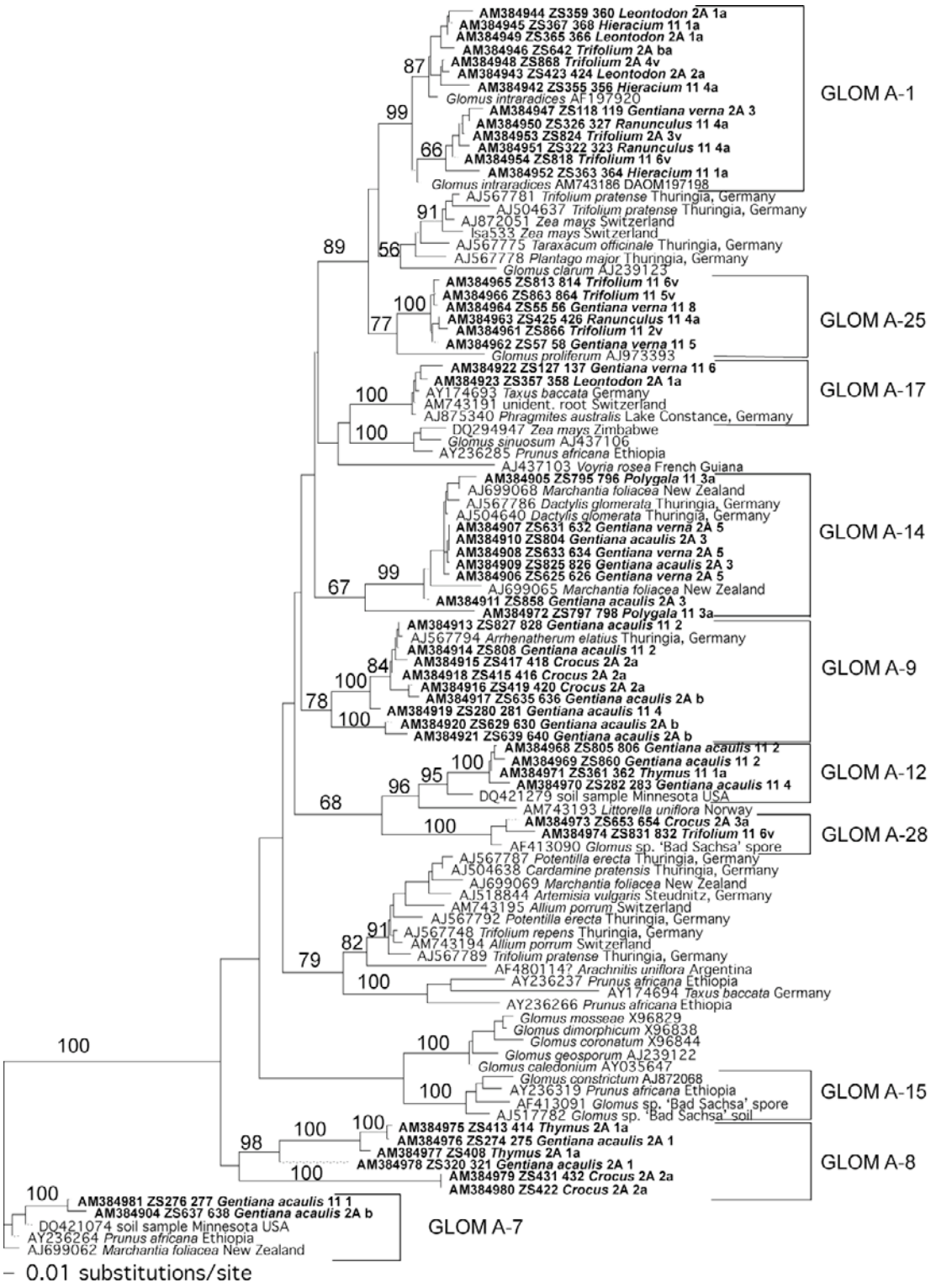

Fig. 7 Phylogenetic tree of *Glomus* group A obtained by neighbor-joining analysis of 387 characters from ITS2 and 5.8S rDNA. Numbers above branches denote neighbor-joining bootstrap values from 1000 replications. The tree was rooted using the sequence type GLOM A-7. Sequences obtained in the present study are shown in boldface and are labeled as described in the legend of Fig. 5. The brackets show the delimitation of the sequence types.

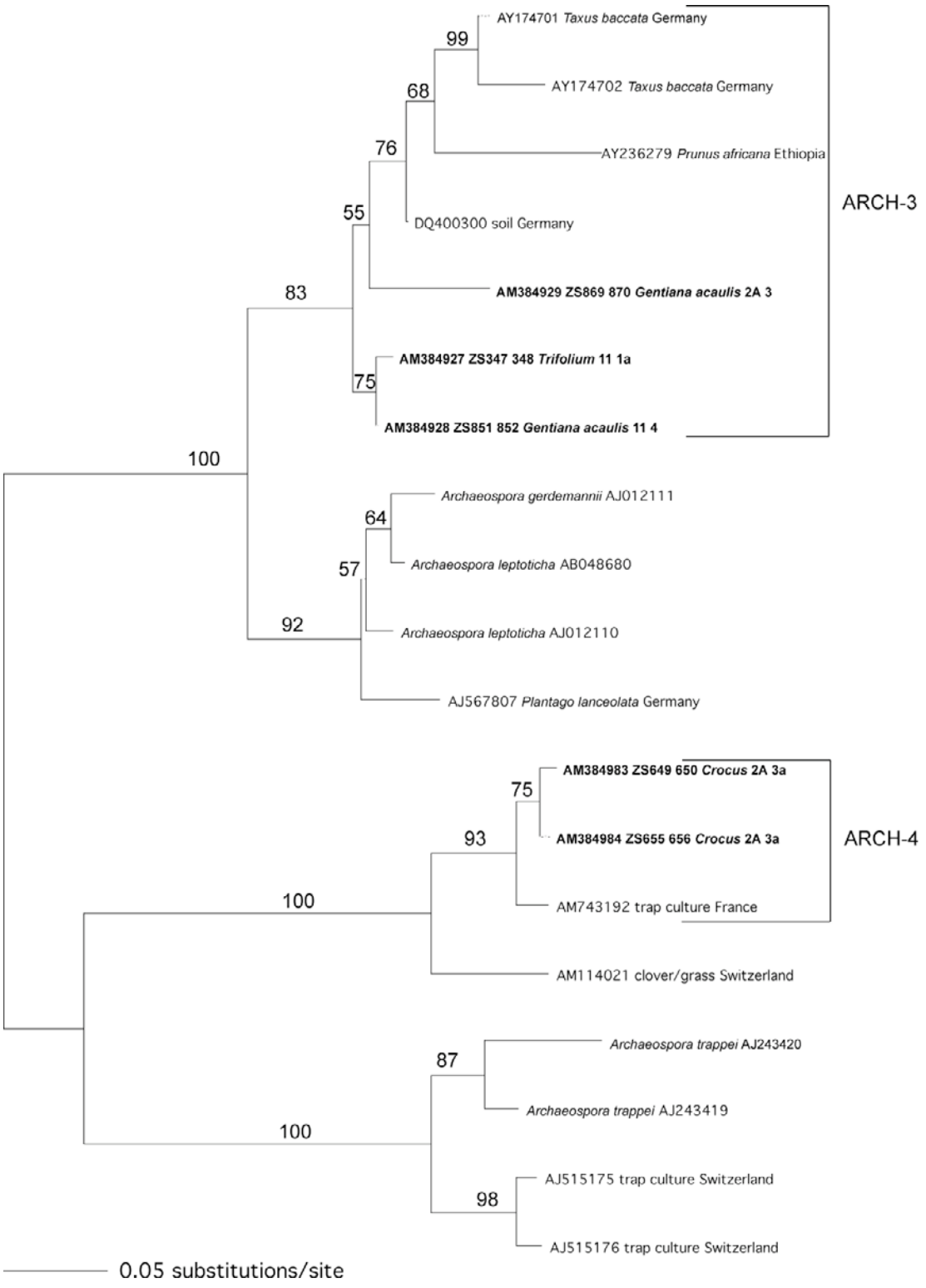

Fig. 8 Phylogenetic tree of Archaeosporaceae obtained by neighbor-joining analysis of 404 characters from ITS2 and 5.8S rDNA. Numbers above branches denote neighbor-joining bootstrap values from 1000 replications. The tree was midpoint-rooted. Sequences obtained in the present study are shown in boldface and are labeled as described in the legend of Fig. 5. The brackets show the delimitation of the sequence types.

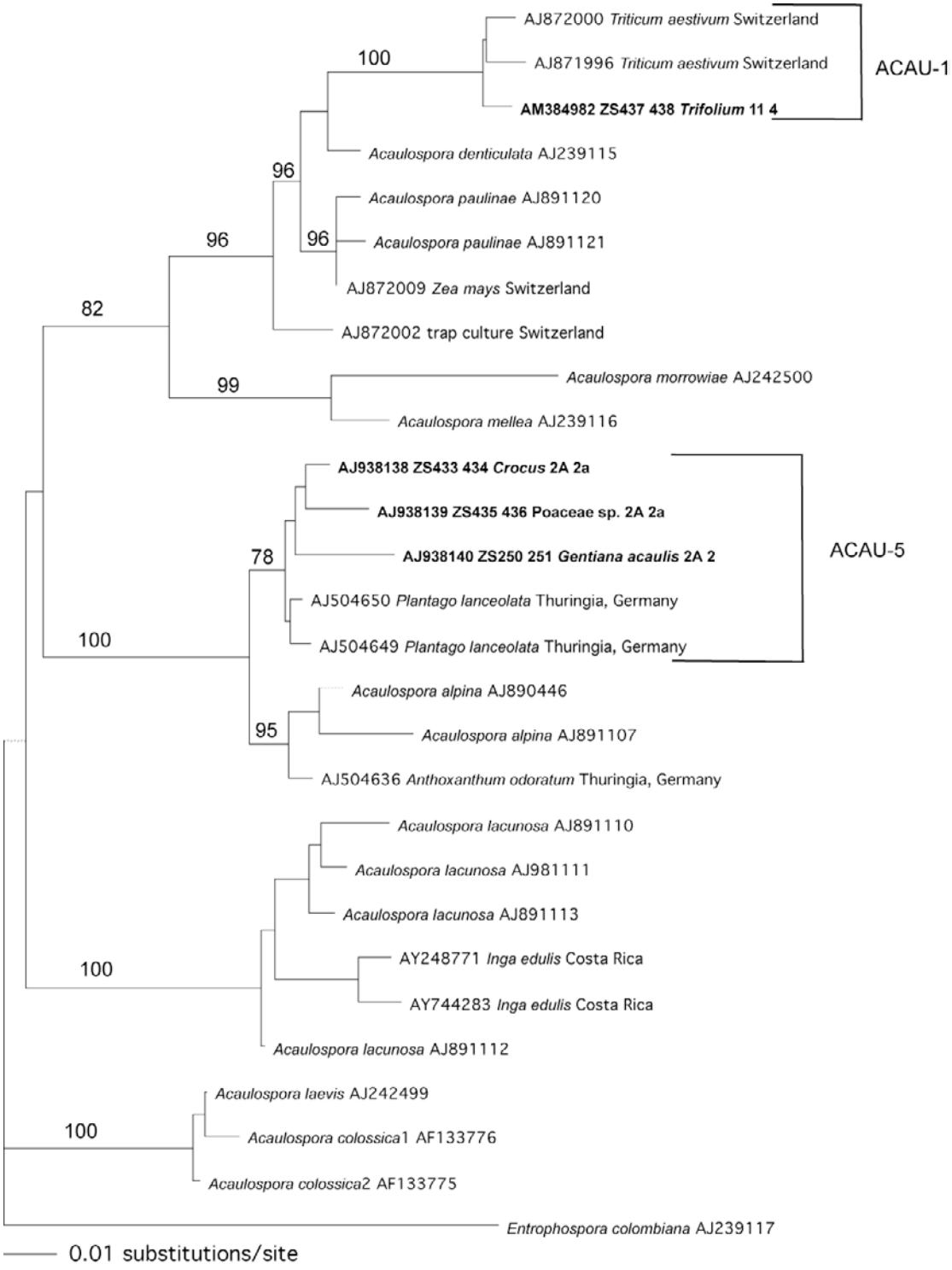

Fig. 9 Phylogenetic tree of Acaulosporaceae obtained by neighbor-joining analysis of 325 characters from ITS2 and 5.8S rDNA. Numbers above branches denote neighbor-joining bootstrap values from 1000 replications. The tree was rooted by *Entrophospora colombiana*. Sequences obtained in the present study are shown in boldface and are labeled as described in the legend of Fig. 5. The brackets show the delimitation of the sequence types.

Tab. 1 Overview of the clone numbers per sequence type in all analyzed samples yielding at least one AMF sequence type or RFLP pattern.[a]

	Sequence type																
	Glomus group A										*Glomus* group B			Acaulosporaceae		Archaeospora-ceae	
Sample description	**GLOM A-1**	**GLOM A-7**	**GLOM A-8**	**GLOM A-9**	**GLOM A-12**	**GLOM A-14**	**GLOM A-15**	**GLOM A-17**	**GLOM A-25**	**GLOM A-28**	**GLOM B-3**	**GLOM B-4**	**GLOM B-5**	**ACAU-1**	**ACAU-5**	**ARCH -3**	**ARCH -4**
***G. verna* 11-8**									1								
***G. verna* 11-5**									2								
***G. verna* 11-2**							3										
***G. verna* 11-6**								9									
***G. verna* 11-5**	9																
***G. verna* 2A-4**	5					1			4								
***G. verna* 2A-3**	8							2	2								
***G. verna* 2A-B**	12																
***G. verna* 2A-5**						5											
***G. acaulis* 11-1**		10															
***G. acaulis* 11-4**				7	1												
***G. acaulis* 11-4**																10	
***G. acaulis* 11-2**				4	3												
***G. acaulis* 2A-2**															3		
***G. acaulis* 2A-1**			3														
***G. acaulis* 2A-4**	6																
***G. acaulis* 2A-C**	11																
***G. acaulis* 2A-B**	1	1		10													
***G. acaulis* 2A-3**						9										11	
***Trifolium* spp. 11-3a**	10											5	1				
***Trifolium* spp. 11-4a**	1													9			
***Trifolium* spp. 11-4a**	12										1	1	1	3			
***Trifolium* spp. 11-1a**	9											1	1			2	
***Trifolium* spp. 2A-1a**	6											6	2				
***Trifolium* spp. 2A-1a**	7											6					
***Trifolium* spp. 2A-Ba**	8										1	7					

	Sequence type																
	Glomus group A										*Glomus* group B			Acaulosporaceae		Archaeospora-ceae	
Sample description	**GLOM A-1**	**GLOM A-7**	**GLOM A-8**	**GLOM A-9**	**GLOM A-12**	**GLOM A-14**	**GLOM A-15**	**GLOM A-17**	**GLOM A-25**	**GLOM A-28**	**GLOM B-3**	**GLOM B-4**	**GLOM B-5**	**ACAU-1**	**ACAU-5**	**ARCH -3**	**ARCH -4**
***Trifolium* spp. 11-5v**	2								4			11					
***Trifolium* spp. 11-6v**	6								1	1		7					
***Trifolium* spp. 11-2v**	10																
***Trifolium* spp. 2A-3v**	11											6					
***Trifolium* spp. 2A-4v**	14																
***Trifolium* spp. 2A-5v**	4																
***Thymus* 11-1a**					5												
***Thymus* 2A-1a**	6		2					1									
***Leontodon* 2A-1a**	10																
***Leontodon* 2A-1a**	4							3									
***Leontodon* 2a-2a**	8																
***Crocus* 2a-2a**			2	8											2		
***Crocus* 2A-3a**										4	10						1
Poaceae spec. 2A-2a	9														2		
Poaceae spec. 2A-Ba	9																
***Hieracium* 11-4a**	10												2				
***Hieracium* 11-1a**	9											6					
***Polygala* 11-3a**						8											
***Ranunculus* 11-4a**	16								1				2		4		

[a]No number in the cell indicates that no clones of the corresponding phylotype were detected in the sample. Sample description: host plant name is followed by the field site code (11 and 2A, respectively), soil core code (1-8 or A-C). Except for *Gentiana verna* and *G. acaulis*, the last letter (a or v) indicates if the host plant was collected in the soil core with *G. verna* (v) or with *G. acaulis* (a).

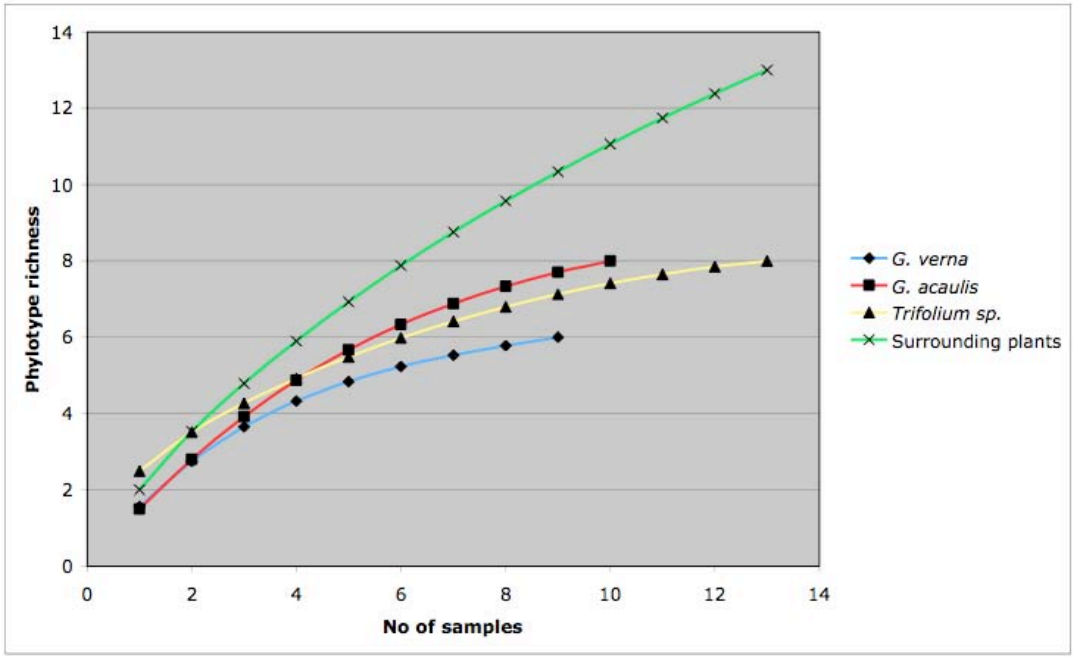

Fig. 10 Sampling effort curves for *Gentiana verna* (n=9), *G. acaulis* (n=10), *Trifolium* spp. (n=13) and the remaining pooled host plants (n=13). Sample order was randomized by 100 replications in EstimateS 7.5 (Colwell 2005).

2.4.2. AMF richness and diversity

The observed absolute numbers of sequence types per root sample were compared using ANOVA. Host plant taxon had a significant influence (P=0.029) on the number of sequence types, whereas the field sites did not (P=0.60). The plant harboring the highest mean number of AMF phylotypes (2.61) was *Trifolium* spp., which differed significantly from *G. verna* and *G. acaulis* (1.44 and 1.50, respectively) according to Fisher's LSD Multiple-Comparison Test. The mean number of AMF phylotypes harbored by the remaining host plants (2.0; pooled data) did not significantly differ from those of either of the host plant species mentioned above.

The Shannon diversity index calculated for different host plant species was 1.99 in the case of *G. acaulis*, 1.69 for *Trifolium* spp. (1.61 for *Trifolium* spp. collected in the soil cores with *G. acaulis* and 1.20 for *Trifolium* spp. collected in the soil cores with *G. verna*), and 1.50 for *G. verna*; for the remaining, pooled host plant species it reached 2.17. The Shannon index calculated for the whole study was 2.38.

2.4.3. AMF communities in different host plant species and field sites

The influence of the host plant species and the field sites (subsequently called "all environmental factors") on the distribution of AMF sequence types was investigated using a multivariate statistical approach. Sample *G. verna* 11-2 was excluded from the analysis as an outlier, because only one sequence type (GLOM A-15) was detected in it - which occurred only once in the whole study. CCA (Canonical correspondence analysis) was focused on *G. acaulis, G. verna* and *Trifolium* spp., as these plant taxa were represented by 9-13 root samples each and thus analyzed in detail. *Trifolium* spp. plants originating from soil cores with *G. verna* or *G. acaulis* were treated as different categories. All environmental factors explained 22.2% of the whole variance, and their effect on the distribution of AMF sequence types was clearly significant (P=0.002). The variance partitioning showed that the host plant identity accounted for 86% of the variance explained by all environmental factors, whereas the field sites accounted only for 13.7% (the remaining 0.3% was explained by the correlation of both of them). Moreover, the influence of the host plants was statistically significant (P=0.002). According to the forward-selection output, *G. acaulis* (P=0.002) and *G. verna* (P=0.004) were the two variables with significant contribution. These results demonstrate that the AMF communities hosted by those two plants were different from each other and also from the AMF harbored by *Trifolium* spp.

The biplot diagram of this CCA (Fig. 11) also demonstrates these results: the centroids representing the field sites are close to each other, indicating that the sites hosted similar AMF communities. In contrast, the centroids representing different host plant species are distant to each other, which demonstrates that they harbored distinct AMF. Fig. 4 also clearly shows which sequence types occurred in which of these three host plants: e.g. GLOM A-7, GLOM A-8, GLOM A-9 and GLOM A-12 were hosted exclusively by *G. acaulis*. The only sequence type common to all three host plants was GLOM A-1, but its relative abundance differed: it was detected in 100% of *Trifolium* spp. samples, but only in 44% of *G. verna* samples and 30% of *G. acaulis* samples (see also Tab. 1).

The AMF communities in the roots of *Trifolium* spp. neighboring *G. acaulis* or *G. verna*, respectively, did not significantly differ from each other. However, a trend towards more similar AMF between *Trifolium* spp. and its neighboring *Gentiana* species was observed, probably due to sharing of some phylotypes (ARCH-3 in *G. acaulis* and their neighboring *Trifolium* spp. and GLOM A-25 in *G. verna* and their neighboring *Trifolium* spp.).

The CCA conducted with the whole dataset with all host plants supported the results of the first CCA: all environmental factors accounted for 33.3% of the whole variance and their influence on the sequence type distribution was significant according to the Monte Carlo

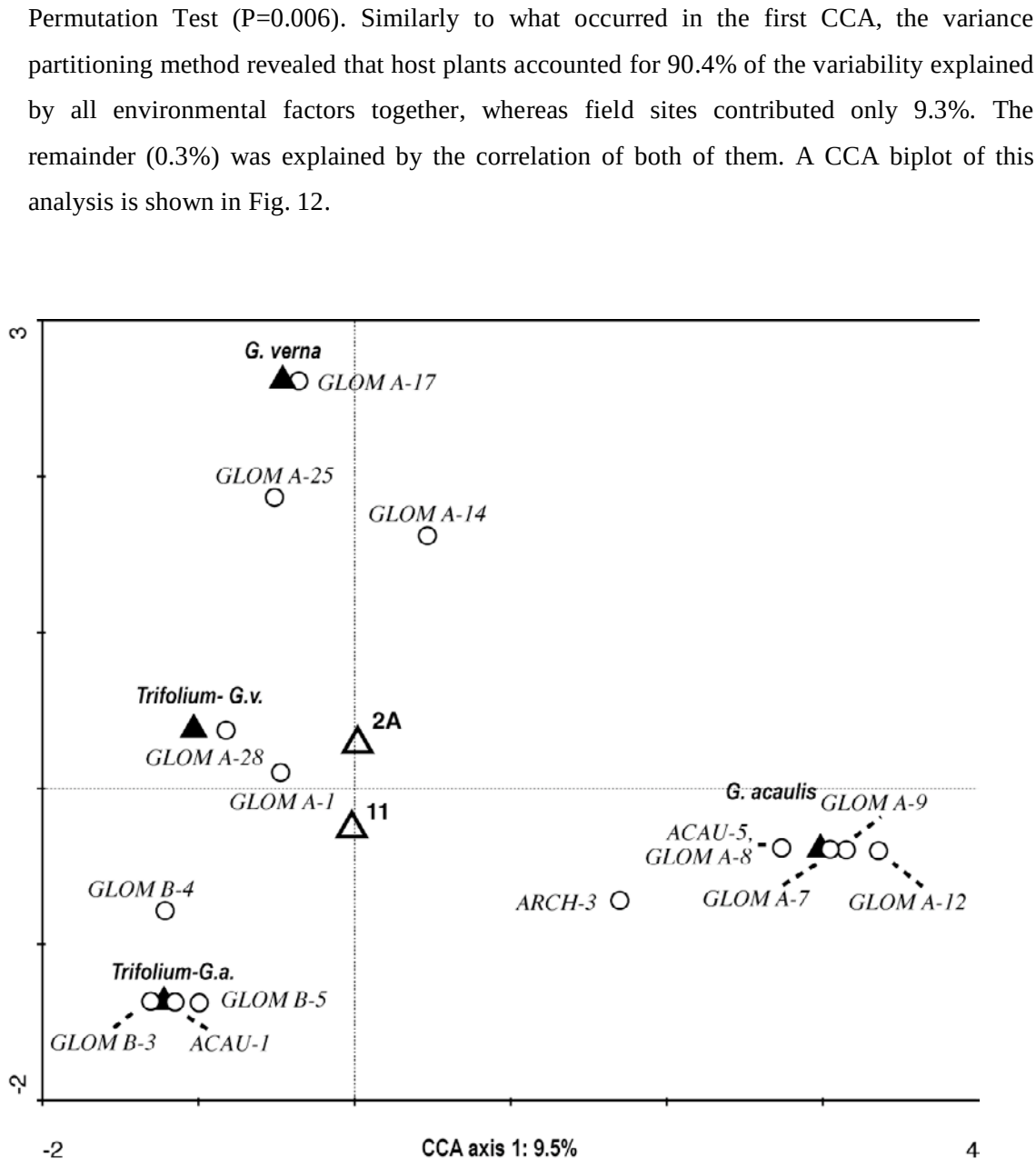

Fig. 11 CCA biplot of the sequence types and environmental factors (using Hill's scaling focused on inter-species distances) of the reduced dataset comprising *Trifolium* spp., *Gentiana verna* and *G. acaulis* samples. *Trifolium* spp. plants originating from soil cores with *G. verna* or *G. acaulis* were treated as different categories. Host plant species are represented by filled triangles, field sites by open triangles and sequence types by circles. The 1st axis accounted for 42.9% of the variability explained by all canonical axes and was significant (P=0.006). The percentages shown on the 1st and 2nd axes correspond to the percentages of variance of AMF sequence types data explained by the particular axis.

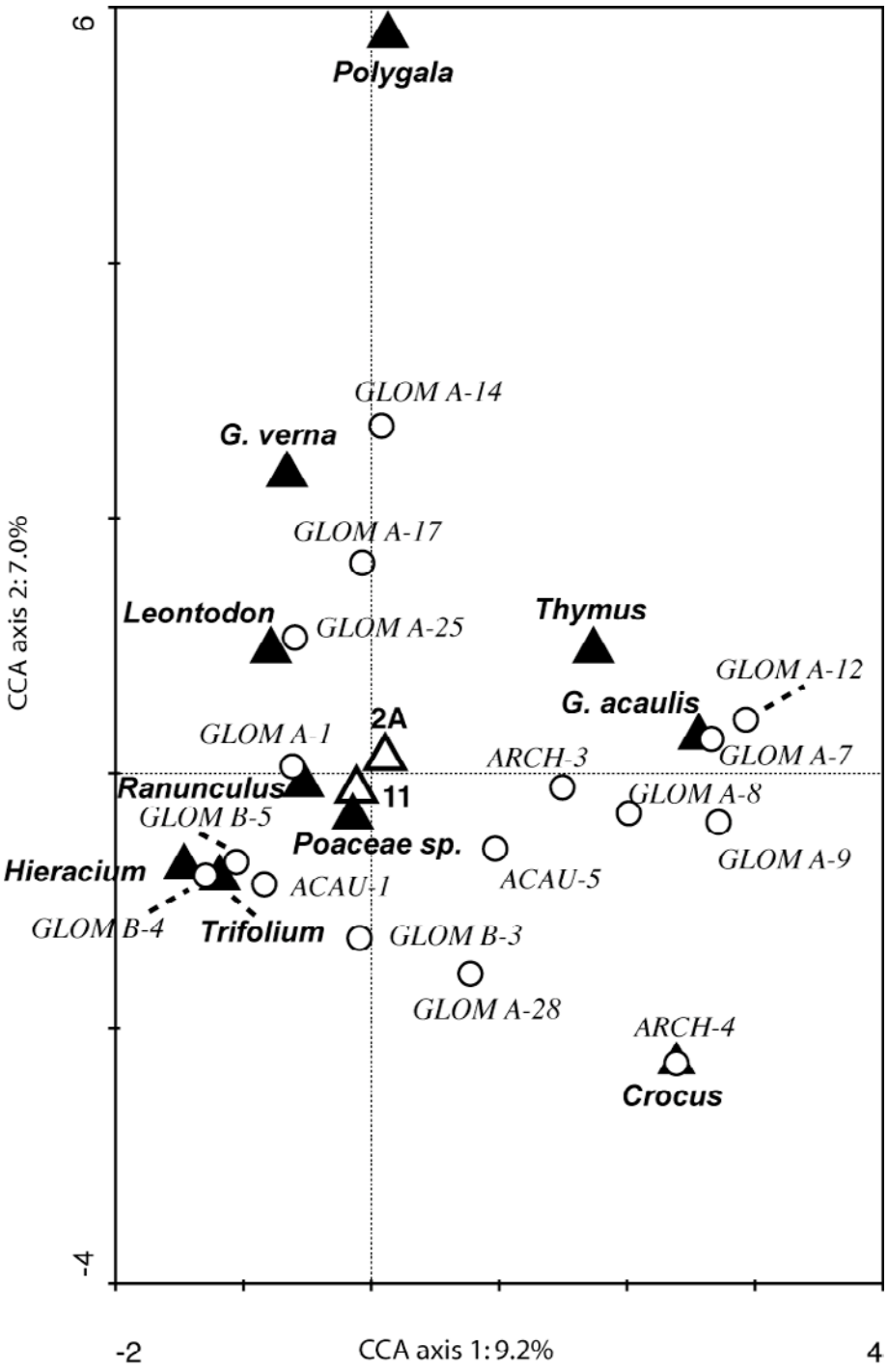

Fig. 12 CCA biplot of sequence types and environmental factors (using Hill's scaling focused on inter-species distances) based on all host plants. Host plant species are represented by filled triangles, field sites by open triangles and sequence types by circles. The 1st axis accounted for 27,5% of the variability explained by all canonical axes and was significant (P=0.042). The percentages shown on 1st and 2nd axes correspond to the percentages of variance of species data explained by the particular axis.

2.5. Discussion

2.5.1. AMF in the European alpine and upper montane forest zone

To our knowledge, this is the first molecular diversity study of AMF in the upper montane forest zone in Europe. Previous studies of this topic used spore morphology or root staining in order to evaluate the diversity of symbiotic fungi and root colonization (Read and Haselwandter 1981; Haselwandter 1987).

AMF diversity has recently been studied using the classical approach based on spore morphology in multiple field sites covering the whole Swiss Alps at altitudes from 1000–3000 m a.s.l. (Oehl et al., unpublished). Part of this study was conducted in several high mountainous grazed meadows close to our study sites in the community of Ramosch and in the neighboring community Sent. Twelve to eighteen AMF species were identified per site (F. Oehl, personal communication), which is in agreement with the 17 phylotypes found in our study. Oehl et al. (2006) observed that species belonging to the genus *Acaulospora* were particularly prominent and more abundant in the Swiss Alps than in the lowlands of Switzerland; those authors also described a new species *Acaulospora alpina,* which was found exclusively in the Alps at altitudes above 1300 m ASL. As our study site near Ramosch was close to one of the study sites investigated by Oehl et al. (2006), it was interesting to see whether we could recover *A. alpina* from colonized roots. The sequence type ACAU-5 (Fig. 9) that we detected was related to *A. alpina* but formed a distinct clade with sequences originating from a mountainous area from central Germany (between 640 and 705 m ASL; Börstler et al. 2006). These findings suggest the possibility of the existence of another *Acaulospora* clade related to *A. alpina* that preferentially occurs in mountainous meadows.

2.5.2. Relationships to phylotypes in other studies

Fifteen out of the 17 AMF phylotypes have been found in previous studies (Tab. 2). Only the sequence types GLOM A-8 and GLOM B-5 were detected for the first time, i.e. no sequences belonging to these groups were found in the EMBL database. These records demonstrate a surprisingly broad ecological amplitude and geographical distribution for most of the AMF sequence types. However, due to our conservative approach in sequence type definition it is possible that we underestimated AMF species diversity and that some sequence types contained more than one species. The molecular delimitation of an AMF species is problematic due to sequence heterogeneity within spores and species (Sanders et al. 1995).

Tab. 2 Overview of all sequence types found in our study and their matches to morphologically described AMF species and molecular phylotypes found in some other studies.

Sequence type	**Morphospecies**	**Reference e.g.**	**Host plant species e.g.**	**Ecosystem/Country**
GLOM A-1	*Glomus intraradices*	e.g. Hijri et al. (2006)	*Zea mays*	Germany, Switzerland
GLOM A-7	-	Wubet et al. (2003a); Russell & Bulman (2005); Waldrop et al. (unpublished)	*Prunus africana*, *Marchantia foliacea*, soil sample	Ethiopia, New Zealand, Minnesota (USA)
GLOM A-8	-	-	-	-
GLOM A-9	-	Börstler et al. (2006)	*Arrhenatherum elatius*	Germany
GLOM A-12	-	Russell et al. (2002); Waldrop et al. (unpublished)	Podocarpaceae root nodules, soil sample	New Zealand, Minnesota (USA)
GLOM A-14	-	Börstler et al. (2006); Russell & Bulman (2005)	*Dactylis glomerata*, *Marchantia foliacea*	Germany, New Zealand
GLOM A-15	*Glomus constrictum*	Hijri et al. (2006); Wubet et al. (2003a), Landwehr et al. (2002)	Pot culture; *Prunus africana*, Spore	Ethiopia, Germany
GLOM A-17	*Glomus badium*	Wubet et al. (2003b); Wirsel (2004); Oehl et al. (2005)	*Taxus baccata*, *Phragmites australis*, spores	Germany, Switzerland
GLOM A-25	*Glomus proliferum*	Declerc et al. (2000); Raab et al. (2005)	Root organ culture	Guadeloupe
GLOM A-28	-	Landwehr et al. (2002)	Spore	Germany
GLOM B-3	-	Wubet et al. (2003a)	*Prunus africana*	Ethiopia
GLOM B-4	-	Börstler et al. (2006)	*Plantago major*	Germany
GLOM B-5	-	-	-	-
ARCH-3	-	Wubet et al. (2003a,b); Hempel et al. (2007)	*Prunus africana*, *Taxus baccata*, soil sample	Ethiopia, Germany
ARCH-4	-	Russell et al. (2002); Hijri et al. (unpublished)	Podocarpaceae root nodules, Trap culture	New Zealand, Switzerland
ACAU-1	-	Hijri et al. (2006)	*Triticum aestivum*	Switzerland
ACAU-5	-	Börstler et al. (2006)	*Plantago lanceolata*	Germany

The high proportion of sequence types without a known counterpart among described morphospecies (76.5%) is consistent with previous predictions (Helgason et al. 2002) that the 200 morphospecies are only a fraction of the true diversity of the Glomeromycota.

Our phylotypes cannot easily be compared to those of other studies using the primer pair NS31/AM1 because those targeted a different 18S rDNA region. In addition, *Glomus* group B is not detected very often using these primers, possibly because of mismatches in the annealing sites.

2.5.3. AMF richness and diversity in our study sites

It is difficult to directly compare results of molecular studies of the AMF diversity in the field, as different research teams target different parts of DNA and define the sequence types inconsistently.

Nonetheless, the overall number of sequence types found in both our sites - seventeen - is in the same range as those of other studies focusing on undisturbed plant species-rich grasslands, which revealed between ten and 24 phylotypes (Vandenkoornhuyse et al. 2002; Scheublin et al. 2004; Börstler et al. 2006; Öpik et al. 2006).

Interestingly, AMF community richness varied on two different levels, which may respond to different factors. The highest AMF richness per sample was detected in *Trifolium* spp. Moreover, *Trifolium* spp. and *G. acaulis* harbored a higher overall AMF richness than *G. verna* (see the sampling effort curves in Fig. 10). The highest overall richness was found in the category "surrounding plants", and it was clearly not characterized exhaustively. The richness per sample, however, was not significantly different compared to *G. verna* or *G. acaulis*. These findings indicate that AMF richness in a plant taxon per root system and across the habitat may not necessarily be linked.

The lower amplification success from *G. acaulis* samples could have been caused by high contents of secondary metabolites like xanthones reported from some *Gentiana* species (Chericoni et al. 2003). A higher sampling effort was necessary to obtain a number of samples yielding PCR products comparable to those from *G. verna* and *Trifolium* spp. A lower efficiency of amplification may potentially have biased the AMF community composition detected in *G. acaulis*. However, this is unlikely for several reasons: i) the sampling effort curve demonstrates that the AMF community in this plant is not less diverse than in *Trifolium* spp., ii) the number of phylotypes found in each sample is not significantly different from the number in *G. verna*. and iii) a systematic bias against some glomeromycotan lineages by possible differences in primer susceptibilities towards the inhibitor substances is unlikely because phylotypes were detected from the same lineages as in *G. verna*.

2.5.4. Host preference versus other factors influencing AMF communities

The sampling effort curve (Fig. 10) shows an interesting trend: several host plant species pooled together hosted more different AMF sequence types than the single plant species or genus, which supports the host preference hypothesis.

Our CCA results (Figs. 11, 12) concerning the strong influence of the plant species on the composition of the AMF community in the roots are in agreement with the results of several studies, which have presented evidence for host preferences in arbuscular mycorrhiza in the past few years (Helgason et al. 2002; Vandenkoornhuyse et al. 2002; Vandenkoornhuyse et al. 2003). In all these cases, host plants harbored diverse communities of glomeromycotan symbionts, usually from different genera or families. In other studies, environmental factors other than host preference appeared to be dominant: site dependency (Öpik et al. 2003); sampling season and soil nitrogen content (Santos et al. 2006); sampling date and field site (Husband et al. 2002a), or age classes of seedlings (Husband et al. 2002b).

Host specificity in the stricter sense, i.e. a host plant being colonized only by a narrow clade of fungal taxa, has been demonstrated only for mycoheterotrophic members of the Gentianaceae (Bidartondo et al. 2002). The *Gentiana* species that we studied clearly show host preference but not host specificity as defined above. In this respect, they are more similar to other green plants from other families than to their mycoheterotrophic relatives. Thus, we did not find evidence for narrowing on a restricted set of symbionts as a possible symptom of a transition to a non-mutualistic symbiosis.

From the fungal point of view, our data indicate that AMF taxa belonging to *Glomus* group B seem to show strong preference for *Trifolium* spp. roots. However, none of these phylotypes was detected in *Trifolium repens* and *Trifolium pratense* in the surroundings of Jena, Germany (Hempel et al. 2007). These findings are in agreement with the fact that none of the studies addressing the topic of host preferences in AMF so far has shown host preference to act across different geographical regions. Therefore the term "local host preference" may be more appropriate to describe this phenomenon.

2.5.5. Ecological consequences of AMF host preference

Scheublin et al. (2004) suggested that host plant species may have various degrees of specificity for AMF species that range from selective specialists to non-selective generalists. None of the plants in our experiment showed a distinctly narrowed spectrum of fungal symbionts comparable to that of mycoheterotrophic Gentianaceae; nevertheless, the plant taxa

analyzed differed in their levels of AMF richness and diversity and contained distinct communities.

Börstler et al. (2006) and Öpik et al. (2006) proposed the concept that some AMF species occur globally, showing high local abundance and low host specificity. *Glomus intraradices* clearly falls into this category as a generalist, because it has been found in a surprisingly broad range of environments. The fact that GLOM A-1 was the only sequence type in our study shared by both gentian species and *Trifolium* spp. strongly supports this notion. However, the second-most-frequent phylotype (GLOM B-4) showed strong evidence of host preference for *Trifolium* spp. The fact that this phylotype was not found in another study analyzing *Trifolium* spp. suggests that local availability of fungal inoculum and other environmental factors may also have an influence on this interaction.

The findings of van der Heijden et al. (1998) showing that AMF diversity is correlated with diversity and yield of emerging plant communities suggest some degree of specificity in the interactions between symbionts in AM. This specificity can be due to preferential colonization or to specific functional interactions (e.g. nutrient transfer). Only the former aspect was addressed in this study and in other molecular studies of host preferences reported so far. The extent to which this phenomenon is responsible for maintenance and coexistence of plant species remains to be shown in future studies.

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Chapter 3

The cultivation bias: different communities of arbuscular mycorrhizal fungi detected in roots from the field, from bait plants transplanted to the field, and from a greenhouse trap experiment

(Accepted by Mycorrhiza)

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Fig. 1 *Inula salicina*, Landskron meadow. Photo by B. Börstler

Fig. 2 *Medicago sativa*, Landskron meadow. Photo by B. Börstler

Fig. 3 *Origanum vulgare*, Landskron meadow. Photo by B. Börstler

Fig. 4 *Bromus erectus*, Landskron meadow. Photo by B. Börstler

3.1. Abstract

The community composition of arbuscular mycorrhizal fungi (AMF) was investigated in roots of four different plant species (*Inula salicina, Medicago sativa, Origanum vulgare* and *Bromus erectus;* Figs. 1-4) sampled in (i) a plant species-rich calcareous grassland, (ii) a bait plant bioassay conducted directly in that grassland and (iii) a greenhouse trap experiment using soil and a transplanted whole plant from that grassland as inoculum. Roots were analyzed by AMF-specific nested PCR, RFLP screening and sequence analyses of rDNA small subunit and internal transcribed spacer regions. The AMF sequences were analyzed phylogenetically and used to define monophyletic phylotypes.

Overall, sixteen phylotypes from several lineages of AMF were detected. The community composition was strongly influenced by the experimental approach, with additional influence of cultivation duration, substrate and host plant species in some experiments.

Some fungal phylotypes, e.g. GLOM-A3 (*Glomus mosseae*) and several members of *Glomus* group B, appeared predominantly in the greenhouse experiment or in bait plants. Thus, these phylotypes can be considered r strategists, rapidly colonizing uncolonized ruderal habitats in early successional stages of the fungal community. In the greenhouse experiment, for instance, *Glomus mosseae* was abundant after 3 months, but could not be detected anymore after 10 months. In contrast, other phylotypes as GLOM-A17 (*Glomus badium*) and GLOM-A16 were detected almost exclusively in roots sampled from plants naturally growing in the grassland or from bait plants exposed in the field, indicating that they preferentially occur in late successional stages of fungal communities and thus represent the K strategy. The only phylotype found with high frequency in all three experimental approaches was GLOM A-1 (*Glomus intraradices*), which is known to be a generalist.

These results indicate that in greenhouse trap experiments it is difficult to establish a root-colonizing AMF community reflecting the diversity of these fungi in the field roots, because fungal succession in such artificial systems may bias the results. However, the field bait plant approach is a convenient way to study the influence of different environmental factors on AMF community composition directly under the field conditions. For a better understanding of the dynamics of AMF communities it will be necessary to classify AMF phylotypes and species according to their life history strategies.

Key words: Arbuscular mycorrhiza; molecular diversity; rDNA; life history strategy; cultivation systems

3.2. Introduction

The diversity of arbuscular mycorrhizal fungi (AMF) can be assessed based on either the spores found in the soil or the fungal mycelium in the roots. AMF spores can be identified either by microscopy or molecular analysis, whereas molecular methods are required to distinguish AMF species in the roots. The production of spores is highly dependent on environmental conditions and on the physiological status and life strategy of the particular mycorrhizal fungus (Smith and Read 1997). A trap culture approach is commonly used to harvest newly-formed spores of AMF from "universal host plants" (e.g. *Plantago lanceolata*, *Trifolium pratense*, *Zea mays*, *Allium porrum*) inoculated using field soil in pot cultures in the greenhouse. It is known that this approach does not reveal the same community composition of AMF species as the direct analysis of spores in the field (Jansa et al. 2002; Oehl et al. 2003). This phenomenon was attributed to selective effects of the trap plant species (Jansa et al. 2002; Ahulu et al. 2006) or to different growth conditions in the greenhouse including the time period of culturing (Oehl et al. 2003).

Molecular methods allow the identification of the symbiotic community colonizing the roots of an individual plant at any given time. Considerable differences between AMF communities present as spores and in the roots in a single field site have been reported (e.g. Clapp et al. 1995; Kowalchuk et al. 2002; Wubet et al. 2003; Renker et al. 2005; Ahulu et al. 2006; Börstler et al. 2006; Hempel et al. 2007).

Based on spore morphology, only about 200 AMF species have been described so far (http://www.tu-darmstadt.de/fb/bio/bot/schuessler/amphylo). This small number was thought to colonize the majority of higher plant species and consequently, their host specificity or preference was thought to be very low (Smith and Read 1997). However, recent molecular studies of AMF field communities (e.g. Husband et al. 2002; Wubet et al. 2004; Börstler et al. 2006) revealed numerous previously unknown phylotypes and, in several cases, the phylotypes inhabiting roots of different plant species in the same habitat differed, indicating some degree of host preference (Helgason et al. 2002; Vandenkoornhuyse et al. 2002; Vandenkoornhuyse et al. 2003; Gollotte et al. 2004; Scheublin et al. 2004; Sýkorová et al. 2007). In contrast, an apparent lack of host preference has been reported by other authors (Öpik et al. 2003; Santos et al. 2006).

Using direct field soil sampling and greenhouse trap cultures, followed by morphological analysis of the AMF spores, low-input grasslands were shown to be the most AMF-diverse among several agroecosystems compared in Central Europe (Oehl et al. 2003) harboring 26-27 AMF species per site. A global survey of molecular studies of root-colonizing AMF by Öpik et al. (2006) identified temperate grasslands as the ecosystem with

the second highest AMF species richness after tropical forests. Read and Birch (1988) identified AMF mycelia as primary sources of inoculum in permanent grasslands.

The aim of this study was to analyze the communities of AMF in roots of four plant species dominant in a plant species-rich calcareous grassland in France (subsequently called target plants) comparing three different experimental approaches: i) direct root sampling in the field (field samples, FS); ii) cultivation of target plants in compartment systems (CS) in the greenhouse using the field soil and a transplanted field plant as inoculum; iii) trapping the AMF in the roots of target plants grown in in-growth cores exposed in the field (bait plants, BP). Our goal was to address whether the same AMF communities could be detected in the different host plants using these three experimental approaches. We also wanted to elucidate whether the AMF phylotypes present in the roots would differ with respect to their ecological preferences and life history strategies. We used the primer set for rDNA small subunit and internal transcribed spacer regions designed by Redecker (2000) allowing us to detect seven genera of the Glomeromycota, which is the largest possible portion of AMF taxon diversity recognized so far.

Fig. 5 The Landskron field site. Photo by D. Redecker

Fig. 6 Compartment systems after 10 months of cultivation in the greenhouse

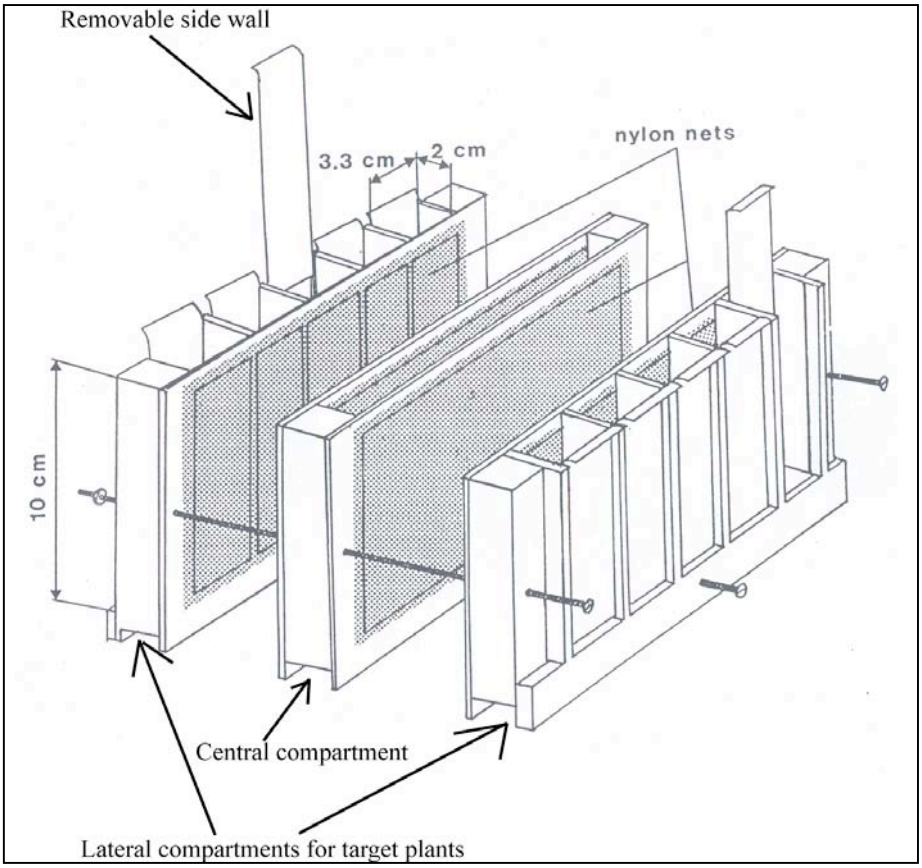

Fig. 7 Scheme of the compartment systems used in the greenhouse bioassay. The big central compartment is separated by nylon mesh from the lateral compartments with target plants (modified from Wyss et al. 1991).

3.3. Materials and methods

3.3.1. Field site

The study site was a low-input species-rich grassland (Fig. 5) close to Leymen in Alsace, France (47°29'16'' N; 7°29'16'' E; ca 490 m above the sea level). It is mown once or twice per year, has not been fertilized during the last 20 years and has very high plant diversity (approximately 80 species) with *Bromus erectus* being the dominant grass. The vegetation type was classified as a Meso-Brometum. The soil pH (measured in H_2O) was 7.4, NaOAc-extractable phosphorus was 12 ng/g, HCl/H_2SO_4-extractable calcium was 1.3g/100g. Humus content was >9% (w/w; laboratory F.M. Balzer, Wetter-Amönau, Germany).

3.3.2. Field samples (FS)

In July 2002 and July 2005, a total of 17 soil cores with a depth of 15 cm were randomly removed in an area of approximately 15 m in diameter in the meadow. Plant roots were washed carefully, separated by plant species and blotted dry using paper tissue. Aliquots of 50 mg consisting of root pieces assembled from a single root system of one species were frozen in liquid nitrogen and stored at -80°C until use. Roots of the following plant species (subsequently called "target plants") were used for further DNA analyzes: the forb *Inula salicina* (Asteraceae, Fig. 1), the legume *Medicago sativa* (Fabaceae, Fig. 2), the forb *Origanum vulgare* (Lamiaceae, Fig. 3) and the grass *Bromus erectus* (Poaceae, Fig. 4). Five samples from five different root systems for each plant species were analyzed. All of the target plants were highly abundant in the field site, but showed a different distribution: *B. erectus* was distributed evenly, *I. salicina* occurred in dense patches, *M. sativa* and *O. vulgare* grew in a scattered pattern.

3.3.3. Greenhouse experiment with compartment systems (CS)

Compartment systems (Wyss et al. 1991; Figs. 6, 7) were used to analyze possible host preferences of AMF and neighbor effects of the target plant species under controlled greenhouse conditions in two different successional stages, ensuring the complete separation of target plant root systems. In September 2003, the central large compartments were filled with a 1:1:1 mixture of autoclaved sand, autoclaved Terragreen (American aluminium oxide, oil dry US special, Lobbe Umwelttechnik, Iserlohn, Germany) and non-autoclaved homogenized soil from the field. In addition, seeds of the plant species *Plantago media* and

Lotus corniculatus had been collected in the field site where they occurred frequently. These seeds were sterilized in 4% sodium hypochlorite for approximately 5 min and pre-germinated in Petri dishes with sterile sand. Two *Bromus erectus* plants taken directly from the field and, in addition, *P. media* and *L. corniculatus* seedlings were planted into the central compartment in order to facilitate propagation of the inoculum. The lateral compartments, separated from the central chamber by a nylon net (60 µm aperture size; Lanz-Anliker AG, Rohrbach, Switzerland) were filled with a 1:1:1 mixture of autoclaved sand, autoclaved Terragreen and autoclaved homogenized soil from the field. Seedlings of target plants grown from the seeds collected in the field site and sterilized as described above were planted singly into one lateral compartment each. Two alternately-placed plant species were cultivated in one compartment system (Fig. 7a). Two compartment systems with the plant combinations *I. salicina/O. vulgare* and *I. salicina/M. sativa* were established and cultivated under greenhouse conditions (12 h light in winter, and 16 h in summer at 24-28 °C; night temperature at least 16 °C). After three months, half of the root system of each target plant was harvested using a removable side-wall while keeping the remainder of the plants intact. The compartments were then refilled with the original substrate; the harvested roots were washed and 3-4 aliquots of 50 mg per root system frozen in -80°C. The second harvest followed in July 2004 (after 10 months), using the whole root systems.

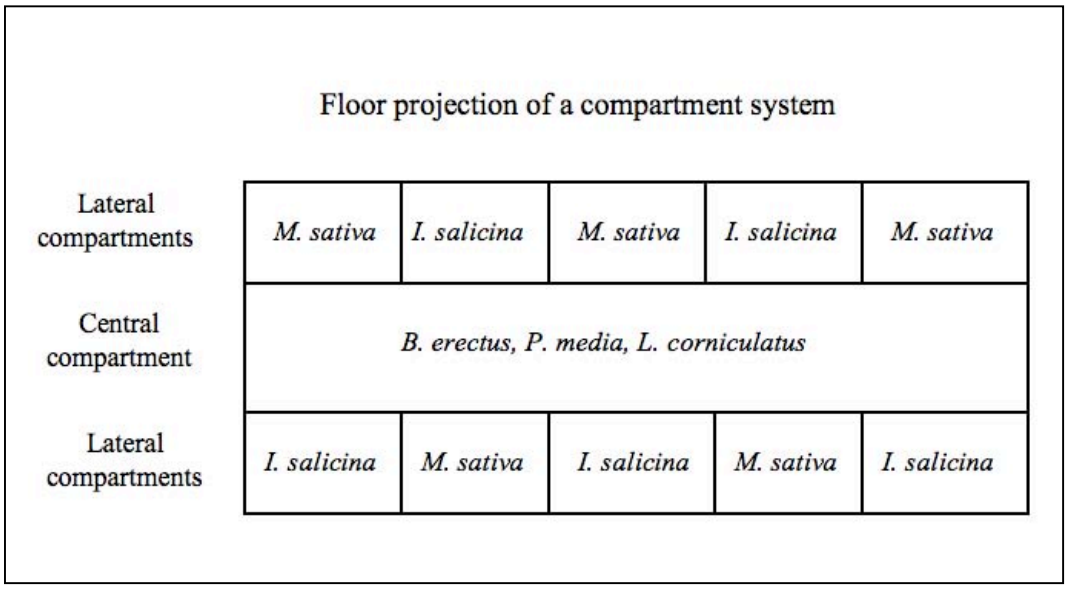

Fig. 7a Scheme of the floor projection of a compartment system with target plant species placement

3.3.4. Bait plants in the field site (BP)

In July 2004, an experiment with bait plants (Fig. 8) was established in order to trap the native AMF community from the field using target plant species under natural field conditions. The purpose was to analyze possible host preferences for AMF in two different successional stages while ensuring the complete separation of the target plant roots from the other plants in the field. The in-growth core system designed by Johnson et al. (2001) was adapted for our study in the following way: plastic bottles (diameter: 53 mm, height: 69 mm; Semadeni, Switzerland) with a screw lid were used. The bottom of each bottle was cut off and a hole with a diameter of ca 4 cm was cut out in the lid. A double nylon net (60 µm aperture size; Lanz-Anliker AG, Rohrbach, Switzerland) was fixated between the bottle and the lid by screwing and the bottles were inverted upside down. Fifteen bottles were filled with a mixture (1:2) of autoclaved sand and autoclaved soil (collected in September 2003 in the field, sieved through 4 mm sieve and homogenized); another fourteen bottles were filled with the same mixture, but the soil was not autoclaved. Five ml of a bacterial filtrate from the non-autoclaved soil were added to all bottles.

Two seedlings of one of the target plant species were planted into each bottle (3-4 repetitions per plant species per substrate, altogether 29 bottles) and cultivated for two months in the greenhouse (Fig. 9) in order to ensure initial growth of the plantlets in the bottles. In September 2004, holes were dug out in a grid with approximately one meter distance between each other in the field site in the same area where the field samples were taken. Bottles were inserted into the holes (Fig. 10) in random order and watered every 4-5 days for three weeks. After three months, soil cores with roots were taken from three bottles per plant species. Holes made by coring were refilled with autoclaved soil from the meadow. Roots were washed and aliquots of 50 mg were frozen at -80°C. The second harvest using the whole root systems was conducted in July 2005, after 10 months exposure in the field.

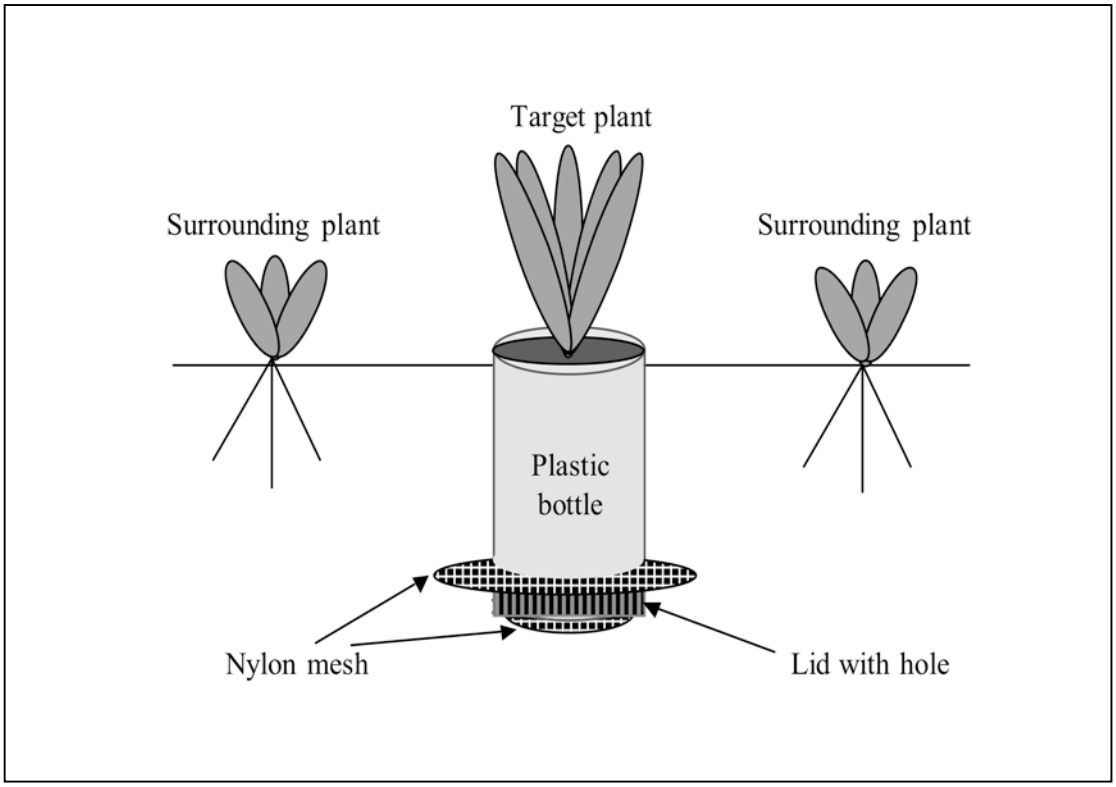

Fig. 8 Scheme of the bait plant bioassay after the transplantation into the field (drawn by B. Börstler)

Fig. 9 Bait plants in the greenhouse before the transplantation into the field

Fig. 10 *Inula salicina* bait plant after the transplantation into the field

3.3.5. DNA extraction and polymerase chain reactions

Roots were ground in liquid nitrogen using a pellet pestle within a 1.5 ml tube. DNA was extracted from roots using the DNeasy Plant Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in two steps, using 50 µl of elution buffer in each step. DNA extracts were diluted 1:10 or 1:100 in TE buffer and used as template for the first step of a nested PCR as described by Redecker (2000). This first round of amplification was performed using the universal eukaryote primers NS5 and ITS4 (White et al. 1990), Taq polymerase from Amersham (Basel, Switzerland) or New England Biolabs (BioConcept, Allschwil, Switzerland), 2 mM $MgCl_2$, 0.5 µM primers and 0.13 mM of each desoxynucleotide. The cycling parameters were: 3 min at 94°C, followed by 30 cycles of 45 sec at 94°C, 50 sec at 51°C and 1 min 30 sec at 72°C. The program was concluded by a final extension phase of 10 min at 72°C.

The PCR products were diluted 1:100 in TE buffer and used as a template in the second round. Five separate PCR reactions were performed using the primer pairs GLOM1310/ITS4i (specific for *Glomus* group A), LETC1677/ITS4i (specific for *Glomus* group B), ACAU1661/ITS4i (Acaulosporaceae), ARCH1311AB/ITS4i (Archaeosporaceae), NS5/GIGA5.8R or NS7/GIGA5.8R or GIGA5.8R/GIGA1313 (Gigasporaceae; Redecker 2000; Redecker et al. 2003). The PCR parameters for the second round differed from the first one only in the annealing temperature (61°C). Moreover, a "hot start" at 61°C was performed manually to prevent non-specific amplification. PCR products were checked on agarose gels (2%:1% NuSieve/SeaKem, Cambrex Bio Science, Rockland, ME, USA) in Tris/Acetate buffer at 120 V for 30 min.

3.3.6. Cloning, restriction fragment length polymorphism analyses and sequencing

PCR products were purified using the High Pure Kit from Hoffman LaRoche (Basel, Switzerland) and cloned into a pGEM-t vector (Promega/Catalys, Wallisellen, Switzerland). Inserts were re-amplified, preferably ten positive clones of each PCR product were digested with *Hinf*I and *Mbo*I and run on agarose gels as described above. Restriction fragment patterns were compared to a database modified from the spreadsheet developed by Dickie et al. (2003). Representative clones of new restriction types were re-amplified, purified using the High Pure Kit and sequenced in both directions. The BigDye Terminator Cycle Sequencing Kit (ABI, Foster City, CA, USA) was used for labeling. Samples were run on an ABI 310 capillary sequencer. Sequences were deposited in the EMBL database under the

accession numbers AM494584-AM494585; AM495115-AM495207; AM497782, AM497783 shown in the phylogenetic trees.

3.3.7. Sequence analyses

Sequences were aligned to previously-published sequences in PAUP*4b10 (Swofford 2001). The glomeromycotan origin of the sequences was initially tested by BLAST (Altschul et al. 1997). Separate ITS alignments were prepared for each of the target groups of the specific primers LETC1677, GLOM1310, ARCH1311AB. In addition, an alignment of the partial 3' end of 18S rDNA small subunit was compiled for the sequences amplified with GLOM1310 and ARCH1311AB (Bidartondo et al. 2002).

Phylogenetic trees were primarily obtained by distance analysis using the neighbor joining algorithm in PAUP*4b10, the Kimura two-parameter model and a gamma shape parameter=0.5. Results were verified by performing maximum likelihood analyses based on parameters estimated in Modeltest 3.5 (Posada 2004).

3.3.8. Definition of sequence phylotypes

Sequence phylotypes were defined in a conservative manner as consistently separated monophyletic groups in the phylogenetic trees. Only those clades were used that were supported by neighbor joining analysis and also present in the respective maximum likelihood tree. In case of GLOM-A and ARCH phylotypes, the clades had to be supported by both 18S partial subunit and ITS trees. We avoided splitting the lineages unless there was a positive evidence for doing so. The sequence phylotypes were designated after the major clade they belonged to, followed by a numerical index (x in the following examples) identifying the type (Hijri et al. 2006): GLOM-Ax (*Glomus* group A), GLOM-Bx (*Glomus* group B) and ARCH-x (Archaeosporaceae). Representative sequences of each phylotype were checked manually for possible chimaeras, which were excluded from further analyses.

3.3.9. Statistical analyses

Presence/absence of AMF phylotypes in each root sample were used to construct the species accumulation curves with 95% confidence intervals, using the analytical formulas of Colwell et al. (2004) in the program EstimateS 8.0 (Colwell 2005). Shannon diversity indices ($H=-\Sigma p_i * \ln(p_i)$) were calculated using the website http://www.changbioscience.com/genetics/shannon.html for each experimental approach and target plant species.

The influence of host plant species and experimental approach on the number of phylotypes found in the root samples was analyzed using the program NCSS (NCSS, Kaysville, UT, USA). In order to investigate the influence of environmental factors (host plant species, experimental approach, harvest, substrate, plant combination) on the distribution of the AMF phylotypes in the root samples, ordination analyses were conducted in Canoco for Windows v. 4.5 (ter Braak and Smilauer 2004) using the presence/absence data for each root sample. Initial Detrended Correspondence Analysis (DCA) suggested a unimodal character of the data response to the sample origin (the lengths of gradients were >4), therefore the Canonical Correspondence Analysis (CCA) was used. The variance partitioning method with permutations in blocks defined by the co-variables was used to compare the influence of groups of environmental factors between each other. For example, host plants were considered as co-variables when the influence of experimental approaches as variables was tested, and reverse. Monte Carlo Permutation Tests were conducted using 499 random permutations. The subsequent forward selection procedure ranked the environmental variables according to their importance and significance for the distribution of the phylotypes.

3.4. Results

3.4.1. PCR yields and phylotypes detected in the root samples

An overview of sampling and phylotypes occurring is presented in Tab. 1. Using our PCR approach with five nested primer sets, 74 of the 97 extracted root samples (76%) yielded 173 PCR products, resulting in 1182 clones after cloning. A total of 130 PCR products (75%; 938 clones) could be assigned to AMF phylotypes. *Inula salicina* root samples from the field and from the second harvests of the compartment systems and bait plants turned out to be the most problematic – only 25% of these DNA extracts yielded PCR amplicons. Eventually, 19 root samples from the field (4-5 replicates/plant species), 20 samples from the bait plants (3 replicates/plant species/harvest) and 31 samples from the compartment systems (5 replicates/plant species/compartment system/harvest) yielded AMF-containing PCR products.

After RFLP screening, 211 clones were sequenced and analyzed phylogenetically. Altogether, 16 different phylotypes were found, nine of which belonged to *Glomus* group A (group definitions according to Schwarzott et al. 2001), six to *Glomus* group B and one to the Archaeosporaceae (Figs. 11, 12, 13, 14; Tab. 1). By far the most abundant phylotype, which was found in 58 root samples, was GLOM A-1 (Figs. 11, 12). It corresponds to the morphologically-defined species *Glomus intraradices*. The second and third most frequent phylotypes were GLOM B-4 and GLOM B-1 (Fig. 13), which could not be assigned to any

morphologically described species, and GLOM A-3 (Figs. 11, 12), which corresponds to *G. mosseae*. No phylotypes belonging to the families Paraglomeraceae, Acaulosporaceae and Gigasporaceae were found.

Out of the 16 phylotypes reported in our study, five are known morphospecies, another six are known only as sequences detected in root or soil samples in other studies and the remaining five are new to science (Tab. 2).

Fig. 11 (figure on the next page) Phylogenetic tree of the Glomeromycota obtained by neighbor-joining analysis of 311 characters of the 18S rDNA subunit. Numbers above branches denote neighbor-joining bootstrap values from 1000 replications. The tree was rooted with *Paraglomus occultum* and *P. brasilianum.* Sequences obtained in the present study are shown in boldface. They are labeled with the database accession number, (e.g. AM495185), internal identification number (e.g. ZS557_558), the host plant species (e.g. *M. sativa*), kind of experimental approach (FS, BP, CS, see text); for FS the harvest year (05 or 02) is indicated; for BP 1st or 2nd harvest are shown (1 or 2); for CS 1st or 2nd harvest (1 or 2) are noted. The parentheses show the delimitation of the phylotypes.

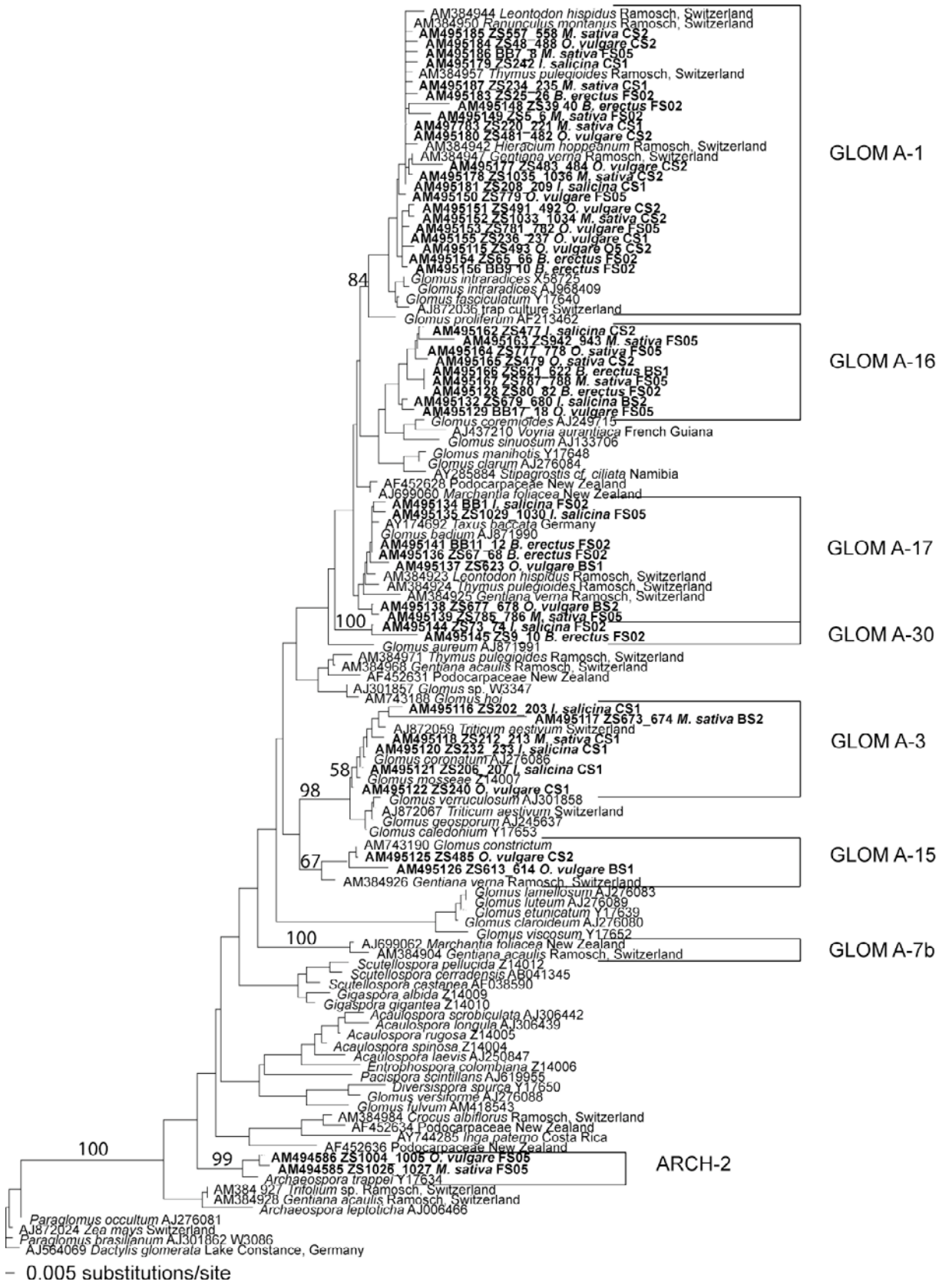

Fig. 11 (legend on the previous page)

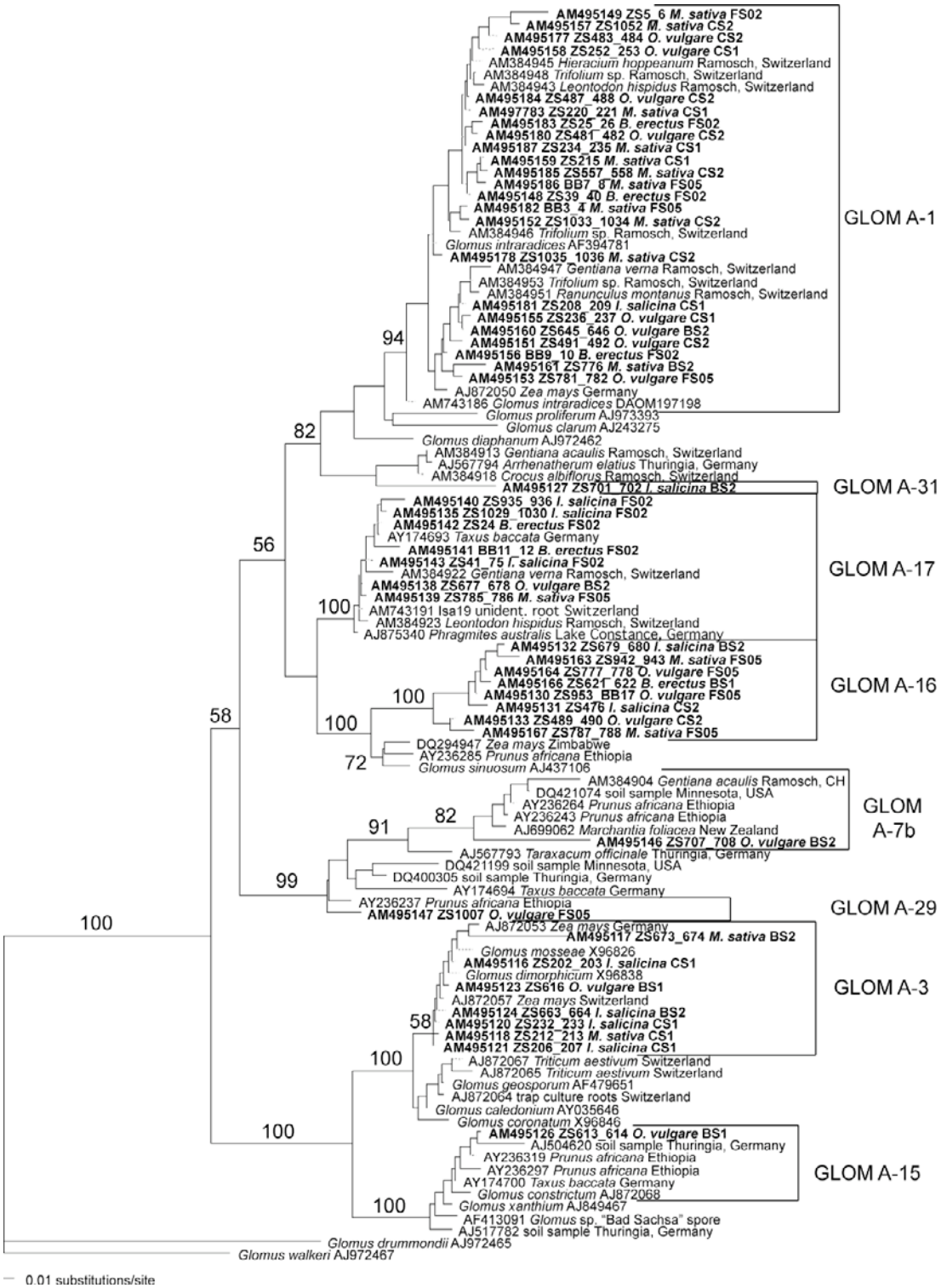

Fig. 12 Phylogenetic tree of *Glomus* group A obtained by neighbor-joining analysis of 387 characters from ITS2 and 5.8S rDNA. Numbers above branches denote neighbor-joining bootstrap values from 1000 replications. The tree was rooted with *Glomus walkeri*. Sequences obtained in the present study are shown in boldface and are labeled like in Fig. 11. The parentheses show the delimitation of the phylotypes.

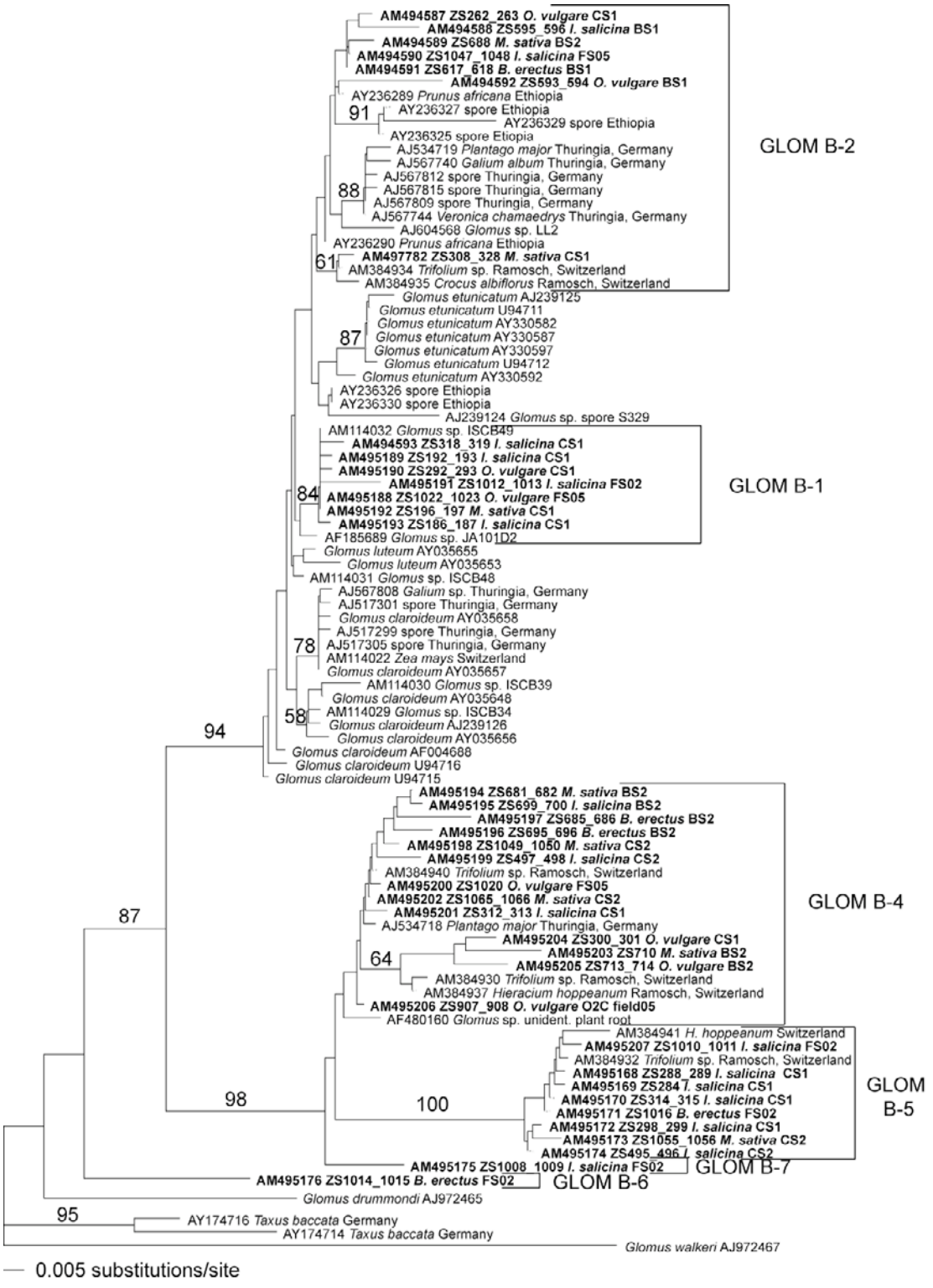

Fig. 13 Phylogenetic tree of *Glomus* group B based on neighbor-joining analysis of 381 characters of ITS2 and 5.8S rDNA sequences. Numbers above branches denote neighbor-joining bootstrap values from 1000 replications. The tree was rooted using *Glomus walkeri*. Sequences obtained in the present study are shown in boldface and are labeled like in Fig. 11. The parentheses show the delimitation of the phylotypes.

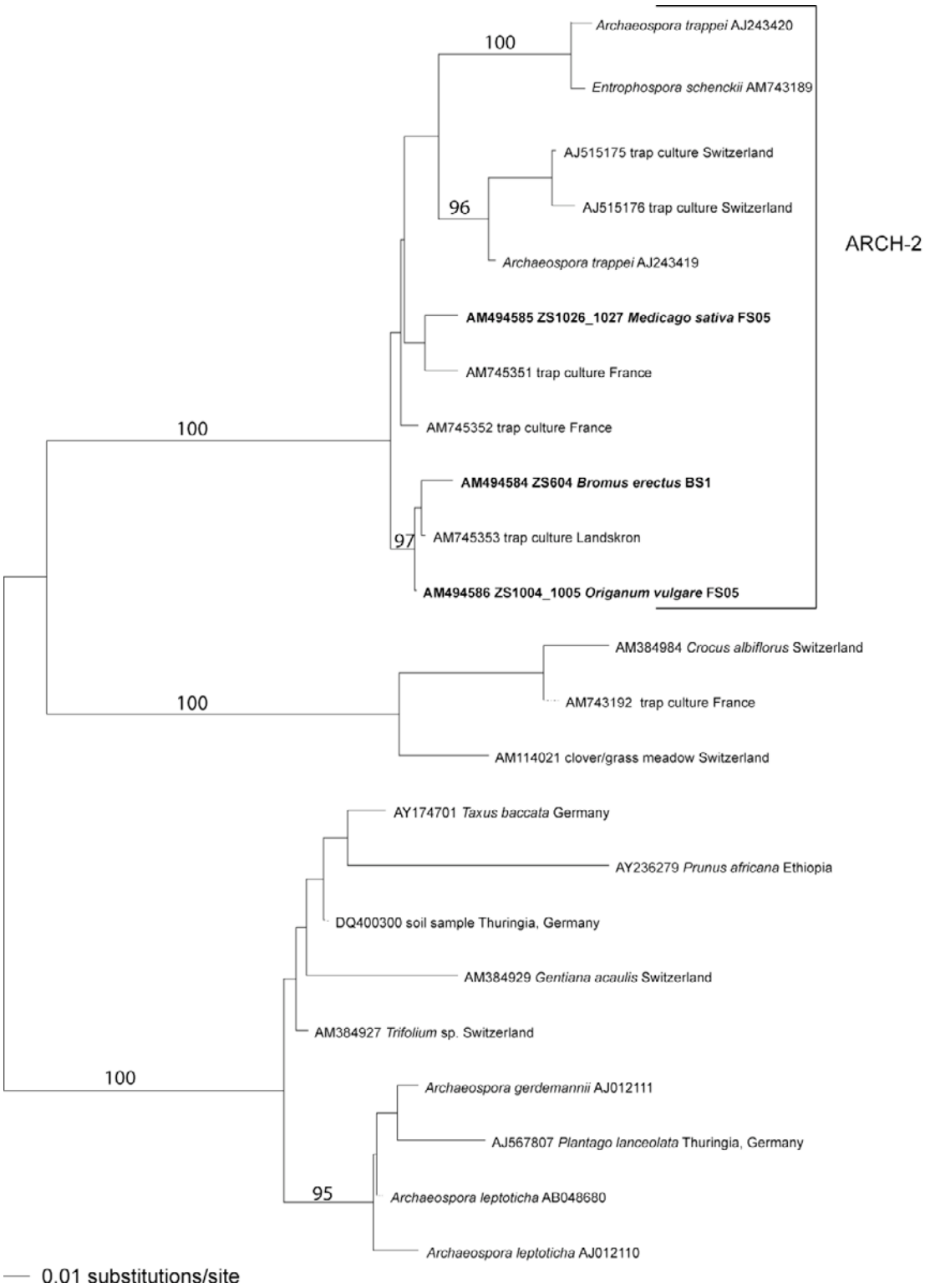

Fig. 14 Midpoint-rooted phylogenetic tree of Archaeosporaceae obtained by neighbor-joining analysis of 328 characters from ITS2 and 5.8S rDNA. Numbers above branches denote neighbor-joining bootstrap values from 1000 replications. Sequences obtained in the present study are shown in boldface and are labeled like in Fig. 11. The parentheses show the delimitation of the sequence types.

Tab. 1 Overview of the clone numbers per sequence type in all analyzed samples yielding at least one AMF sequence type or RFLP pattern. Each line corresponds to a root sample taken from a single plant.

Sample description				Sequence types															
				Glomus group A									*Glomus* group B						Archaeo-sporaceae
Experim. approach	Host plant		Harvest	Glom A-1	Glom A-3	Glom A-7b	Glom A-29	Glom A-31	Glom A-30	Glom A-15	Glom A-16	Glom A-17	Glom B-1	Glom B-2	Glom B-4	Glom B-5	Glom B-6	Glom B-7	Arch-2
Compartment systems	*Inula*	Combination[b] I-O	1st harvest (after 3 months)	5															
				8											5	5			
				2	1										3	5			
				5	2														
				6															
	Origanum			9									9						
				11									2	7					
				11											13				
	Inula	Combination I-M		10	1								7		2				
				10	4								7						
				8	2								5						
					10														
					13								1		7	1			
				5	1								1						
				5	2								9		4				
	Medicago			9											8				
				13															
				12															
					10								2						
				12									1	6					
	Inu -la	Comb. I-O	2nd harvest (after 10 months)	6							5				9	4			
	Origanum			5							4				7	1			
				10															
				8						1					1				
				8							2		5		7				
				9															
	Medicago	Comb. I-M		13												2			
				9											5	1			
				8											5	5			
				12											7				
				9											9				
Field	*Bromus*		July 02	9															
				2					1										
				5															

Sample description			Sequence types															
			Glomus group A									*Glomus* group B						Archaeo-sporaceae
Experim. approach	Host plant	Harvest	Glom A-1	Glom A-3	Glom A-7b	Glom A-29	Glom A-31	Glom A-30	Glom A-15	Glom A-16	Glom A-17	Glom B-1	Glom B-2	Glom B-4	Glom B-5	Glom B-6	Glom B-7	Arch-2
			1							2	4							
			7								1				4	4		
	Inula		4					1										
											10	1			1		1	
			5								1							
	Inula	**July 05**	8								2		8					
	Medica-go	**July 02**	1															
	Medica-go	**July 05**	9								2							
			7							1	1							
			9															1
			7							3								
	Origanum		5							5				1				
			10															
			9			1								10				7
			9							1		10						
			8															
Bait plants	*Inula*-A[c]	**1st harvest (after 3 months)**											4					
	Inula-N			5						1	2							
	Inula-N												2	1				
	Brom.-N									11			13					2
	Brom.-A													1				
	Brom.-A		8															
	Orig.-N										10	3	7					
	Orig.-A		8															
	Orig.-A			8					2					12				
	Medi.-N		6	1														
	Inula-A	**2nd harvest (after 10 months)**	13							2			1		1			
	Inula-N		1	7			1			5			1	9				
	Brom.-N		8									1	2		1			
	Brom.-A		12											2	1			
	Brom.-A													7	2			
	Orig.-A		10											5				
	Orig.-A		7		1						3			8				
	Medi.-A		9	1									5	5				
	Medi.-N		7															
	Medi.-A		8											12				

[a]No number in the cell indicates that no clones of the corresponding phylotype were detected in the root sample.
[b]Compartment systems description: combination of target plants (e.g. I-O corresponds to *Inula-Origanum* combination).
[c]Bait plants description: host plant name is followed by N (non-autoclaved substrate) or A (autoclaved substrate).

Tab. 2 Overview of all sequence types found in our study and their matches to morphologically described AMF species and molecular phylotypes found in some other studies

Sequence type	Morphospecies	Reference examples	Host plant species examples	Ecosystem/Country
GLOM A-1	*Glomus intraradices*	Hijri et al. (2006)	*Zea mays*	Germany, Switzerland
GLOM A-3	*Glomus mosseae*	Hijri et al. (2006)	*Zea mays*	Germany, Switzerland
GLOM A-7b	-	Wubet et al. (2004); Russell & Bulman (2005); Waldrop et al. (unpublished); Sýkorová et al. (2007); Appoloni et al. (submitted)	*Prunus africana, Marchantia foliacea*, soil sample; *Gentiana acaulis; Agrostis scabra, Agrostis stolonifera*	Ethiopia, New Zealand, Minnesota (USA), Switzerland Yellowstone (USA), Iceland
GLOM A-15	*Glomus constrictum*	Hijri et al. (2006); Wubet et al. (2004), Landwehr et al. (2002); Sýkorová et al. (2007)	Pot culture; *Prunus africana*, spore; *Gentiana verna*	Ethiopia, Germany, Switzerland
GLOM A-16	-	-	-	-
GLOM A-17	*Glomus badium*	Wubet et al. (2003); Wirsel (2004); Oehl et al. (2005); Sýkorová et al. (2007)	*Taxus baccata, Phragmites australis*, spores, *Gentiana verna, Leontodon hispidus*	Germany, Switzerland
GLOM A-29	-	Wubet et al. (2003 and 2004); Waldrop et al. (unpublished); Hempel et al. (2007)	*Taxus baccata*, *Prunus africana*, soil sample	Germany, Ethiopia, Minnesota (USA)
GLOM A-30	-	-	-	-
GLOM A-31	-	-	-	-
GLOM B-1	sister group to *G. luteum*	Hijri et al. (2006); Sudarshana et al. (unpublished)	Pot culture	
GLOM B-2	sister group to *G. etunicatum*	Wubet et al. (2004), Börstler et al. (2006); Sýkorová et al. (2007)	*Prunus africana*, spore; *Plantago major, Galium album*, spores; *Crocus albiflorus, Trifolium* sp.	Ethiopia, Germany, Switzerland
GLOM B-4	-	Börstler et al. (2006); Sýkorová et al. (2007); Bidartondo et al. (2002)	*Plantago major, Trifolium* sp., *Hieracium hoppeanum*, unidentified plant root	Germany, Switzerland
GLOM B-5	-	Sýkorová et al. (2007)	*Trifolium* sp., *Hieracium hoppeanum*	Switzerland
GLOM B-6	-	-	-	-
GLOM B-7	-	-	-	-
ARCH-2	*Archaeospora trappei* ?	Hijri et al. (2006); Appoloni et al. (submitted)	Trap cultures; *Agrostis scabra*	Switzerland, Yellowstone (USA)

3.4.2. AMF richness and diversity

The sampling effort curves (Fig. 15a) showed that for the compartment systems (CS), the number of analyzed root samples was sufficient to characterize almost exhaustively the phylotypes present in the roots, as the curve clearly approaches saturation. In contrast, the curves for field samples (FS) and bait plants (BP) are not as clearly saturated but still approaching a plateau. This can be attributed to the higher complexity of the AMF community in these samples and to the lower number of samples analyzed (19 and 20, respectively) compared to the compartment systems (31 samples). To detect one more new phylotype, the analysis of additional 4-5 field or bait plant samples would have been necessary. Species accumulation curves calculated for each plant species across all experimental approaches (Fig. 15b) show the strongest saturation in *M. sativa*, where only nine AMF phylotypes were found.

The observed absolute numbers of phylotypes per root sample were compared using ANOVA. Neither host plant species nor experimental approach nor their interaction had a significant influence on the number of phylotypes/sample (P=0.3; P=0.083 and P=0.07, respectively). The host plant harboring the highest mean number of AMF phylotypes/root sample (2.9) was *I. salicina*, followed by *O. vulgare* (2.6), *B. erectus* (2.3) and *M. sativa* (2.1). The mean number of AMF phylotypes/sample detected in the field was 2.3, whereas it was 2.6 in the bait plant approach and 2.5 in the compartments.

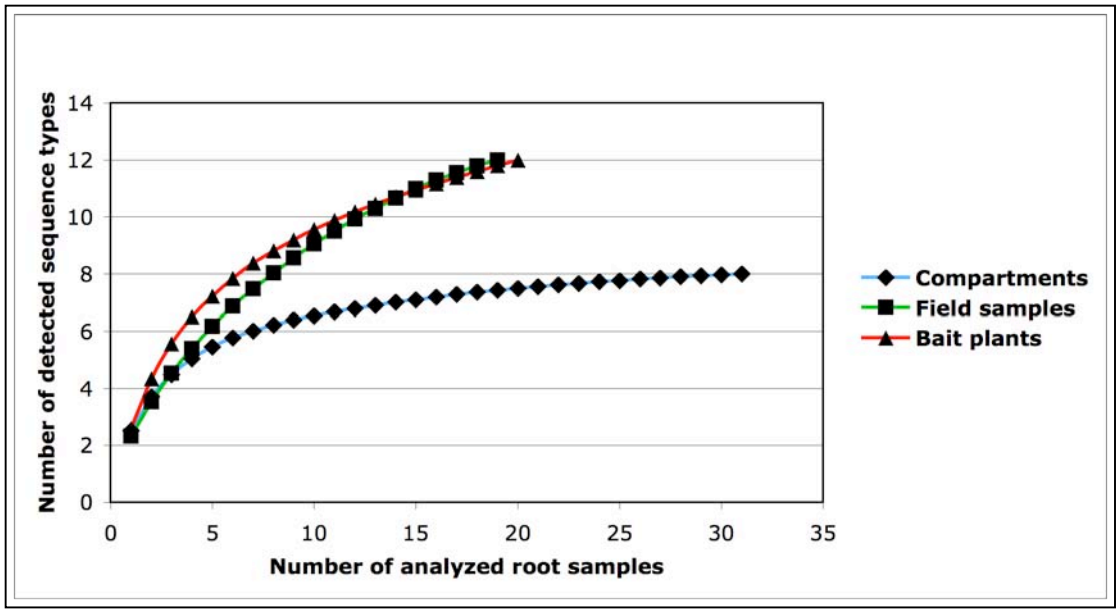

Fig. 15a

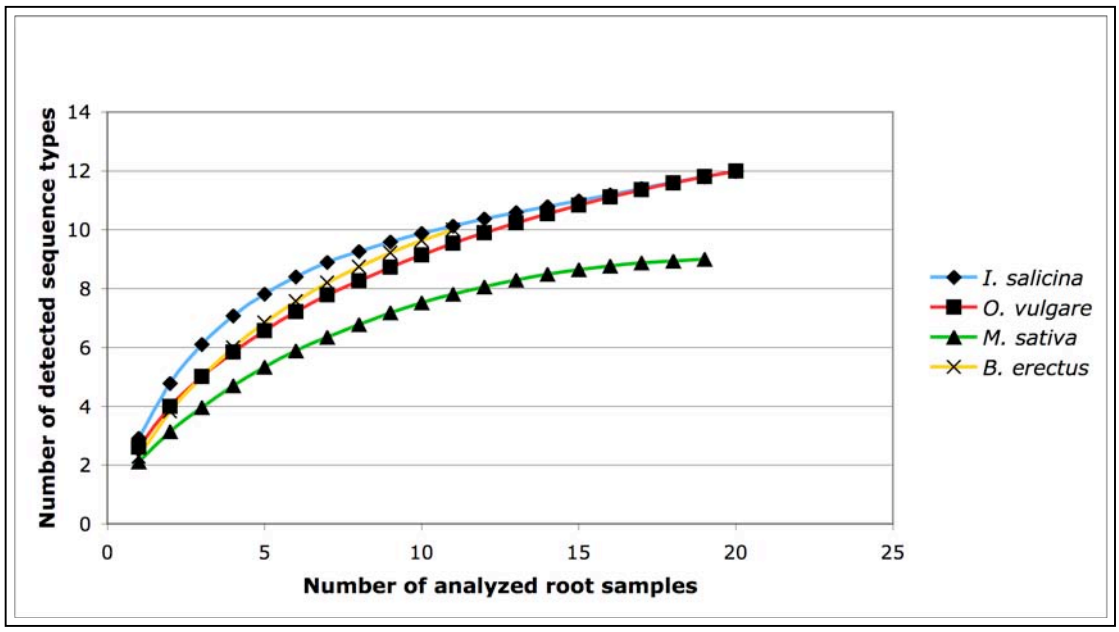

Fig. 15b

Figs. 15a, b Sampling effort curves for **a)** compartment systems (n=31), field samples (n=19) and bait plants (n=20); **b)** for each host plant species: *I. salicina* (n=20), *O. vulgare* (n=20), *M. sativa* (n=19) and *B. erectus* (n=11). The curves were computed analytically in EstimateS 8.0 (Colwell 2005).

3.4.3. AMF community composition in different experimental approaches and host plant species

The influence of the kind of experimental approach and the host plant species (subsequently called “all environmental factors”) on the distribution of AMF phylotypes in the root samples was investigated using a multivariate statistical approach. Phylotypes GLOM A-7B, GLOM A-29, GLOM A-31, GLOM B-6 and GLOM B-7 were excluded from the analysis, because they were detected only once in the whole study.

The initial CCA (Canonical correspondence analysis) performed using all samples revealed that all environmental factors explained 15% of the whole variance and that their effect on the distribution of AMF phylotypes was clearly significant (P=0.002). The forward selection procedure ranked the environmental factors as following: field samples (P=0.002), compartment systems samples (P=0.012) and *O. vulgare* (P=0.028). These results indicate that the root samples originating from field and compartment systems differed significantly from each other and also from the bait plants root samples, and that *O. vulgare* differed from samples originating from all other host plants. The influence of other host plant species on the distribution of the phylotypes was not significant. The variance partitioning showed that the experimental approach accounted for 63% of the variance explained by all environmental factors, whereas the host plant species accounted only for 34%. The remaining 3% was explained by the correlation of both groups of factors.

The biplot diagram of this CCA (Fig. 16) also demonstrates these results: the centroids representing the three experimental approaches are distant from each other, forming a triangle, which demonstrates that the roots contained distinct AMF. In contrast, the centroids representing the host plant species are located inside in this triangle (except for *O. vulgare*) indicating that they hosted more similar AMF communities. The location of the phylotypes in the plot (Fig. 16) indicates in which experimental system they were detected. The relative abundance of each phylotype (Tab. 1) also contributed to its position in the plot. GLOM A-3, for instance, was detected only in CS and BP, GLOM A-17 and ARCH-2 in FS and BP. Most of the remaining phylotypes were present in all three experimental approaches, but their relative abundance differed: GLOM B-4, for instance, was present in 50% of samples from CS and BP, but only in 11% of the samples from FS. GLOM B-1 occurred in 35% of CS samples but only in 10% of FS and BP samples. GLOM A-16 was found in only 10% of the CS samples, but in 25-26% of the FS and BP samples. For the abundance of the remaining phylotypes see Tab. 1. The only phylotype present at relatively high abundance in all three experimental approaches was GLOM A-1.

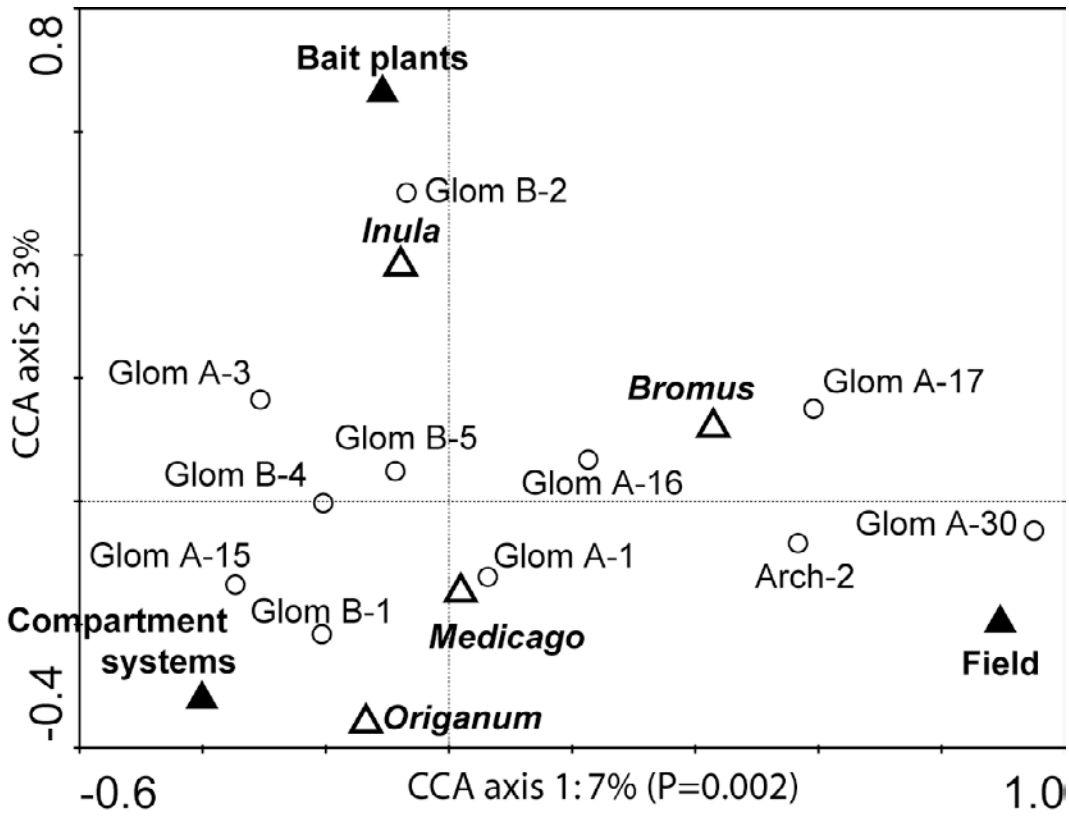

Fig. 16 CCA biplot of the phylotypes and environmental factors (using Hill's scaling focused on inter-species distances) of all samples from all three experimental approaches. Only phylotypes that occurred more than once in the whole study were included in the analysis. The three experimental approaches are represented by filled triangles, host plant species by open triangles and phylotypes by circles. The 1st axis accounted for 42,9% of the variability explained by all canonical axes and was significant (P=0.002). The percentages shown by 1st and 2nd axis correspond to the percentage of variance of AMF phylotypes data explained by the particular axis.

3.4.4. Field samples – effect of host plant species and sampling year

A CCA was also performed with data from field samples only (Fig. 17). It revealed that the host plants and the two sampling years as environmental factors explained 30% of the whole variance and their effect was not significant at the P=0.05 level (P=0.066). The variance partitioning method revealed that the influence of the host plant species (66%) on the AMF community composition was about twice the influence of the sampling year (29%). The only variable with significant influence according to the forward selection procedure was *O. vulgare* (P=0.032), probably due to specific presence of GLOM B-4 and absence of GLOM A-17, which was present in all remaining host plant species. These CCA results unfortunately are biased by the unequal numbers of samples per host plant species from each sampling year (see also CCA biplot in Fig. 17 and Tab. 1) and therefore by strong correlations. Furthermore, when the two sampling years were considered as the only environmental factors, their influence was significant (P=0.024), which may also be caused by the specific presence of *O. vulgare* samples only in the harvest from the year 2005.

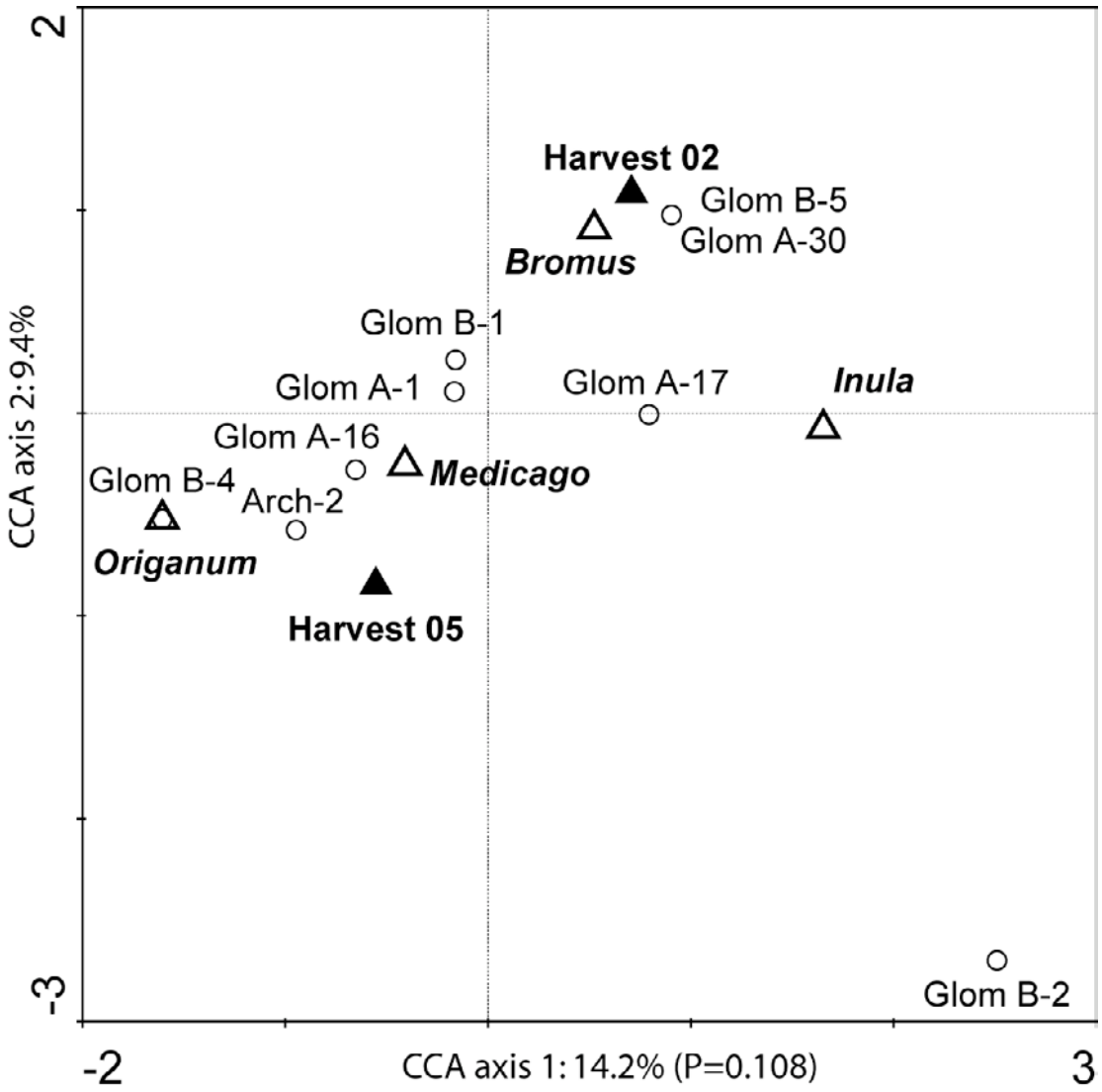

Fig. 17 CCA biplot of sequence types and environmental factors (using Hill's scaling focused on inter-species distances) of the data from the field. Harvests are represented by filled triangles, host plant species by open triangles and sequence types by circles. The 1st axis accounted for 48% of the variability explained by all canonical axes and was not significant (P=0.108). The percentages shown by 1st and 2nd axis correspond to the percentage of variance of AMF sequence types data explained by the particular axis.

3.4.5. Compartment systems: influence of host plant species, plant species combination and the duration of cultivation

A CCA was also performed with data from compartments systems only (Fig. 18). All environmental factors accounted for 29% of the whole variance in the CS samples and their effect was clearly significant (P=0.002). The forward selection procedure revealed the cultivation duration (P=0.002) as a significant factor. The host plant species *I. salicina* was not significant (P=0.062). Variance partitioning showed that from the variance explained by all environmental factors, the cultivation duration in fact accounted for 47%, the host plant species accounted for 39%, and the plant species combination in the CS (*I. salicina/O. vulgare* or *I. salicina/M. sativa*) explained only 14%. The influence of host plant species was significant (P=0.006) considering the other factors as covariables, but not significant (P=0.134) excluding other factors. Fig. 18 clearly shows that the centroids of the two harvests are located on the first canonical axis (P=0.002) far apart from each other, whereas the centroids of the host plant species are distributed along the second canonical axis (vertically) with *I. salicina* located far from the other two host plant species. The centroids of the plant combinations are in the middle of the biplot indicating that both combinations hosted similar AMF communities.

Interestingly, the phylotypes GLOM A-3 and GLOM B-2 were present only in samples form the first harvest and the abundance of GLOM B-1 sharply dropped from the first to the second harvest, indicating that these phylotypes could be the fastest colonizers of a new niche, but disappeared later. In contrast, phylotypes GLOM B-5 and GLOM A-16 were more often or exclusively detected in samples from the second harvest, which suggests that these are AMF typical for older, more mature ecosystems Interestingly, they were also detected in the field samples and bait plants. Other phylotypes like GLOM A-1 or GLOM B-4 were present in samples from both harvests more or less equally, indicating their generalist character.

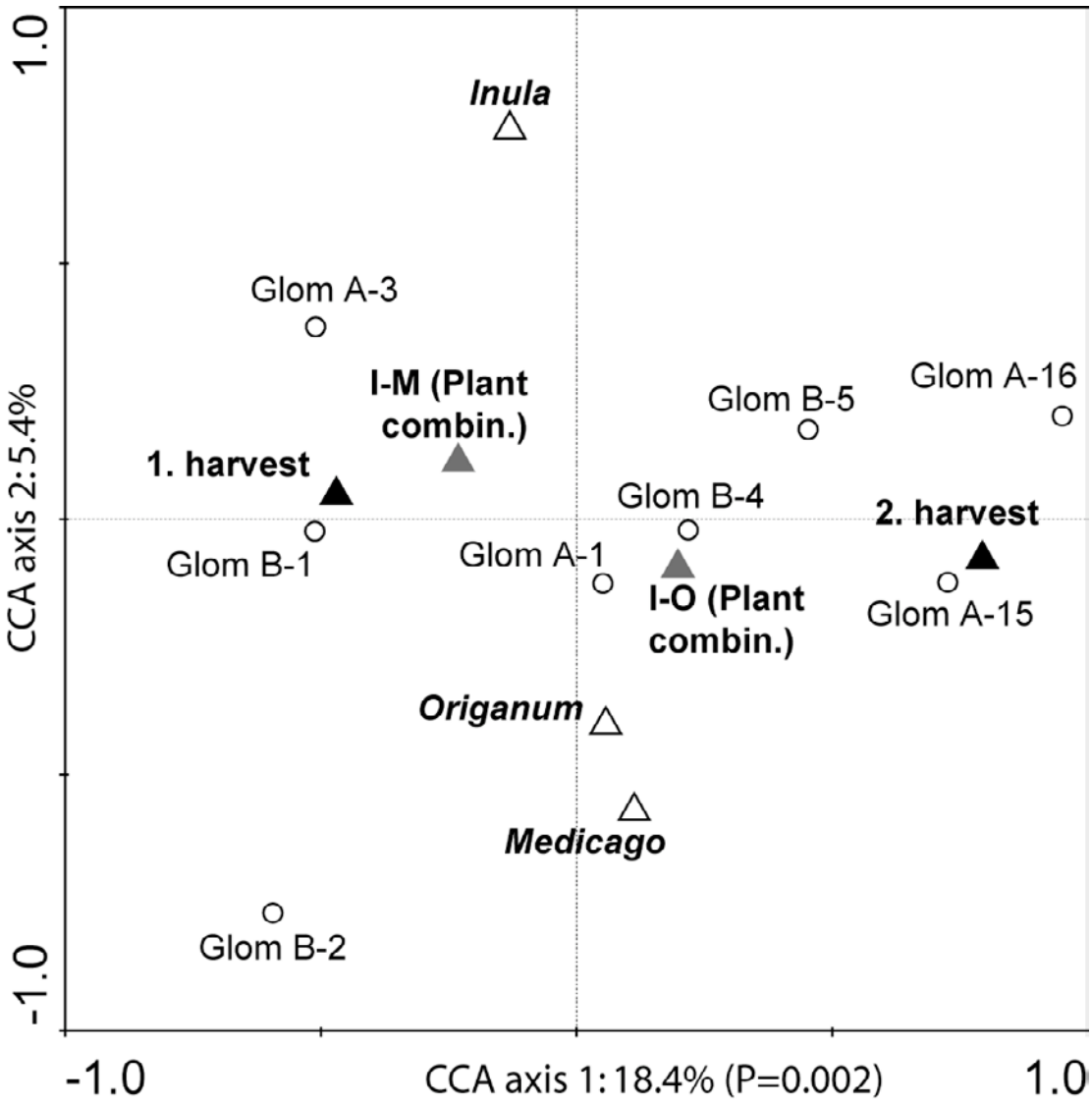

Fig. 18 CCA biplot of sequence types and environmental factors (using Hill's scaling focused on inter-species distances) of the data from the compartment systems. Harvests are represented by filled black triangles, plant combinations by filled grey triangles, host plant species by open triangles and sequence types by circles. The 1st axis accounted for 63% of the variability explained by all canonical axes and was significant (P=0.002). The percentages shown by 1st and 2nd axis correspond to the percentage of variance of AMF sequence types data explained by the particular axis.

3.4.6. Bait plants – influence of host plant species, duration of cultivation and substrate treatment

The initial growth of the plantlets was more vigorous in the non-autoclaved substrate than in autoclaved soil (data not shown), which may be caused by the toxic ions released during the autoclaving process. After the transplantation into the field, the bait plants were generally thriving, except for *M. sativa*, which showed limited growth, with few roots; three plants even died until the second harvest.

A CCA was performed with the bait plant samples only (for biplot see Fig. 19). It showed that all environmental factors explained 35% of the whole variance and their effect was significant (P=0.01). The forward selection identified the substrate type (P=0.03) and the host plant *B. erectus* (P=0.042) as significant factors. The variance partitioning revealed that the host plant species explained 58% of the variance explained by all environmental factors, the substrate type accounted for 19% and the cultivation duration explained only 13%. Ten percent was explained by correlations of these factors. When considered alone, the influence of the substrate and the host plants was significant.

An interesting phenomenon was the species richness per sample in the two different substrates: in the non-autoclaved treatment, there were substantially more phylotypes per sample already after the first harvest, with an average of 2.6 phylotypes/sample in comparison to 1.4 in the autoclaved treatment. The same was observed after the second harvest (3.7 versus 3.0). Overall, the samples from the second harvest were slightly higher in species richness (10 phylotypes detected) than samples from the first one (9 phylotypes detected, Tab. 1). The overall number of phylotypes in the autoclaved substrate (10) did not differ from the non-autoclaved soil.

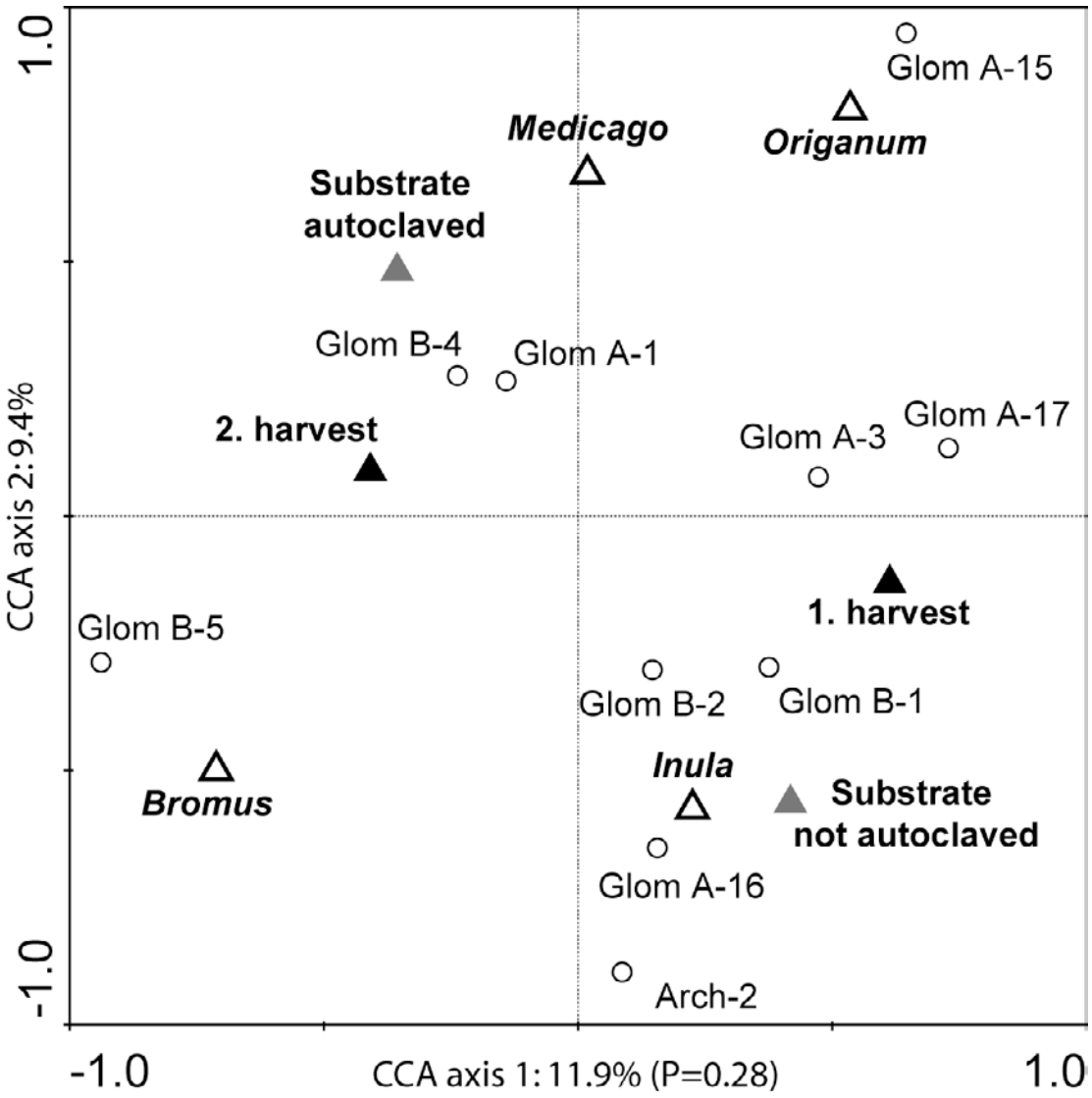

Fig. 19 CCA biplot of sequence types and environmental factors (using Hill's scaling focused on inter-species distances) of the data from the bait plants. Harvests are represented by filled black triangles, original substrate (autoclaved or not-autoclaved) by filled grey triangles, host plant species by open triangles and sequence types by circles. The 1st axis accounted for 34% of the variability explained by all canonical axes and was not significant (P=0.28). The percentages shown by 1st and 2nd axis correspond to the percentage of variance of AMF sequence types data explained by the particular axis.

3.5. Discussion

To our knowledge, this is the first study specifically addressing the influence of culturing methods on AMF community diversity using molecular methods. Our results demonstrate that the culturing techniques we used had a much stronger influence on AMF communities in the roots than host specificity.

Generally, the number of twelve phylotypes found in our field site is within the range of 10-24 phylotypes found by other authors applying molecular methods in temperate grasslands (Öpik et al. 2006). However, this number is considerably lower than the 24 morphospecies found using the microscopic investigation of spore morphology in the same field site (Oehl et al. 2003). It should be emphasized that spore-based methods and root-based molecular analysis characterize two different but related parameters of the soil biota: the spores reflect the inoculum potential which may be rather long-lived and usually does not perfectly correspond to the momentarily active fungal community within the roots (Renker et al. 2005; Börstler et al. 2006; Hempel et al. 2007) which is characterized by molecular methods.

Another possible reason for the lower apparent diversity we detected was the fact that we focused on only four out of at least 60 potential host plant species in the site. This subset of taxa may not harbor the whole AMF community of this field site. Some degree of host preference of different host plant species has been reported (e.g. Vandenkoornhuyse et al. 2003; Gollotte et al. 2004) and in another field site we showed that the diversity of detected AMF phylotypes increased with the number of plant species analyzed (Sýkorová et al. 2007). Similar to the present study, Oehl et al. (2003) did not find any species of the Acaulosporaceae in the field site and the respective trap cultures. *Scutellospora calospora*, a member of the Gigasporaceae, which were not detected at all by molecular methods, was among the rarest morphospecies in the spore-based study and the only representative of its family. Notably, the relatively high number of six phylotypes from *Glomus* group B in the present study exceeds the local diversity detected for this group in any previously published study.

Many studies based on spore morphology have demonstrated that not necessarily the same AMF morphospecies are found in a field site and in greenhouse cultures set up using soil from this site ("trap cultures", Jansa et al. 2002; Oehl et al. 2003; Oehl et al. 2004), although there is usually a considerable overlap between the two species groups. The period of time the cultures are grown also appears to be important, as some species only sporulate after extended cultivation, e.g. 20 months (Oehl et al. 2004).

The fungi sporulating early in trap cultures could potentially be representatives of the r strategy (Pianka 1970), which dominate resource-rich uncolonized habitats in early successional stages of the fungal community. K strategists would follow the opposite strategy of slow growth under resource-limited conditions and occurrence in late successional stages. However, it is difficult to draw direct conclusions about the life history strategy of the fungi detected by their spores as differences in sporulation behavior may conceal these characteristics. Although r strategists typically invest heavily into their reproduction, it is possible that some r strategists are not prolific sporulators. Moreover, species abundantly producing spores in the field or in the greenhouse do not always dominate the AMF community in the field roots (e.g. Ahulu et al. 2006).

Molecular studies have demonstrated differences in AMF communities in the roots between natural/seminatural and arable/disturbed sites, but attributed these differences mainly to environmental factors like high nutrient concentration, ploughing, fertilizer and fungicide input as well as low crop diversity or crop rotation in arable sites (Helgason et al. 1998; Daniell et al. 2001; Jansa et al. 2002; Hijri et al. 2006). As a form of recurring disturbance, ploughing was identified as factor potentially affecting AMF communities, but succession in AMF communities was not addressed in this context.

The analysis of the distribution of the phylotypes across culturing approaches and different harvesting times revealed some highly interesting patterns (see Tab. 3). Most strikingly, GLOM A-3 (*G. mosseae*) was never detected in FS, but it occurred in 25% of the BP and 50% of the samples of the first harvest of the CS. Apparently, it later disappeared from the CS, most likely displaced by other fungi throughout the succession in the system. The presence of the spores of this morphospecies in the field site was already reported by Oehl et al. (2003), confirming that it was present predominantly as inoculum that could colonize the roots of BP and CS. These data strongly suggest that *G. mosseae* is a typical early-stage colonizer and an r strategist adapted to disturbed systems. This life history strategy is consistent with its occurrence in arable soils (Helgason et al. 1998; Daniell et al. 2001; Hijri et al. 2006), where it has to be adapted to frequent soil disturbance and low host plant diversity and therefore faces similar environmental conditions like in CS and BP.

Showing the opposite trend, GLOM-A-17 (*Glomus badium)* was never found in the CS, but was occasionally found in the BP and frequently in the FS. It was previously detected in the field site by an approach based on spore morphology (Oehl et al. 2003) and was reported to be widespread in European grasslands (Oehl et al. 2005), which is consistent with our observations of its preference for undisturbed systems. Similarly, GLOM-A-16 occurred frequently in FS and BP, but was not found in the first harvest of the CS. ARCH-2 was never found in CS, but occasionally in the FS and BP. We conclude from the data that these

phylotypes preferentially occur in more mature root/soil ecosystems and later stages of succession. As the competition for nutrient resources can be expected to increase under these conditions we suggest they can be classified as K strategists, though to different extents.

The occurrence patterns of other phylotypes were not as striking but still showed a tendency to preferentially occur in either cultivated or natural environments (see Tab. 3). For instance, phylotypes GLOM A-15 (*G. constrictum*), GLOM B-1 (sister group of *G. luteum*), GLOM B-2 (sister group of *G. etunicatum*), GLOM B-4 and GLOM B-5 occurred predominantly in CS and BP, which indicates their ecological preferences for early successional stages. However, it should be noted that there were also apparent generalists exemplified by *G. intraradices*, which was the most frequently detected phylotype in all systems. Several phylotypes occurred only once, therefore not allowing to assign them reliably.

The BP approach revealed an equally high diversity of AMF phylotypes as the FS. It detected both phylotypes predominantly present in FS (like GLOM A-17, GLOM A-16 or ARCH-2) and in CS (GLOM A-3, GLOM B-4 or GLOM B-5). Therefore, this approach seems to be useful to study the diversity of both AMF actively colonizing roots and present as inoculum in the field, and represents a valuable tool to evaluate the influence of different environmental factors on AMF community composition directly under field conditions. If using the trap culture approach to evaluate AMF diversity, long-term cultivation is advisable to minimize the possible exclusion of AMF appearing late in succession. When samples are taken from plants naturally growing in the field, a broad range of host plant species should be sampled to avoid possible effects of host preference (Sýkorová et al. 2007).

For a better understanding of the dynamics of AMF communities it will be necessary to classify AMF phylotypes and species according to their life history strategies. The present study provides some first steps in this direction. Our findings also emphasize that in short-term greenhouse experiments only a certain subset of AMF species, mainly comprising r strategists, is colonizing roots. This succession in the system is particularly important to consider for planning, setting up and inoculating experiments using multispecies AMF consortia.

Tab. 3 Relative abundance of the sequence types in root samples from different experimental approaches (calculated as % from presence/absence data of each sequence type in each root sample)

Sequence type	Relative abundance in the compartment systems (% of samples)		Relative abundance in the bait plants (% of samples)		Relative abundance in the field (% of samples)
	1st harvest	2nd harvest	1st harvest	2nd harvest	
GLOM A-1	85	100	30	100	95
GLOM A-3	50	0	30	20	0
GLOM A-7b	0	0	0	10	0
GLOM A-29	0	0	0	0	5
GLOM A-31	0	0	0	10	0
GLOM A-30	0	0	0	0	11
GLOM A-15	0	9	10	0	0
GLOM A-16	0	27	20	20	26
GLOM A-17	0	0	20	10	37
GLOM B-1	50	9	10	10	11
GLOM B-2	10	0	40	40	5
GLOM B-4	35	73	30	70	11
GLOM B-5	15	45	0	40	11
GLOM B-6	0	0	0	0	5
GLOM B-7	0	0	0	0	5
ARCH-2	0	0	10	0	11

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Appendix 1

Sebacinales - an order of basidiomycetes with a uniquely wide spectrum of mycorrhizal types - are co-occurring with arbuscular mycorrhizal fungi in plant root samples

(This preliminary study was done in cooperation with Michael Weiß from the University of Tübingen; some analyses shown in this chapter were conducted by Susann Appoloni during her MSc thesis.)

A1.1. Summary

Sebacinales are an order of the Basidiomycota recently established by Weiß et al. (2004). Its members are widely distributed and known to form various types of mycorrhizal associations of different morphology (ecto-, ectendo-, orchid, ericoid, jungermannoid) with a broad range of host plant species (Selosse et al. 2002a, Selosse et al. 2002b, Kottke et al. 2003, Setaro et al. 2006, Selosse et al. 2007). Only a limited number of pure cultures of the Sebacinales are available, which causes a problem for the construction of molecular markers and for the implementation of manipulative experiments. One of the species, which is axenically cultivable and therefore being investigated as a model organism of the Sebacinales, is *Piriformospora indica* (Verma et al. 1998). It associates with AMF host plants and promotes their growth and resistance to fungal diseases (Varma et al. 1999). In contrast to AMF, preliminary data suggest that *P. indica* requires host cell death for the proliferation in the roots (Deshmukh et al. 2006).

In this study, we used Sebacinales-specific primers for the internal transcribed spacer (ITS) 2 to amplify and sequence the D1/D2 regions of the nuclear 28S rDNA subunit. We analyzed root DNA extracts originating from seventeen different AMF-host plant species from several ecosystems with different human impact in Europe and North America. Our results show that fungi from the order Sebacinales were present in each but two sites in the root samples of fourteen plant species tested, where they co-existed with the Glomeromycota. All but one sequence belonged to the subgroup B (according to Weiß et al. 2004), where they clustered with sequences from the Ericaceae, liverworts, *P. indica* and green orchids. In agreement with the results of a study investigating the presence of the Sebacinales in the Ericaceae (Selosse et al. 2007), the phylogenetic analysis of our sebacinoid sequences did not

reveal any patterns according to their host plant species or geographical origin. Nevertheless, to confirm these results, the analysis of a larger sample set from larger number of host plant species originating from more field sites would be advisable. The aspect of functional interaction between the Sebacinales and their host plants (e.g. nutrient transfer) was not addressed in this work and remains to be investigated in future studies.

A1.2. Introduction

A1.2.1. Systematic position of the order Sebacinales within the kingdom Fungi

The order Sebacinales was established recently by Weiß et al. (2004) using molecular methods. These authors revealed that Sebacinales occupy a basal position within the Hymenomycetidae, which is a subclass of Hymenomycetes (i.e. Agaricomycotina), with *Geastrum* as a sister group (Fig. 1). Agaricomycotina, Ustilaginomycotina and Pucciniomycotina are the three subphyla of the phylum Basidiomycota (James et al. 2006, Hibbett et al. 2007). The subphylum Agaricomycotina includes almost two-thirds of known members of all Basidiomycota, including the vast majority of mushroom-forming fungi, but also jelly fungi and yeasts.

Previously, the phylum Basidiomycota was divided into two classes: Homo- and Heterobasidiomycetes (Oberwinkler 1982, Hibbett 2001). Heterobasidiomycetes were characterized by basidiospores capable of forming secondary spores and septate or aseptate basidia; whereas Homobasidiomycetes have aseptate basidia and basidiospores exclusively germinating directly. The Sebacinales were placed in the Heterobasidiomycetes (Weiß et al. 2004). This classification turned out to be artificial with Heterobasidiomycetes being paraphyletic. The classification into three subphyla mentioned above is now widely accepted. Nevertheless, the terms Homo- and Heterobasidiomycetes are still in use.

The findings of Weiß et al. (2004) suggest that the Sebacinales are the most basal group in the Basidiomycota with mycorrhizal members. Early diverging lineages in the Agaricomycotina include parasitic and saprotrophic fungi. The mycorrhizal basidiomycetes seem to have multiple, independent evolutionary origins from these saprotrophic ancestors (James et al. 2006). In contrast, Selosse et al. (2002b) and Weiß et al. (2004) proposed the hypothesis that the common ancestor of the Hymenomycetidae was mycorrhizal. In this hypothesis, the distribution of mycorrhizal taxa within the basidiomycetes could be explained by multiple independent origins of saprotrophism rather than by convergent evolution of mycorrhizas.

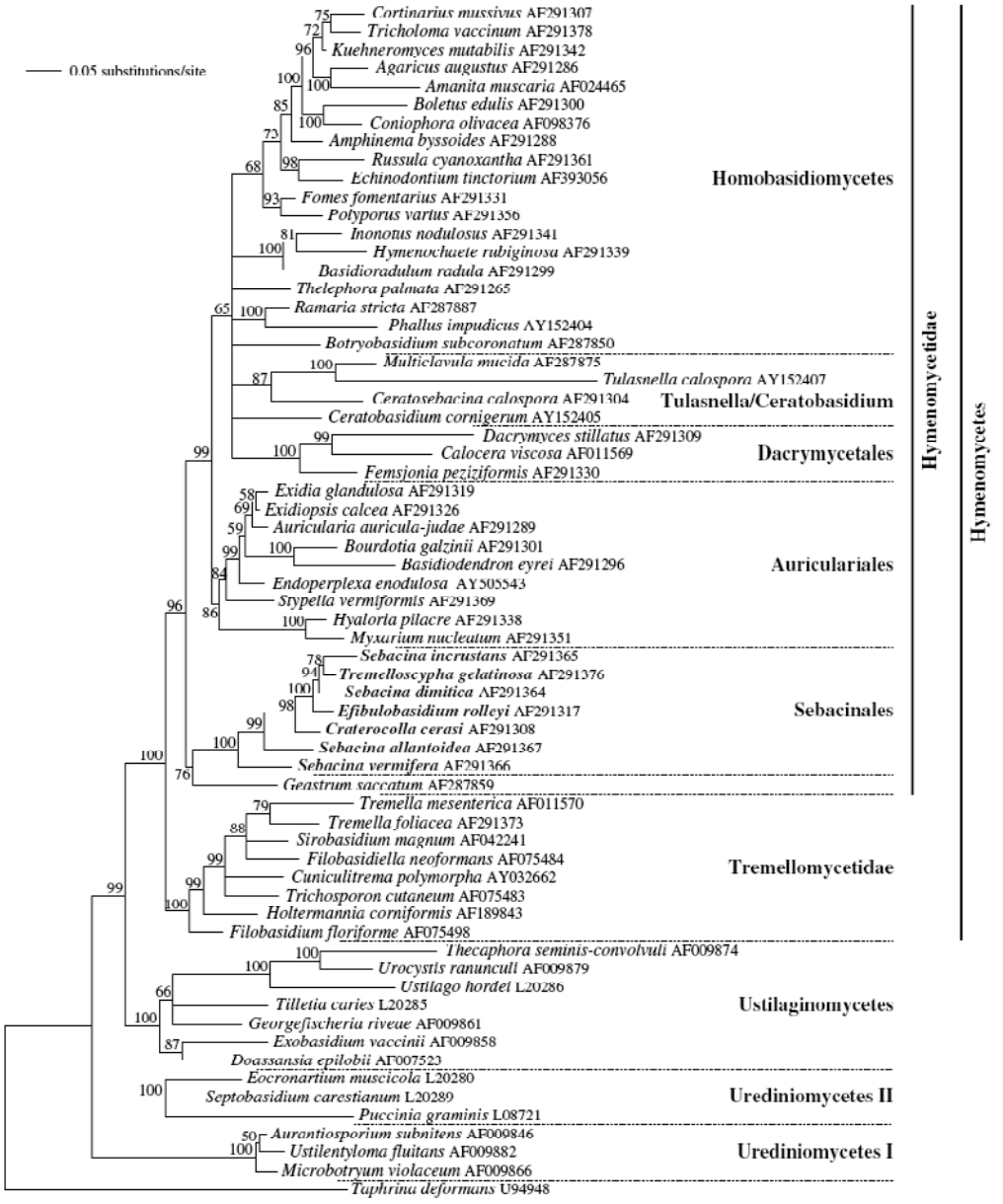

Fig. 1 Phylogenetic placement of the Sebacinales within the basidiomycetes. From Weiß et al. (2004)

A1.2.2. The order Sebacinales: morphology and known genera

Some members of Sebacinales form inconspicuous basidiomes (e.g. in the form of a crust on forest litter, Fig. 2) and were previously considered as saprophytes or parasites. They were placed into the order Auriculariales on the basis of their ultrastructural and microscopic features (Bandoni 1984). However, using molecular analyses of the phylogenetic relationships in Auriculariales, Weiß and Oberwinkler (2001) showed that the Sebacinales do not belong to the Auriculariales.

Weiß et al. (2004) defined Sebacinales morphologically by the combination of longitudinally septate basidia, imperforate parenthosomes (i.e. a derivative of the endoplasmic reticulum) surrounding the septal pores (Fig. 3) and both the lack of clamp connections (structures formed during cytokinesis on some basidiomycetous hyphae) and cystidia (large sterile cells in the hymenium of a basidiomycete between clusters of basidia). Only a few genera are known from this order: e.g. *Sebacina*, *Tremellodendron*, *Efibulobasidium*, *Tremelloscypha*.

Fig. 2 Basidiome of *Sebacina* cf. *incrustans*. Photo by D. Redecker

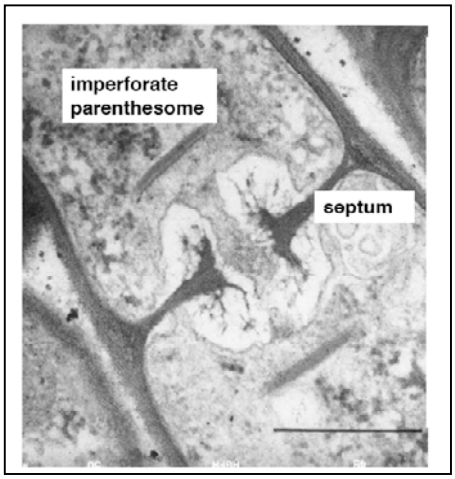

Fig. 3 Dolipore with straight, imperforate parenthosome of a sebacinoid fungus. From Kottke et al. (2003)

Interestingly, a recently described root-endophytic fungus *Piriformospora indica* (Verma et al. 1998) also belongs to the Sebacinales (Weiß et al. 2004). This fungus was discovered in an arbuscular mycorrhizal spore from desert soil in India and was shown to provide strong host plant growth-promoting activity during its symbiosis with a broad spectrum of AMF host plants (Verma et al. 1998). Furthermore, it induced plant resistance to fungal diseases and tolerance to salt stress (Waller et al. 2005). In contrast to many *Sebacina* spp., *P. indica* can be easily propagated on various media in the absence of a host plant.

Rhizoctonia is a highly polyphyletic form genus encompassing various asexual fungal stages and thought to form orchid mycorrhizas (Roberts 1999). Molecular analysis placed one of the *Rhizoctonia* isolates of Williams (1985) to Sebacinales close to *P. indica* among isolates of the *S. vermifera* complex (Weiß et al. 2004). Although fungi with *Rhizoctonia* anamorphs are known to be difficult to get to produce sexual stages, some of the isolates produce teleomorphs belonging to the *Sebacina vermifera* species complex.

The lack of useful macro- and microscopic characters causes a problem for an accurate morphological delimitation of species or even genera. Moreover, only a limited number of pure cultures of *Sebacina* spp. are available (mainly from the *S. vermifera* complex, Warcup 1988), which might be due to a strictly symbiotic life strategy. As a consequence, only a limited number of the sequences of morphologically-defined sebacinoid species are available. However, an increasing number of sebacinoid sequences obtained from environmental root samples (e.g. Setaro et al. 2006) indicates that the Sebacinales may be a larger taxon than previously thought, containing many still undescribed species.

Using ITS and 28S rDNA sequences, some authors observed different (Setaro et al. 2006) or homogeneous (Selosse et al. 2007) sebacinoids in a single root sample. However, it is not clear whether this represents inter- or intraspecific variation (Selosse et al. 2007). Therefore, molecular species delimitation is so far unclear. In addition, the genus *Sebacina* sp., as currently defined morphologically, is not monophyletic (Weiß et al. 2004). In conclusion, more morphological, anatomical and molecular investigations of the Sebacinales are necessary.

A1.2.3. Broad diversity of mycorrhizal strategies within the Sebacinales

Recently, many ribosomal DNA sequences from plant roots were published that can be assigned to the Sebacinales, which indicates that they may be involved in a wide spectrum of mycorrhizal types. They have been detected to build ectomycorrhizas (Selosse et al. 2002a), orchid mycorrhizas (Warcup 1988, Selosse et al. 2002b), ericoid mycorrhizas (Allen et al. 2003, Selosse et al. 2007), jungermannoid mycorrhizas (Kottke et al. 2003) and

ectendomycorrhizas (Setaro et al. 2006). This broad diversity of mycorrhizal strategies present in the Sebacinales is unique.

The morphology of symbiotic structures of the Sebacinales differs according to the type of mycorrhiza they form in a particular plant species. In ectendomycorrhizas (the cavendishioid, Setaro et al. 2006; and arbutoid type, Selosse et al. 2007), the Sebacinales intraradically build fine intercellular hyphae and large intracellular hyphal coils, whereas on the root surface, they form a hyphal sheath consisting of thin septate hyphae. In ectomycorrhizas, these fungi form true ectomycorrhizae with hyphal mantle and Hartig net (Selosse et al. 2002a) and in orchid roots they form intracellular coils (Selosse et al. 2002b). *P. indica* forms pear-shaped chlamydospores in the root hairs. It grows inter- and intracellularly in the root cortex forming coils, branches and round bodies, but does not enter the central cylinder of the AMF host plants (Varma et al. 1999, Waller et al. 2005). Preliminary data suggest that in contrast to AMF, *P. indica* requires host cell death for its proliferation in the roots (Deshmukh et al. 2006). However, the main part of the root develops further and is not necrotized.

Within the order Sebacinales, Weiß et al. (2004) distinguished two subgroups using the D1/D2 region of the nuclear 28S LSU rDNA. Subgroup A contains sequences from basidiomes (fruitbodies), from ectomycorrhizas and heterotrophic orchids. Subgroup B contains sequences from axenic cultures of *Sebacina vermifera* originating from roots of green autotrophic orchids, sequences from ericoid mycorrhizas, liverwort rhizoids and *P. indica*. Interestingly, achlorophyllous orchids (*Neottia nidus-avis*) showed high specificity, being associated with Sebacinales from the subgroup A (Selosse et al. 2002b), whereas green orchids associated with Sebacinales from the subgroup B and also with other fungi (Shefferson et al. 2005).

Surprisingly, apart from *P. indica*, there is only sporadic information on the association of the Sebacinales with AMF-host plant species or their interaction with AMF. Williams (1985) observed an endophytic association of a multinucleate rhizoctonia with grasses as well as legumes in several pot cultures of AMF and obtained rhizoctonia isolates from AMF spores, vesicles, or colonized roots. Similar isolates were obtained in Australia from diverse herbaceous host plants in the field or by baiting the soil with *Lolium perenne* and *Trifolium subterraneum* in greenhouse pots (Milligan and Williams 1987). These findings can be interpreted in the way that rhizoctonias might be saprophytes growing as opportunists on decaying mycorrhizas or even as parasites of mycorrhizal fungi. Interestingly, inoculation with these rhizoctonias affected plant growth only in the presence of AMF (Williams 1985).

The aim of this work was to elucidate whether the fungi from the order Sebacinales i) are also present in the roots of herbs known to form arbuscular mycorrhizal symbiosis; ii)

show any specificity or preferences towards their host plants; iii) show any distribution patterns according to their geographic origin.

A1.3. Material and methods

A1.3.1. Root sampling and DNA extraction

Some root samples collected for AM fungal community analyses (see Chapters 2 and 3) were also used for the investigations of the presence of Sebacinales. For field site characteristics, sampling details and DNA extraction procedure see Chapters 2.3 and 3.3. Furthermore, samples originating from following agriculturally managed field sites were analyzed: from the DOK experiment in Basel-Land, Switzerland; a maize monoculture field near Rheinweiler, Germany; and a leek field in Muri, Switzerland (for field site details, agricultural practice, sampling description and explanation of sample labeling see Hijri et al. 2006). Additionally, Susann Appoloni analyzed several samples originating from thermal sites in Yellowstone National Park, USA and from Iceland (Appoloni 2006). An overview of all analyzed samples is provided in Tab. 1.

Tab. 1 Overview of all root samples analyzed for the presence of the Sebacinales. The column "PCR product in the 2nd nested PCR" represents the results of nested PCR reactions with the primer pairs ITS1F/TW14 and Seb3ITS/NL4.

Sample origin	Root sample description: plant species (internal code)	PCR product in the 2nd nested PCR: yes (Y)/ no (N)*	Ligation number	Number of clones screened	No of different RFLP patterns after the digestion with enzymes *Hinf*I and *Mbo*I	Closest BLAST hits	Sequence types of the Glomeromycota detected in the same root sample∇
Landskron, FR - bait plant (1st harvest)	*Bromus erectus* (BA2)	N	-	-	-	-	GLOM B-4
Landskron, France - bait plants (2nd harvest)	*Bromus erectus* (BA1)	Y (DS)+	-	-	-	Sebacinales sp. (ZS725_726)	GLOM B-4; B-5
	Medicago sativa 1 (MA2)	Y (DS)+	-	-	-	Sebacinales sp. (ZS727_728)	GLOM A-1; B-4
	Bromus erectus (BA2)	Y (DS)+	-	-	-	Sebacinales sp. (ZS730)	GLOM A-1; B-4; B-5
	Inula salicina (IA4)	N+	-	-	-	-	GLOM A-1; A-16; B-2; B-5
	Origanum vulgare (OA3)	N+	-	-	-	-	GLOM A-1; B-4
	Inula salicina (IN3)	N+	-	-	-	-	GLOM A-1; A-3; A-31; A-16; B-2; B-4
	Bromus erectus (BN2)	N+	-	-	-	-	GLOM A-1; B-1; B-2; B-5
	Origanum vulgare (OA1)	N+	-	-	-	-	GLOM A-1; A-7; A-17; B-4
	Medicago sativa 2 (MN2)	Y (DS)+	-	-	-	Sebacinales sp. (ZS731_732)	-
	Medicago sativa (MA3)	N	-	-	-	-	GLOM A-1; A-3; B-2; B-4
	Origanum vulgare (ON4)	N	-	-	-	-	-
	Inula salicina (IN2)	N	-	-	-	-	-
	Medicago sativa 3 (MN2)	Y (DS)	-	-	-	Sebacinales sp. (ZS743_744)	GLOM A-1
	Inula salicina (IN2)	N	-	-	-	-	-
	Origanum vulgare (ON4)	N	-	-	-	-	-
Landskron, France – field samples	*Origanum vulgare* (O2-C)	Y (DS)	-	-	-	Sebacinales sp. (ZS745_746)	GLOM A-1; A-16; B-4
	Origanum vulgare (O3-D)	Y (DS)	-	-	-	Sebacinales sp. (ZS747_748)	GLOM A-1
	Medicago sativa (M3-A)	Y (DS)	-	-	-	Bad quality of the sequence	GLOM A-1; A-17
	Medicago sativa (M4-A)	Y (DS)	-	-	-	Sebacinales sp. (ZS751_752)	GLOM A-1; A-16; A-17
Ramosch, Switzerland	*Gentiana acaulis* (R-11-2)	N	-	-	-	-	GLOM A-9; A-12
	Gentiana verna (R-2A-5)	N+	-	-	-	-	GLOM A-14
	Polygala vulgaris (R-11-4a)	N+	-	-	-	-	-
	Gentiana acaulis 1 (R-2A-c)	Y (DS)+	-	-	-	Sebacinales sp. (ZS733_734)	GLOM A-1
	Gentiana acaulis 2 (R-2A-b)	Y (DS)+	1288	10	2 (9x like Sebacinales sp. ZS789_790)	Sebacinales sp. (ZS759_760; ZS789_790) + bad sequence after DS	GLOM A-1; A-7; A-9
	Trifolium sp. (R-2A-ba)	Y (DS)+	-	-	-	Sebacinales sp. (ZS737_738)	GLOM A-1; GLOM B-3; GLOM B-4

Sample origin	Root sample description: plant species (internal code)	PCR product in the 2nd nested PCR: yes (Y)/ no (N)*	Ligation number	Number of clones screened	No of different RFLP patterns after the digestion with enzymes *Hinf*I and *Mbo*I	Closest BLAST hits	Sequence types of the Glomeromycota detected in the same root sample∇
	Poaceae sp. 1 (R-2A-ba)	Y (DS)+	1289	8	4 (2x like Sebacinales sp. ZS761_762; 2x like Sebacinales sp. ZS763_764)	Sebacinales sp. (ZS761_762; ZS763_764; 766) + bad sequence after DS	GLOM A-1
	Gentiana acaulis (R-11-3)	N	-	-	-	-	-
	Crocus albiflorus (R-2A-3a)	N	-	-	-	-	GLOM A-28; GLOM B-3
	Gentiana acaulis (R-2A-a)	N	-	-	-	-	-
DOK experiment, Switzerland	*Triticum aestivum* K64a	Y (double band; DS)	1393	5	4 (1x like *Cryptococcus* ZS978_979)	*Dioszegia* (ZS976_977); *Cryptococcus* (ZS978_979) + bad sequence after DS	Not analyzed
	Triticum aestivum K64b	Y (double band; DS)	1394	7	6 (2x like *Cryptococcus* ZS978_979)	*Cortinarius/Parasola* chimaera? (ZS980, 981); *Cryptococcus/Coniochaeta* chimaera? (ZS982, 983) + bad sequence after DS	Not analyzed
	Zea mays M40a	Y (DS)	1395	9	4 (5x like Sebacinales sp. ZS986_987)	Sebacinales sp. (986_987); *Phialocephala* (ZS984_985) + Sebacinales sp. (ZS964) after DS	Not analyzed
	Zea mays K62b	Y (double band; DS)	1396	7	3 (1x like *Cryptococcus* ZS988_989; 2x like ZS990_991)	*Cryptococcus* (ZS988_989; ZS990_991) + *Cryprococcus* (ZS967) after DS	Not analyzed
	Zea mays O18c	Y (DS)	1397	9	5 (4x like Sebacinales sp. ZS994_995; 1x like *Xylaria* ZS992_993)	Sebacinales sp. (994_995) + Sebacinales sp. (ZS968_969) – DS; *Xylaria* (ZS992_993);	Not analyzed
	Zea mays O18b	Y (DS)	1398	10	6 (4x like Sebacinales sp. ZSZS996_997)	Cryptococcus (ZS998_999); Sebacinales sp. (ZS996_997) + Sebacinales sp. (ZS970_971) after DS	Not analyzed
Leek field, Switzerland	*Allium porrum* KB II	N	-	-	-	-	Not analyzed
	Allium porrum KA V	N	-	-	-	-	Not analyzed
Maize field "R", Germany	*Zea mays* R2	N	-	-	-	-	Not analyzed
	Zea mays 1; R4	Y (DS)	1399	10	5 (2x like Sebacinales sp. ZS1000_1001; 2x like ZS1002_1003)	Sebacinales sp. (ZS1000_1001; ZS1002_1003)	Not analyzed
Rabbit Creek,	*Agrostis scabra* 1; 6-A2; 19.4°C	Y (DS)	-	-	-	Sebacinales sp. (SA531_532)	GLOM A-1; A-11

Sample origin	Root sample description: plant species (internal code)	PCR product in the 2nd nested PCR: yes (Y)/ no (N)*	Ligation number	Number of clones screened	No of different RFLP patterns after the digestion with enzymes *Hinf*I and *Mbo*I	Closest BLAST hits	Sequence types of the Glomeromycota detected in the same root sample∇
Yellowstone, USA	*Agrostis scabra* 39-A5; 36.4°C	Y (DS)	-	-	-	*Cryptococcus* (SA533_534)	GLOM A-7a; PARA-3
	Dichanthelium lanuginosum 77-D7; 54°C	Y (DS)	-	-	-	Bad quality of the sequence	-
	Agrostis scabra 97-A7; 29.5°C	Y (DS)	-	-	-	Bad quality of the sequence	GLOM A-1; A-24; A-26
	Dichanthelium lanuginosum 1; 110-D3; 29°C	Y (DS)	-	-	-	Sebacinales sp. (SA539_540)	GLOM A-11; A-26; A-27; ACAU-6
	Dichanthelium lanuginosum 2; 126-D1; 33.1°C	Y (DS)	-	-	-	Sebacinales sp. (SA541_542)	GLOM A-1; GLOM A-27
	Agrostis scabra 2; 197-A3; 25.5°C	Y (DS)	-	-	-	Sebacinales sp. (SA543_544)	GLOM A-7b; A-11; A-27
	Dichanthelium lanuginosum 3; 87-D5; 47.7°C	Y (DS)	-	-	-	Sebacinales sp. (SA551_552)	GLOM A-1; A-11; A-26; ACAU-7; PARA-1
Jen's spot, Yellowstone, USA	Poaceae sp. 81; 27°C	Y (DS)	-	-	-	Bad quality of the sequence	GLOM A-1; A-7b; A-11; A-26; PARA-1
Insect Hell Central, Yellowstone, USA	Poaceae sp. 134; 25°C	Y (DS)	-	-	-	Sebacinales sp. (SA547_548)	GLOM A-1; A-13; A-26; A-27
Ölkelduháls, Iceland	*Agrostis stolonifera* Ö11A; 28.7°C	Y (DS)	-	-	-	Sebacinales sp. (SA549_550)	GLOM A-1; PARA-1

* Explanatory notes:
DS = direct sequencing without cloning
+ this sample also yielded PCR product in the 2nd nested PCR reaction using primer combination NS5/ITS4 (1st nested PCR) and SebITS3/ITS4i (2nd nested PCR)
∇ Sýkorová et al. (2007a); Sýkorová et al. (2007b); (Appoloni 2006)

A1.3.2. Nested PCR reactions

Initially, a quick check of the presence or absence of the Sebacinales was performed in several samples from Landskron and Ramosch (Tab. 1). The dilutions 1:100 of first nested PCR products (using primers NS5/ITS4, for details see Chapters 2.3.3 and 3.3.5) were used as templates for the second nested PCR round, where Sebacinales-specific primer ITS3Seb (with annealing site at the 3' end of the 5.8S rDNA subunit, Setaro et al. 2006) and a universal primer ITS4i (Redecker et al. 2003) were employed. For PCR conditions see Chapters 2.3.3 and 3.3.5. PCR products were checked on agarose gels, purified and sequenced in both directions as described in Chapters 2.3.4 and 3.3.6. The resulting short fragment representing mainly the ITS2 region with a length of ca 270 bp could be tested by BLAST (Altschul et al. 1997) but not analyzed phylogenetically due to its variability.

Therefore, all samples were then analyzed using the universal fungal primer ITS1F and the universal primer TW14 in the first nested PCR and the Sebacinales-specific primer ITS3Seb and universal fungal primer NL4 in the second PCR round. For all primer sequences see Setaro et al. (2006) and for the PCR conditions Chapters 2.3.3 and 3.3.5. The resulting fragment - ITS2 region and the 5' end of the 28S rDNA subunit (D1/D2 region) of the nuclear rDNA - had an expected length of ca 900 bp and was suitable for the phylogenetic analysis. PCR products were checked on agarose gels, purified and sequenced in both directions (for details of these methods see Chapters 2.3.4 and 3.3.6). Nine PCR products (Tab. 1) from Ramosch, the maize field R and the DOK experiment were purified and cloned, clones were re-amplified, digested with restriction enzymes *Mbo*I and *Hinf*I and some of them purified and sequenced (for details of these methods see Chapters 2.3.4 and 3.3.6).

Sequences were aligned to previously published sequences in PAUP*4b10 (Swofford 2001). A phylogenetic tree was obtained by distance analysis (neighbor joining; Saitou and Nei 1987) in PAUP*4b10 using the Kimura two-parameter model and a gamma shape parameter=0.5. Results were verified by performing maximum likelihood analysis based on parameters estimated in Modeltest 3.5 (Posada 2004) as well as Bayesian analysis performed in MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003). Four chains were run over 1×10^6 generations with a burnin value of 2500.

A1.4. Results

A1.4.1. PCR products obtained

The primer combination ITS3Seb and ITS4i yielded PCR products in 15 out of 22 analyzed root samples. The PCR products were sequenced directly and the BLAST comparison revealed that the sequences belong to the Sebacinales. However, as these PCR products were too short for a phylogenetic analysis, all samples were analyzed using the primers ITS1F/TW14 in the first and ITS3Seb/NL4 in the second nested PCR. Only these longer fragments suitable for the phylogenetic analysis will be discussed in further text.

Directly sequenced products of the second nested PCR (with the primers ITS3Seb/NL4) were predominantly of good quality, and according to BLAST and phylogenetic analyses they belonged to the Sebacinales. In case the direct sequencing did not yield a sequence of a good quality, PCR products were subjected to cloning. Five to ten clones screened per cloned PCR product yielded two to six different RFLP patterns. The five sequenced clones from the two samples from Ramosch contained only Sebacinales. However, the seven PCR products from arable sites showed a different phenomenon: their sequenced clones belonged not only to the Sebacinales, but also to other Basidio- and Ascomycota. Thus, the cloning was a good solution when the quality of the direct sequencing output was bad, which might have been caused by a simultaneous presence of several sebacinoid phylotypes (showing also different RFLP patterns) or sebacinoid and non-sebacinoid fungi in a single root sample.

An overview of all analyzed samples, PCR and sequencing products is provided in Tab. 1. Fungi from the order Sebacinales were found in each but two field sites, in a total in 45% of the analyzed root samples. The ratio of positive (i.e. Sebacinales-containing) to negative samples differed in each site: Sebacinales were detected in 64% of samples from Yellowstone and Iceland; in 40% of samples from Landskron and in 33% of samples from the agriculturally managed sites (DOK experiment, leek and maize field) as well as from Ramosch.

A1.4.2. Phylogenetic analysis

The results of the neighbor joining (Fig. 4), maximum likelihood (ML) and Bayesian analyses showed that all but one sequence belonged to the subgroup B (sensu Weiß et al. 2004). Several clusters are apparent within this subgroup using all three approaches. The clusters with high neighbor joining branch support (>90) were also recovered by the maximum

likelihood and Bayesian algorithm. The sequences belonging to the subgroup B clustered well with sequences from the GenBank obtained from *S. vermifera* isolates, ericoid plants as well as green orchids and liverworts. The only sequence from *Zea mays* belonging to the subgroup A clustered well with *Efibulobasidium albescens*.

In two cases (*D. lanuginosum* and *M. sativa*), the sequences obtained from different individuals of the same plant species were identical over all of the 524 characters of the conserved part of the LSU included into the phylogenetical analyses. However, other sequences from these plant species were also found in other clusters in the neighbor joining, ML and Bayesian tree. No clear geographical pattern was observed: e.g. sequences from Iceland, Swiss Alps and Yellowstone belonged all to a single well-supported cluster together with GenBank sequences from orchids from Australia or Ecuador as well as ericoid plants from Ecuador.

After RFLP screening of eight to ten clones, the cloned PCR products yielded two different RFLP patterns in case of *G. acaulis* from Ramosch, five in case of *Z. mays* from the R field, and by Poaceae sp. from Ramosch four. Sequences of clones originating from a single root sample in two cases (*Zea mays*: ZS1000_1001 and ZS1002_1003; *G. acaulis*: ZS789_790 and ZS759_760) clustered relatively close to each other. They differed in 33 and 9 characters, respectively. The sequences obtained from the Poaceae sp. root sample (ZS763_764 and ZS761_762) differed in 11 characters and did not cluster together in the neighbor joining tree. In contrast, in the ML and Bayesian tree, they grouped together in one cluster, which was however only weakly supported.

Fig. 4 (Fig. on the next page) Phylogenetic tree of the Sebacinales obtained by neighbor joining analysis of 524 characters of the D1/D2 region of the 28S rDNA subunit. The left number above the each branch line denotes the bootstrap value obtained from 1000 replicates of the neighbor joining analysis; the right number after the slash indicates the credibility value from the Bayesian analysis. The tree was rooted with *Geastrum saccatum* and *Auricularia auricula-judae*. Sequences obtained in the present study are shown in color. They are labeled with internal identification number (e.g. ZS789 790), the host plant species (e.g. *Gentiana acaulis*), field site name and country. Sequences in the same color originate from root samples from the same field site. Multiple sequences originating from a single root sample are labeled with the same number (which is placed between plant species and field site name, see also Tab. 1). The parentheses show the delimitation of the two subgroups defined by (Weiss et al. 2004).

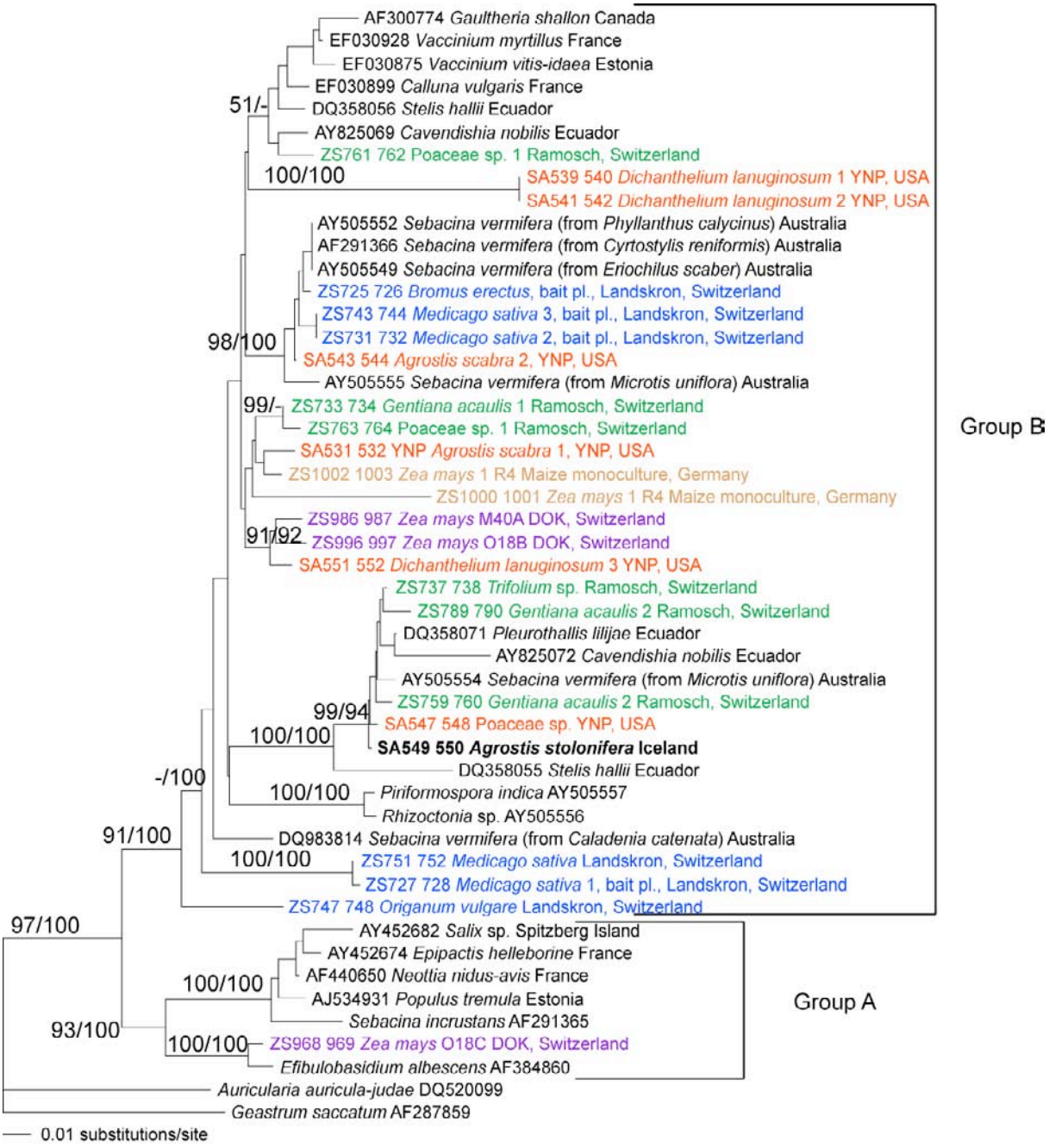

Fig. 4 (legend see previous page)

A1.5. Discussion

To our knowledge, this is the first study on the molecular diversity of the Sebacinales in the AMF host plants. Our results show that these fungi were present in the majority of analyzed root samples from almost all field sites and that they coexisted with different lineages of the Glomeromycota in these plants (Tab. 1). No Sebacinales were detected in the leek field in Switzerland and Jen's spot in USA, probably due to low number of samples analyzed (two and one, respectively). In all remaining field sites, Sebacinales were detected in different percentages of samples analyzed. Thus, this fungal order indeed seems to be ubiquitous.

In agreement with our results, previous studies reported different sebacinoid sequences in the same site or in the same host plant species at the same site (e.g. Selosse et al. 2007) or even a single root sample (Setaro et al. 2006). These authors as well as others (Weiß et al. 2004) did not reveal any pattern related to the geographical origin of the root samples, which corresponds well to the results of our study. Therefore, we can conclude that the Sebacinales might probably show no clear geographical pattern and also no strong preferences or even specificity towards their host plant species. However, this conclusion could be biased by the fact that the delimitation of a molecular species within this order is still unclear (Selosse et al. 2007). Nevertheless, further investigations like analysis of more host plant species from more field sites, more root samples per host plant species per field site and cloning of the PCR products would be necessary to confirm these conclusions.

The broad diversity of mycorrhizal strategies present in the Sebacinales might enable hyphal linkage and nutrient transfer between different organisms. For example, Sebacinales from the subgroup A may connect mycoheterotrophic orchids and surrounding ectomycorrhizal trees (Selosse et al. 2002b). Our sequences clustered well with GenBank sequences obtained from green orchids, ericoid plants and *P. indica* in the subgroup B. As orchids or ericoid plants were growing in some of our field sites (Landskron and Ramosch, respectively), we can hypothesize on possible hyphal connections between them and our AMF host plants. Setaro et al. (2006) noticed that *Cavendishia nobilis*, a member of the Ericaceae forming a specific type of ectendomycorrhiza (cavendishioid mycorrhiza) with Sebacinales from the subgroup B, grows in a tropical rain forest community in Ecuador dominated by arbuscular mycorrhizal plants (Kottke et al. 2004). These AM plants have not been investigated yet, but in case they would also host the Sebacinales, we can hypothesize on possible hyphal connections between them and *C. nobilis* and it would fit well together with our observations of arbuscular mycorrhizal herbs hosting Sebacinales from the subgroup B. However, as the sebacinoid host plants from the subgroup B are green, the physiological

meaning of possible hyphal connections would be different from the mycoheterotrophic orchids and surrounding trees mentioned above.

The only morphological investigations of intraradical structures of a sebacinoid fungus in AMF host plant roots were conducted using *P. indica* and barley by Varma et al. (1999) and Deshmukh et al. (2006). These authors did not observe typical arbuscules like those formed by the Glomeromycota. Deshmukh et al. (2006) however, suggest that this fungus causes protoplast plasmolysis and thus host cell death. As *P. indica* can increase biomass and grain yield of crop plants, as well as their resistance to fungal diseases and abiotic stress (Waller et al. 2005), further investigations are necessary to elucidate the function and physiology of this symbiosis.

As we did not conduct any ultrastructural and physiological investigations, we cannot conclude that the Sebacinales were growing indeed intraradically and we can just speculate on the function of these fungi for their host plants and of the plants for the fungi. Several scenarios are theoretically possible: i) Sebacinales may form a “standard” mycorrhiza with bi-directional nutrient transfer, where both the plant as well as the fungus profit from each other; ii) they may be saprophytes growing as opportunists on decaying mycorrhizas or even parasites of arbuscular mycorrhizal fungi; iii) they may be root epi- or endophytes or even parasites without any relationship to AMF.

In conclusion, members of the Sebacinales have been detected molecularly in root samples of host plants belonging to all main mycorrhiza types. As their mycelia may be shared by different root systems, we might expect a major role in interplant interactions. Nevertheless, the ecological potential and function of these associations have not been studied yet. Physiological investigations of nutrient transfer between the putative symbiotic partners using labeled carbon, nitrogen and phosphorus could elucidate this question. This approach should be supplemented also with molecular and ultrastructural analyses, with attempts to get more Sebacinales in pure cultures to be used in greenhouse experiments. The mechanisms of the coexistence or interaction of Sebacinales with other mycorrhizal fungi in the roots remain a question for future research as well. As mentioned above, Sebacinales might grow on the surface of AMF-colonized roots, they might be mycoparasites, they might form dual colonization of non-overlapping root areas with other mycorrhizal fungi or they might outcompete and replace other fungi in the roots. Selosse et al. (2007), for instance, observed Sebacinales coexisting with an ascomycete in a single cortical cell of an ericoid plant root.

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Appendix 2

Acaulospora alpina, a new arbuscular mycorrhizal fungal species characteristic for high mountainous and alpine regions of the Swiss Alps

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A2.1. Abstract

Acaulospora alpina sp. nov. forms small (65–85 µm in diameter), dark yellow to orange-brown spores laterally on the neck of hyaline to subhyaline sporiferous saccules. The spores have a three-layered outer spore wall, a bi-layered middle wall and a three-layered inner wall. The surface of the second layer of the outer spore wall is ornamented, having regular, circular pits (1.5–2 µm in diameter) that are as deep as wide and truncated conical. A 'beaded' wall layer as found in most other *Acaulospora* spp. is lacking. The spore morphology of *A. alpina* resembles that of *A. paulinae* but can be differentiated easily by the unique ornamentation with the characteristic pits and by the spore color. A key is presented summarizing the morphological differences among *Acaulospora* species with an ornamented outer spore wall. Partial DNA sequences of the ITS1, 5.8S subunit and ITS2 regions of ribosomal DNA show that *A. alpina* and *A. paulinae* are not closely related. *Acaulospora lacunosa*, which has similar color but has generally bigger spores, also has distinct rDNA sequences. *Acaulospora alpina* is a characteristic member of the arbuscular mycorrhizal fungal communities in soils with pH 3.5–6.5 in grasslands of the Swiss Alps at altitudes between 1800 and 2700 m above sea level. It is less frequent between 1300 and 1800 m above sea level, and it has so far not been found in the Alps below 1300 m nor in the lowlands of Switzerland.

Key words: Alps**,** Acaulosporaceae, *Acaulospora paulinae*, *Acaulospora lacunosa*, Glomeromycetes, key, molecular identification, mycorrhiza, spore morphology, phylogeny, taxonomy

A2.2. Introduction

At high altitudes, in the mountainous and alpine regions of the Swiss Alps extending from 1000–3000 m above sea level (a.s.l.), we have observed an astonishingly high diversity of arbuscular mycorrhizal (AM) fungal species (Oehl, unpublished). Spores of about 60 known species of the Glomeromycota (Schüssler et al 2001) could be identified from different grasslands growing on soils that had developed on siliceous and calcareous bedrocks. Some of the species were new and have recently been described (Oehl and Sieverding 2004, Oehl et al 2005a). Among the AM fungi, species belonging to the genus *Acaulospora* were particularly prominent and relatively much more abundant than in the lowlands of Switzerland. Here we describe a new *Acaulospora* species under the epithet *A. alpina* that was found exclusively in the Alps at altitudes >1300 m a.s.l.

The genus *Acaulospora* was described by Gerdemann and Trappe (1974) who also presented the first key for the two species known at that time. The key differentiated a species known to produce spores with a smooth surface (*A. laevis*) from another one with an ornamented surface (*A. elegans*). Today we know 18 *Acaulospora* spp. with smooth spore surfaces and 15 *Acaulospora* spp. (including *A. alpina*) with ornamentation of the outer spore wall. Schenck et al (1984) presented the latest key to the ornamented species of *Acaulospora*. They used spines, tubercles, ridges, folds, pits or cracks as differentiating features for the spore wall ornamentations. We use similar characteristics and we present an updated key for *Acaulospora* spp. with ornamented spore walls.

In recent years molecular biological tools have been applied to identify AM fungi (Clapp et al 1995, Redecker 2000, Oehl et al 2005a). Environmental rDNA sequences are rapidly increasing in number in the public data bases. However, only a few DNA sequences of *Acaulospora* originating from morphologically-characterized spores are available. This is also true for the highly variable rDNA Internal Transcribed Spacer (ITS) region, which is a useful tool to distinguish many species-level AM fungal taxa (Redecker et al 2003). Some of these database sequences show strong similarity to fungal groups other than the Glomeromycota and are more likely to originate from contaminant organisms (Millner et al 2001). Therefore, there is a clear need for rDNA sequences from described *Acaulospora* species.

New species can only be adequately characterized when sequences of morphologically similar species are included in the analysis. To identify the phylogenetic position of *A. alpina*, the sequence of the ITS1, 5.8S rDNA and ITS2 region was determined not only from this new species but also from *A.*

paulinae Blasz. (Blaszkowski 1988) and *A. lacunosa* J.B. Morton (Morton 1986), which produce morphologically similar spores. Sequences obtained for *A. alpina* were also compared to environmental sequences of *Acaulospora* spp. available from the public data bases.

A2.3. Materials and methods

A2.3.1. Soil sampling

Soil samples were taken from mountainous and alpine grasslands in the Swiss Alps from altitudes of 1000–3000 m a.s.l.; the soils had developed on different geological bedrocks from nutrient poor Jurassic sandstones over granite and gneiss rocks to carbonatic and dolomitic limestones and ultrabasic serpentinites. Undisturbed soil cores from 0-10 cm depth were collected at several times between July and September of 2003. Spores of AM fungi were separated from the soil samples by a wet sieving process as described by Sieverding (1991).

A2.3.2. AM fungal bait cultures

Bait cultures were established directly after sampling as follows: 1000 mL pots were half filled with 500 g of an autoclaved substrate (Terragreen; American aluminium oxide, Oil Dry US special, type III R; Lobbe Umwelttechnik Iserlohn, Germany) -Loess mixture 3:1; pH-KCl 6.2; organic carbon 0.3 %; available P (Na-acetate) 2.6 mg kg^{-1}; available K (Na-acetate) 350 mg kg^{-1}. 50 g dry weight field samples were placed at one side on the top of the substrate and covered with another 300 g of autoclaved substrate. Above the soil inocula, about 5–7 seeds of each of the four trap plants, *Plantago lanceolata* L., *Lolium perenne* L., *Trifolium pratense* L. and *Hieracium pilosella* L. were sown. We added 0.2 mL of a culture broth with *Rhizobium trifolii* (DSM 30138, from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) grown in liquid DSMZ 98 medium at 27 C for 12 h to the two-wk-old *Trifolium pratense* plants in each pot. An automated watering system (Tropf-Blumat, Weninger GmbH, A-6410 Telfs) was installed and the cultures were kept in the greenhouse of the Institute of Botany in Basel under ambient natural light and temperature conditions until the end of 2004. The formation of spores in the bait cultures was checked between June and December 2004 at bimonthly intervals as described by Oehl et al (2003, 2004). The new fungus only infrequently produced spores in these bait cultures. All trials of mono-species cultures, either initiated with single or multi-spores, so far failed to establish a successful symbiosis.

A2.3.3. Morphological analyses

The described morphological characteristics of spores and sporiferous saccules and their subcellular structures are based on observations of specimens mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG; Koske and Tessier 1983), in a mixture of PVLG and Melzer's reagent (Brundrett et al 1994), a mixture of lactic acid to water at 1:1, Melzer's reagent, and in water. The terminology of the spore structure is that of Stürmer and Morton (1999), which was adapted by INVAM (International Culture Collection of Arbuscular and Vesicular-Arbuscular Endomycorrhizal Fungi, see homepage: www.invam.caf.wvu.edu), except that we use different abbreviations for the walls and wall layers. In detail, we call the outer 'spore wall' layers of the Acaulosporaceae sw1–3, the first flexible inner wall iw1 of Stürmer and Morton (1999), the 'middle wall' (mw), and the second flexible inner wall iw2 of Stürmer and Morton (1999) the 'inner wall' (iw). Photographs in Figs. 1–9 and 11–19 were taken with a digital camera (Olympus model DP70-CU) on a compound microscope (Zeiss Axioplan). To improve the quality of the pictures taken of the ornamentation of different *Acaulospora* spp., the software Auto-Montage Essentials 5.00 (Olympus) was used (technique used in Figs. 1–3; 7, 8, 14, 15). Specimens mounted in PVLG and the mixture of PVLG and Melzer's reagent were deposited at Z+ZT (Zürich, Switzerland), FB (Freiburg, Germany) and OSC (Corvallis, USA) herbaria.

A2.3.4. Molecular analyses

DNA crude extracts were produced as described by Redecker et al. (1997) from spores of *A. alpina* originating from a grassland on a Humic Cambisol at Spadla Alp (at 2700 m a.s.l. near Sent, Engiadina Bassa, Canton Grischun; soil pH-5.0 measured in water) and from isotypes isolated at Grand Muveran (at 2600 m a.s.l. near Ovronnaz/Martigny, Canton Valais; pH 5.6), from Tschima da Flix (at 2400 m a.s.l. near Sur, Surses, Canton Grischun; pH 6.0) and at Stützalp (at 1900 m a.s.l. near Davos, Canton Grischun; pH 6.1). DNA was extracted from approximately 10 spores from each location.

Extracts of single spores were used as templates for a two-step polymerase chain reaction (Redecker et al 2003) using the primers NS5/ITS4 and ACAU1661/ITS4i, respectively (Redecker 2000). PCR products were purified with a High Pure PCR Product Purification Kit (Roche, Mannheim, Germany), cloned into pGEM-T (Catalys, Wallisellen, Switzerland), reamplified from the clones and digested with *Mbo*I and *Hin*fI restriction enzymes (Fermentas, Vilnius, Lithuania). Samples with different RFLP patterns were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) for labeling. Samples were run on an ABI 310 capillary sequencer (Applied Biosystems). Sequences of *A. alpina* were submitted to the EMBL database under the accession numbers AJ890446 and AJ891101 - AJ891109. To compare the sequences obtained from *A. alpina* to other *Acaulospora* spp. of similar spore morphology, an isolate of *A. paulinae* and

the BEG78 isolate of *A. lacunosa* were included in the analysis. The isolate of *A. paulinae* originated from a soil sample taken from a meadow with *Arrhenaterum elatius* L. as the characteristic grass species, at Wintzenheim-La-Forge (Alsace, France) in April 2003. The *A. lacunosa* isolate originated from a temperate forest in New Hampshire, USA. Sequences were submitted to the EMBL database under the accession numbers AJ89114-AJ891121 (for *A. paulinae*) and AJ891110 - AJ891113 (for *A. lacunosa*).

The sequences were aligned in PAUP*4b10 (Swofford 2001) in a dataset comprising rDNA ITS1, 5.8S subunit and ITS2 of other fungi from the family Acaulosporaceae. From a total of 700 positions in the alignment, 315 positions were selected that were in unambiguous alignment. The ITS1 region contains numerous insertions/deletions in long stretches of A or T, which causes serious alignment problems; therefore this region was excluded from the analysis. In all phylogenetic analyses, the sequence of the AM fungus *Entrophospora colombiana* was used as an outgroup. The appropriate sequence evolution model for maximum likelihood analysis (HKY+G) was determined using Modeltest 3.5. (Posada 2004). Bayesian analysis was performed in MrBayes 3.0 (Ronquist and Huelsenbeck 2003). Four chains were run over 3.6 x 10^6 generations with a burnin value of 2000. Neighbor-joining analysis was performed using the Kimura 2-parameter model and a gamma shape parameter of 0.5. Alternatively, maximum likelihood distances obtained by the HKY+G model were used for neighbor joining, which yielded the same tree topology. Bootstrap analysis (Felsenstein 1985) was performed to estimate the robustness of the phylogeny.

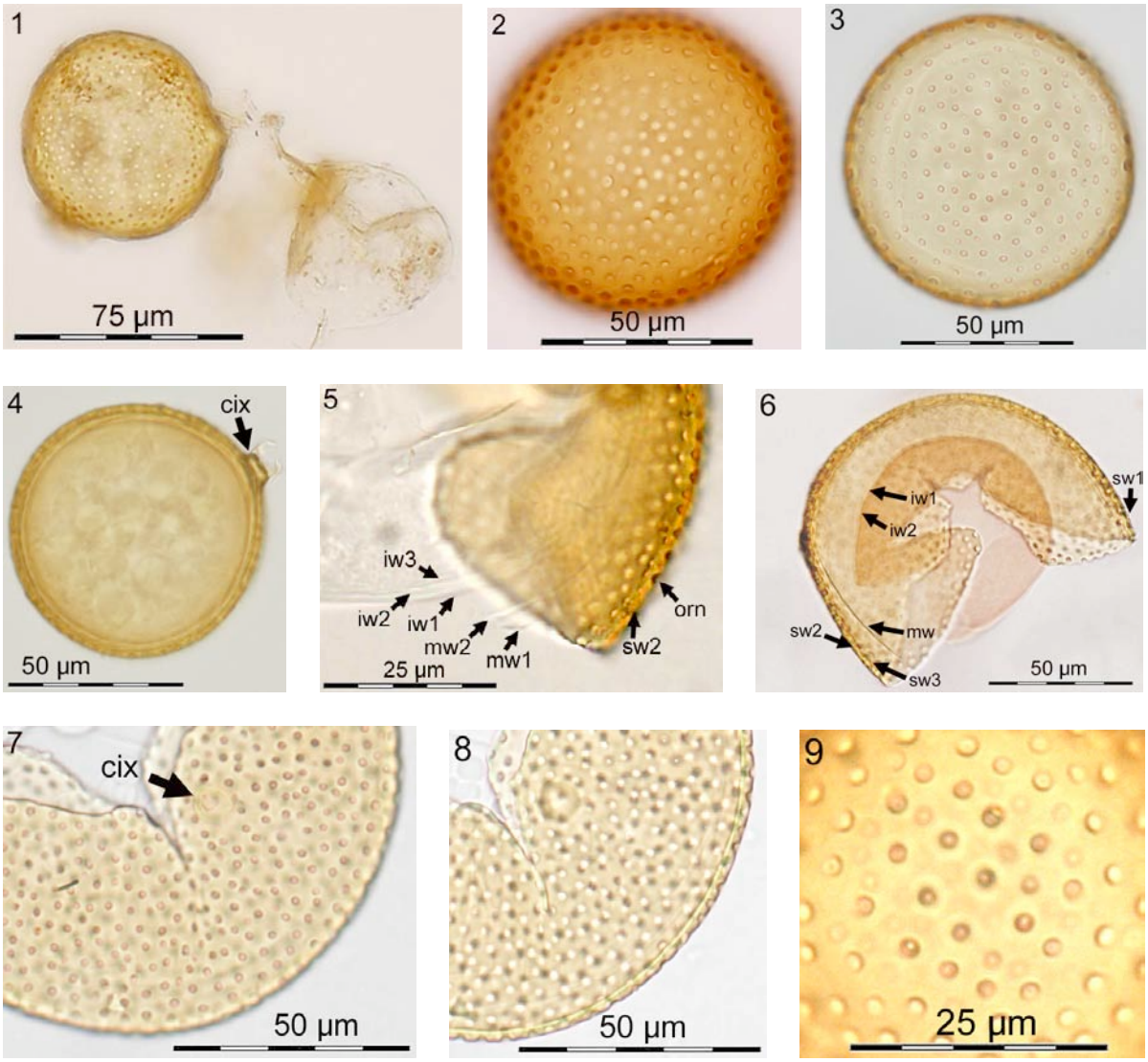
A2.4. Taxonomic analysis

A2.4.1. Latin diagnosis

Acaulospora alpina Oehl, Sykorova & Sieverd. sp. nov. (Figs. 1–9)

Sacculus sporifer hyalinus aut pallido-luteus, globosus vel subglobosus, 65–92 µm in diametro et formationi sporae praecedens. Sporae singulae lateraliter formatae ad hypham in 40–80 µm distantia ad sacculum terminalem, flavae vel fulvae vel fulvo-aurantiae vel aureae vel aurantio-brunneae, (53–) 65–85 (–97) µm in diametro, globosae vel subglobosae vel ovoideae vel ellipsoideae vel irregulares (53–) 60–81(–91) × 62–87 (–110) µm. Sporae tunicis tribus: tunica exterior, media et interior. Tunica exterior in totum 2.5–4.0 µm crassa, stratis tribus: stratum exterius hyalinum, tenue et evanescens; stratum medium laminatum vel unitum, flavum vel fulvum vel fulvo-aurantium vel brunneo-aurantium, depressionibus subtilibus, rotundis, 1.5–2.2 (–2.8) µm in diametro, et conicis, 2.0–2.5 µm profundis, in interiorem strati huius insculptis; stratum interius flavum vel fulvum, subtile. Tunica media tenuis stratis duobus et tunica interior stratis tribus, uterque tunicae hyalinae et flexibiles.

Tunica interior 1.2–3.0 µm in totum; solo stratum medium tunicae interioris pallide colorans reagente Melzeri. Typus hic designatus # 41–4101: Z+ZT.



Figs. 1–9 *Acaulospora alpina* —photographed from type specimen. **1.** Spore and sporiferous saccule, scale bar = 75 µm. **2., 3.** Spore showing regular ornamentation, scale bar = 50 µm. **4.** Spore with cylindric pedicel around cicatrix (cix), scale bar = 50 µm. **5.** Cracked spore, with three walls (sw, mw, iw); outer spore wall three-layered (sw1–3) with pitted ornamentation (orn) on sw2; flexible middle wall (mw) with two usually adherent layers (mw1 and mw2; here separated) and inner wall (iw) with three tightly adherent layers (iw1–3), scale bar = 25 µm. **6.** Inner wall (iw2) staining pale purple in Melzer's reagent; iw3 often difficult to observe even in broken spores, scale bar = 50 µm. **7.** Cracked spore showing circular pits on sw2 and the cicatrix (cix), scale bar = 50 µm. **8.** Wall layer sw2 with truncated cone-shaped pits, scale bar = 50 µm. **9.** Outer wall at higher magnification showing regular, round pits on sw2, scale bar = 25 µm. All photos by Fritz Oehl.

A2.4.2. Morphological description

Sporiferous saccule is hyaline, globose (about 65–80 µm in diameter) to subglobose, 65–75 × 75–92 µm, with one wall layer that is generally 1.0–2.1 µm thick (Fig. 1); formed at the end of a hypha in 40–80 µm distance from the spore that arises thereafter. The saccule usually collapses after the spore wall has formed and is usually detached from mature spores in soil samples.

Spores (Figs. 1–4) form laterally on the subtending hypha of the sporiferous saccule. The spores are dark yellow, orange to brown, globose to subglobose, (53–) 65–85 (–97) µm in diameter, rarely ovoid to irregular, (53–) 60–81 (–91) × 62–87 (–110) µm in diameter.

Outer spore wall consists of three layers (sw1, sw2 and sw3), in total 2.5–4.0 µm thick (Figs. 5, 6). Outer layer (sw1) is hyaline, unit, 0.5–1.0 µm thick, sloughing, evanescent and thus, usually absent in mature spores. Second layer (sw2) is light to dark yellow to yellow-orange to orange-brown, laminated, 2.0–3.0 µm thick including the ornamentation with regular, round and truncated conical pits that are 1.5–2.2 (–2.8) µm in diameter and at least as deep as wide (Figs. 7–9). Due to their truncated cone shape, the pits often appear to have a dark central point, but there is no second depression or projection within the pit. The distance between the pits is (3.0–) 4–6 (–7) µm. The inner spore wall layer (sw3) is concolorous with sw2, 0.5–1.3 µm thick, usually tightly adherent to sw2 and often difficult to observe when < 1.0 µm. None of these wall layers stains in Melzer's reagent.

Middle wall is hyaline, bi-layered and rather thin; in total 0.5–1.2 µm; both layers (mw1 and mw2) are semiflexible (Fig. 5), tightly adherent to each other and thus, often appearing as being one wall layer (Fig. 6). None of the layers reacts to Melzer's.

Inner wall is hyaline, with three layers (iw1-3) that are 1.2–3.0 µm thick in total (Fig. 5). The iw1 is about 0.5 µm thick, and not 'beaded'; iw2 is 1.2–2.0 µm thick; iw3 is about 0.5 µm thick and usually very difficult to detect due to the close adherence to iw2. Only iw2 shows a light, pale pink reaction to Melzer's reagent (Fig. 6) usually visible only in cracked spores and not observed in all specimens.

Cicatrix (Figs. 4, 7) remains after detachment of the connecting hypha (Fig. 4), (5–) 7–12 µm wide. The layer sw2 often continues for a small distance [0.8–2.2(–3) µm] into the detaching hypha forming a short cylindrical pedicel around the pore. Although the pore itself is not ornamented, the tapering pedicel wall often has the pitted ornamentation of sw2. The pore is closed by some of the inner laminae of sw2 and by sw3.

Etymology. Latin, *alpina*, referring to the Swiss Alps where the species was first found.

A2.4.3. Specimens examined

SWITZERLAND. GRISCHUN: Sent, Alp Spadla, at 2000–2700 m a.s.l. (HOLOTYPE: Z+ZT); GRISHUN: Pontresina, Diavolezza at 2000–2700 m; GRISCHUN, San Murezzano (St. Moritz), Corviglia at 2700 m; GRISHUN: La Punt, Passo D'Alvra-Piz Üertsch, at 2300–2600 m, GRISHUN: Sur, Tschima da Flix, at 2000–2500 m; GRAUBÜNDEN: Davos, Parsennhütte and Stützalp, at 1800–2300 m (ISOTYPE: OSC); GRAUBÜNDEN: Chur-Haldenstein, at 1620–2300 m; GRISHUN: Sumvitg-Surrein, Alp Nadels, at1950–2500 m; TICINO, Olivone, Piz Corvo-Paso di Lucomagno, at 1800–2500 m; TICINO: Airolo, Passo di Gotthardo, at 1800-2000 m; URI and VALAIS: Realp-Oberwald, Furkapass, at 1850–2650 m; BERN: Axalp, Axalphorn, at 1700–2300 m; BERN: Grindelwald, Grosse Scheidegg/ Gemschberg, at (1350–)1800–2500 m (ISOTYPE: FB); VALAIS: Ovronnaz, Grand Muveran, at 1720–2600 m; VALAIS: Champez, Le Cartogne, at (1350–)1800–2600 m; VALAIS: Col de Grand St. Bernhard, Pointe de Drône, at 2300–2500 m a.s.l.

A2.4.4. Commentary

Spores of *A. alpina* were abundantly isolated from the rhizosphere of alpine grasslands (soil pH 3.5–5.5) with vegetation dominated by *Carex curvula* All. or *Nardus stricta* L. Spores were less frequent in alpine grassland soils with pH > 6.0 and plant species communities dominated by *Carex ferruginea* Scop., *Carex sempervirens* Vill. or *Sesleria caerulea* (L.) Scop., or in lower altitude grasslands (1500–1800 m a.s.l.) with plant species communities dominated by *Nardus stricta* or by *Trisetum flavescens* (L.) P. Beauv. The new species was found in a broad range of soils that developed on acidic sandstones, siliceous gneiss and granite rocks, up to ultrabasic serpentinite and calcareous 'Bündner Schiefer' schists and carbonatic and dolomitic limestones.

A2.4.5. Molecular biological analysis

Sequences of approximately 550 bp length were obtained, comprising ITS1, the 5.8S rDNA subunit and ITS2. Phylogenetic analysis firmly placed all sequences of *A. alpina* into the genus *Acaulospora* and in a single clade, which is clearly distinct from the other *Acaulospora* spp. that have been analyzed. In particular, *A. lacunosa* and *A. paulinae* are not closely related to *A. alpina* or each other. The sister group of *A. alpina* is made up of environmental sequences obtained from roots from the Schiefergebirge mountains of Thuringia, Germany (Renker et al 2003) and an alpine meadow near Ramosch (Canton Grischun, Switzerland) (Fig. 10). One of the environmental sequences from *Anthoxanthum* roots (ASP504636) appears to be an outlier, grouping somewhat intermediate between *A. alpina* and the other sequences obtained from roots. These environmental sequences are different from those of *A. alpina* as indicated by the bootstrap values. Together, *A. alpina* and the environmental sequences form a monophyletic clade, which is supported by the bootstrap distance

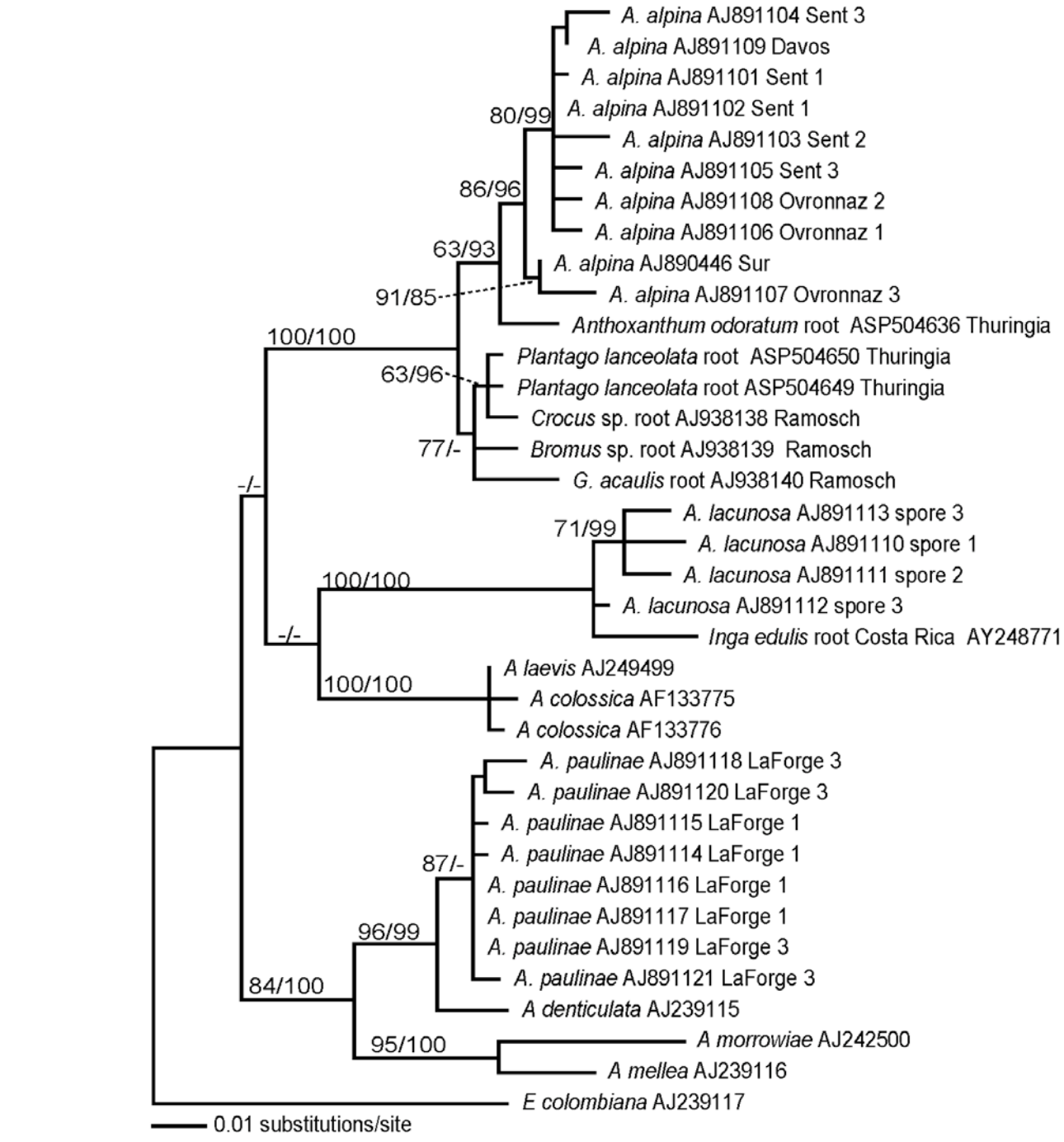


Fig. 10 Phylogenetic tree of *Acaulospora* spp. obtained by maximum likelihood analysis of 5.8S rDNA and ITS2 sequences. The left number above the line of each branche denotes the bootstrap value obtained from 1000 replicates of neighbor-joining analysis (Felsenstein, 1985); the right number after the slash indicates the credibility value from Bayesian analysis. Sequence labels show the source organism, the database accession number and the locality. Multiple spores analyzed from the same site are numbered 1, 2, 3.

A2.5. *Acaulospora* spp. with ornamented outer spore walls

Including *A. alpina*, 15 *Acaulospora* spp. have been described that have ornamented outer spore walls. These are: *A. elegans* Trappe & Gerd. (Gerdemann and Trappe 1974), *A. scrobiculata* Trappe (Trappe 1977), *A. bireticulata* F.M. Rothwell & Trappe (Rothwell and Trappe 1979), *A. spinosa* C. Walker & Trappe (Walker & Trappe 1981), *A. foveata* Trappe & Janos and *A. tuberculata* Janos & Trappe (Janos and Trappe 1982), *A. rehmii* Sieverd. & S. Toro and *A. denticulata* Sieverd. & S. Toro (Sieverding and Toro 1987), *A. taiwania* H.T. Hu (Hu 1988), *A. undulata* Sieverd. (Sieverding 1988), *A. cavernata* Blasz. (Blaszkowski 1989), *A. excavata* Ingleby & C. Walker (Ingleby et al. 1994), and *A. lacunosa* and *A. paulinae*. Below we present a key, to help to distinguish the ornamented *Acaulospora* spp. For this report, species that have depressions or pits on the outer spore wall are of particular interest, and we include colored photographs for those species (Figs. 11-19). We did not have access to a specimen of *A. taiwania*, thus, a photo of this species is not presented. Photographs of the pitted *Acaulospora* spp. spores were generally taken from type or isotype material. We included *A. denticulata* in the picture series because it has a pit (cavity) in each of the broad projections of which the ornamentation of this species consists. It is, however, a member of the group of species with spines or projections on the spore surface.

A2.5.1. Key: *Acaulospora* Spp. with ornamented outer spore walls

1. Spores with spines or polygonal projections with or without a reticulum 2
1. Spores with depressions (pits) or cerebriform folds 6
    2. Spores' spines or projections with a reticulum 3
    2. Spores' spines or projections without a reticulum 4
3. Reticulum three-layered enclosing polygonal projections ±1 × 1 µm; spores generally 150–200 µm . . . . . .*A. bireticulata*
3. Reticulum one-layered, overlaid over crowded, densely-organized spines ±2 µm high; spores 140–280 µm . . . . . .*A. elegans*
    4. Spores with fine spines or tubercles 5
    4. Spores with circular to oblong projections, 4–5(–9) µm wide and up to 3.2 µm high; each projection with a center cavity . . . . . .*A. denticulata* (Fig. 11)
5. Spores with fine crowded, densely organized spines, 1–4 µm tall, 1 µm at base and tapering to 0.5 µm at the tip . . . . . *A. spinosa*

5. Spores with fine tubercles 0.7–3.5 µm long and 1.5 µm broad at the base, tapering to 0.7–1.1 at the rounded tip, irregular distances (0.5–3 µm) between single tubercles . . . . . .*A. tuberculata*

6. Spores with pits. 7

6. Spores with cerebriform folds . . . . . .*A. rehmii* (Fig. 12)

7. Spores in sporocarps, spores 75–80 µm in diameter, ornamentation of 0.5–1µm wide, 4–5 side pits, 1.2 × 0.5–1 µm across, ridges form mesh . . . . *A. taiwania*

7. Spores formed singly in soil, not in sporocarps 8

8. Pits of irregular shape 9

8. Pits of regular round shape 10

9. Spores 100–240 µm in diameter, subhyaline to light olive, circular to ellipsoid to y-shaped pits, 1.0–1.5 × 1.0–3 µm in diameter . . . . . *A. scrobiculata* (Fig. 13)

9. Spores 100–180 µm in diameter reddish-yellow to yellow-brown, with irregular, saucer-shaped pits, 0.2–3 × 0.2–6 µm in diameter . . . . . *A. lacunosa* (Fig. 14)

10. Spores with regular round pits, spores regularly < 100 µm in diameter 11

10. Spores with regular round pits, spores regularly > 100 µm in diameter 12

11. Spores hyaline to subhyaline 13

11. Spores yellow to orange brown, truncated cone shape pits of widest diameter of 1.5–2.2 µm . . . . . *A. alpina* (Figs. 2, 3)

12. Spores regularly 100-180 µm in diameter 14

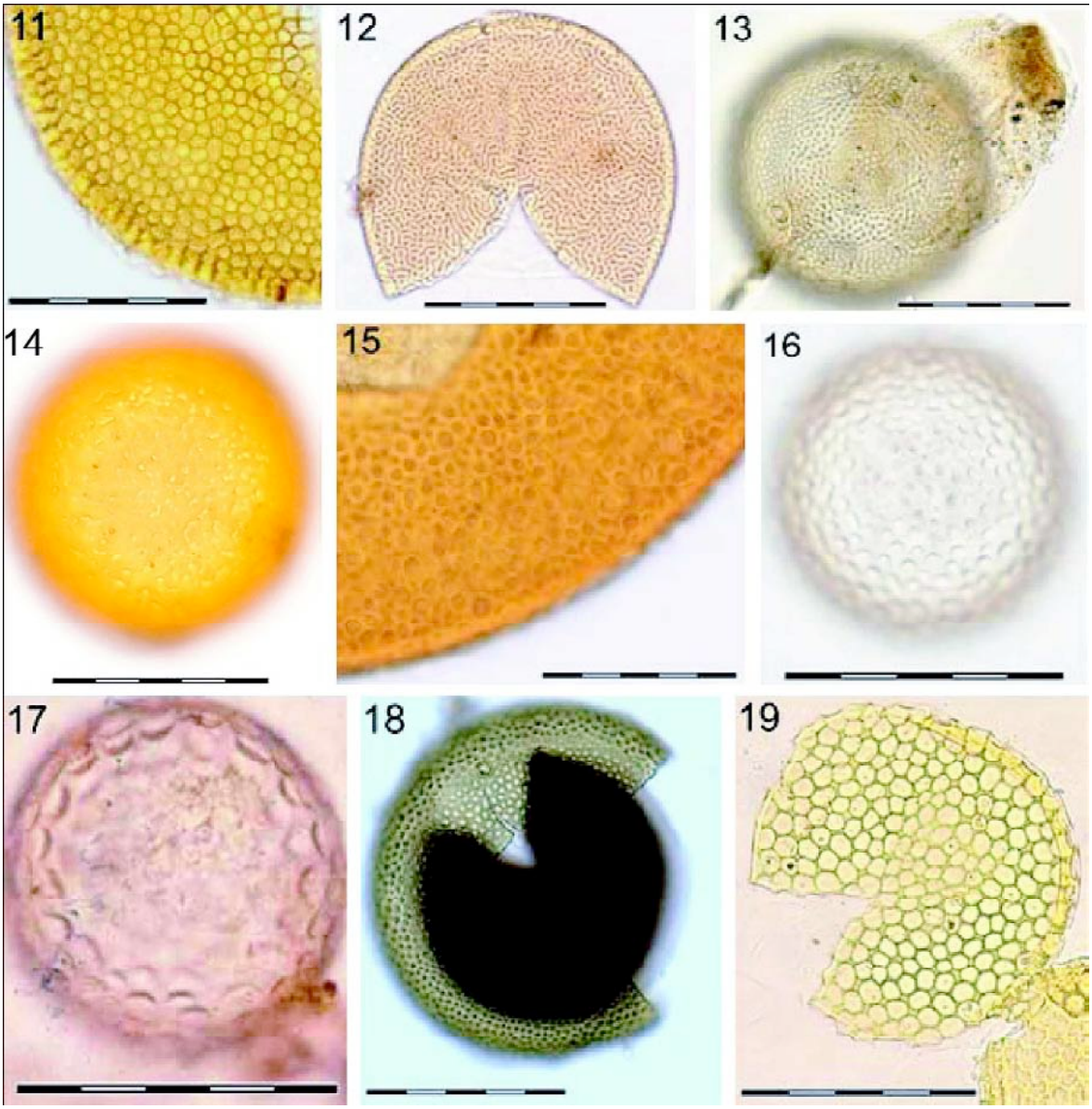
12. Spores regularly >185 µm in diameter with concave round pits of widest diameter 4–10 µm . . . . . *A. foveata* (Fig. 15)

13. Spores hyaline to subhyaline, concave round pits of widest diameter < 3.5 µm . . . . . *A. paulinae* (Fig. 16)

13. Spores hyaline to subhyaline, concave round pits of widest diameter >3.5 µm . . . . . .*A. undulata* (Fig. 17)

14. Spores yellow brown, 115–170 µm in diameter with concave round pits of widest diameter 2–5µm . . . . . .*A. cavernata* (Fig. 18)

14. Spores ochre to brown, 100–180(–200) µm diameter with concave round pits of widest diameter 4–20 µm . . . . . *A. excavata* (Fig.. 19)



Figs. 11–19 *Acaulospora* spp. with ornamented outer spore walls (sw). **11.** *A. denticulata* (ISOTYPE, OSC #46,713) with polygonal knobby projections with a central depression on the rounded tops, scale bar = 50 µm. **12.** *A. rehmii* (ex type, C–116–6, Sieverding collection) with ridges and depressions appearing as cerebriform folds, scale bar = 100 µm. **13.** *A. scrobiculata* (ISOTYPE, BOLIVIA, Santa Cruz de la Sierra, Oehl collection) with regular or y-shaped pits, scale bar = 100 µm. **14.** *A. lacunosa* (isotype obtained from the International Bank for the Glomeromyceta, BEG78) with irregular, saucer-shaped pits, scale bar = 100 µm. **15.** Big-spored *A. foveata* (culture C–48–1, Sieverding collection, described in Schenck et al (1984), scale bar = 100 µm. **16.** *A. paulinae* (ISOTYPE, SWITZERLAND, Therwil (Basel), deposited by Oehl at Z+ZT) with round concave pits, scale bar = 75 µm. **17.** *A. undulata* (ex type, Sieverding collection) with large depressions (undulations), scale bar = 75 µm. **18.** *A. cavernata* (ISOTYPE, GERMANY, Black Forest, Glottertal; deposited by Oehl at Z+ZT) with round pits, 3–5 µm wide, inner wall layer stained purple in Melzer's reagent, scale bar = 100 µm. **19.** *A. excavata* (ISOTYPE, OSC #83,345) with large and deep globose depressions, scale bar = 100 µm.

A2.6. Discussion

Spores of the genus *Acaulospora* share several features. With the exception of *A. undulata* and *A. myriocarpa* Spain, Sieverd. & N.C. Schenck (Schenck et al 1986) (see below), they have three walls (Stürmer and Morton 1999): an outer spore wall (sw), a middle wall (mw) and an inner wall (iw) using our spore wall terminology. The innermost wall (iw) is the wall from where spores germinate and a, so-called germination orb may be involved in the germination process (Spain 1992). The outer wall generally is three-layered, the middle wall is bi-layered and the germinal inner wall is two- or three-layered. Some of these layers are often difficult to discern, e.g., the innermost layer of the outer wall, and some of the layers of the inner wall. Also, the mounting medium can have a strong influence on the visibility of fine wall layers and, therefore, difficult to see layers should be observed in water (Spain 1990).

Despite these common features in all Acaulosporaceae, the spores of the new species, *A. alpina*, can be distinguished easily from all others by the unique surface ornamentation and by a combination of several other morphological characteristics. These are the small spore size, the dark yellow to orange-brown spore color, the apparent absence of a 'beaded' layer in the inner wall and the weak, sometimes absent, staining reaction of iw2 in Melzer's reagent.

Three AM fungal species have similarities in spore morphology with *A. alpina*. *Acaulospora taiwania* shares spore size and spore color, but forms the spores in sporocarps and not singly in the soil as *A. alpina*. Furthermore, the ornamentation on the spore wall of *A. taiwania* consists of 4–5 sided pits that give the appearance of a mesh. The morphological definition of *A. paulinae* is broad and overlaps with *A. alpina*. However, the ornamentation structures of *A. paulinae* are coarser, less regular and consist of concave pits or depressions (Fig. 16), and not of truncated conic depressions as in *A. alpina* (Figs. 5, 8). Also, spores of *A. paulinae* have a significant 'beaded' inner wall layer, and the innermost layer stains strongly in Melzer's reagent. Spores of *A. lacunosa* are similar in color to those of *A. alpina* but they are bigger. Moreover, *A. lacunosa* has irregular depressions on the spore surface, a beaded inner wall layer, and one of the inner wall layers stains dark purple in Melzer's reagent.

Spores size of *A. undulata* is similar to that of *A. alpina*. However, their spore color is white to creamy and the round concave pits are generally wider in diameter. Furthermore, in *A. undulata* the middle wall is lacking and the inner wall bears some similarity to the inner wall of some species of the genus *Archaeospora* J.B. Morton & D. Redecker (Morton and Redecker 2001). The root infection structures of *A. undulata* stain only weakly with trypan-blue, and vesicles were extremely scarce (E. Sieverding, unpublished observations). These features are typical for members of the genus

Archaeospora. Spores of *A. myriocarpa* also lack the middle spore wall (see above) and the root infection structures of *A. mycriocarpa* (Schenck et al 1986) resemble those of Archaeosporaceae (Morton and Redecker 2001), too. Based on these observations it is possible that *A. undulata* and *A. myriocarpa* are both members of Archaeosporaceae.

Phylogenetically, both *A. paulinae* and *A. lacunosa* are clearly separated from *A. alpina* (Fig. 10). These three species are not related to each other. Comparison of sequences obtained from spores to those from field-collected roots allows additional insights into the occurrence and ecological range of AM fungal taxa. The closest relatives to *A. alpina* were detected in roots from a site close to one of our spore sampling sites (Ramosch, Engadin) and from a mountainous grassland (710 m a.s.l.) in Central Germany (Renker et al 2003) (Fig. 10). These data suggest that species related to *A. alpina* may occur at alpine as well as lower-altitude mountainous areas. Our analyses also show that previously unnamed environmental sequences from Costa Rica (AY248771) apparently belong to *A. lacunosa*. Some other previously published sequences from this species (Millner et al 2001) are not related to *A. lacunosa*, and even not to the Glomeromycota. It is likely that the sequences belong to non-Glomeromycota fungi inhabiting AM fungal spores.

Acaulospora lacunosa was described from lower pH soil and soils with high aluminium concentration in West Virginia (Morton 1986). *Acaulospora paulinae* was reported to be widespread in grasslands and arable lands of Poland (Blaszkowski 1993). We found *A. paulinae* frequently in grasslands and arable lands of the Upper Rhine lowland in France, Germany and Switzerland (Oehl et al., 2003, 2004, 2005b) and lower mountainous regions, but rather restricted to de-carbonated soils with pH of 4–6.5. With increasing altitude in the Alps, spores of *A. paulinae* were found in decreasing spore numbers, but spores were found even up to 3000 m a.s.l. (www.nfp48.ch/projekte/projectdocs/17/Wiemken.pdf). In contrast, spores of *A. alpina* were most abundant in grasslands of the high mountainous and alpine regions between 1900–2600 m a.s.l. Above 2700 m a.s.l. the species was rarely found. So far it has not been detected in the lowlands nor, in the Swiss Alps, in mountainous grasslands at altitudes below 1300 m a.s.l.

A2.7. Acknowledgements

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A2.8. References

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Chapter 4

General discussion

4.1. Overall contributions of these studies to science

To our knowledge, this is the first molecular study of root colonizing AMF communities in the European upper montane zone. The finding that host preferences of green gentians for AMF are much lower in comparison to their mycoheterotrophic relatives is new to science as well. In agreement with other studies conducted in species-rich grasslands, clear differences were observed among AMF communities harbored by different plant species co-occurring in a single field site.

Furthermore, different AMF communities were detected in two plant species-rich grasslands in the French lowland on the edge of the Jura mountains and in the Swiss Alps, which indicates some geographical patterns of AMF. In the roots of the same target plant species in the lowland site, different AMF communities were revealed depending on culturing methods of these plants. These results indicate distinct ecological strategies of different AMF species and phylotypes. This finding is of high importance for the general interpretation of greenhouse and field AMF assays.

For the first time, we detected fungi from the order Sebacinales co-occurring with the Glomeromycota in the roots and speculate on their role and relationship to AMF.

4.2. AMF taxa detected by our experimental approach in both investigated field sites

Overall, by far the most abundant group of the Glomeromycota detected in the root samples was *Glomus* group A. This is not surprising, as this subgroup includes the highest number of species of AMF (http://www.lrz-muenchen.de/~schuessler/amphylo/). Its high abundance and diversity is also in agreement with other studies targeting grassland ecosystems, where different phylotypes from this group were the dominant taxa in the investigated field sites (Öpik et al. 2006).

Glomus group B was the second most abundant taxon in both our studies. It occurred predominantly in *Trifolium* sp. samples from the alpine site as well as samples from the greenhouse and field bait plants from the Landskron study. We improved the PCR specificity

for this group using the LETC1677 primer, which turned out to be more efficient than the previously used LETC1670. *Glomus* group B is known for the difficulties in distinguishing its species and in defining sequence types (Rodriguez et al. 2005). Despite this fact, we delimited several highly abundant sequence types (GLOM B1, B-2, B-4 and B-5) in our samples, some of them new to science. AMF belonging to *Glomus* group B were detected in several studies targeting field roots as well as spores in different parts of the world (Jansa et al. 2002; Oehl et al. 2003; Renker et al. 2005; Ahulu et al. 2006; Börstler et al. 2006; Wubet et al. 2006). However, as the majority of other molecular studies focusing on the AMF communities in the field roots (e.g. Vandenkoornhuyse et al. 2002; Scheublin et al. 2004; Santos et al. 2006) used the primer pair AM1/NS31, which has a mismatch in the primer annealing site for the *Glomus* group B, the global diversity and frequency of occurrence of this group it the roots has probably been underestimated. Interestingly, species belonging to this group often appear in trap plant cultures and sporulate there already after several months of greenhouse cultivation (Jansa et al. 2002; Oehl et al. 2004; Oehl et al. 2005).

Sequence types belonging to the family Archaeosporaceae according to its old delimitation (Morton and Redecker 2001) were present in both sites investigated. However, in the Landskron site, only sequences belonging to the clade *A. trappei* were detected. Interestingly, the study of Oehl et al. (2003) did not reveal any spores of *A. trappei* in this field. The sequences detected in our study clustered well with sequences found by Hijri et al. (2006) in roots from trap cultures from different agriculturally used field sites located close the Landskron meadow. These results may indicate predominant intraradical occurrence of this species in the Landskron site. In the Ramosch site, sequences from two other clusters were detected. Both of them would belong to the newly established genus *Appendicispora* (Spain et al. 2006; Walker et al. 2007) and seem to have a broad occurrence. The sequence type ARCH-3 comprises sequences from Ethiopia (Wubet et al. 2003) and Germany (Hempel et al. 2007) and the other one (ARCH-4) has been found in Switzerland (Hijri et al. 2006), USA (Appoloni 2006) and Costa Rica. Similarly to the *Glomus* group B, we can only speculate on the global occurrence and distribution of the Archaeosporaceae and Appendicisporaceae in the roots, as most studies have been conducted using primer pairs, which do not amplify these families.

Although our primer set enables the detection of the broadest range of taxa of all primer sets used (Redecker 2006), we did not find the following taxa: *Glomus* group C (Diversisporaceae), Pacisporaceae, Paraglomeraceae and Gigasporaceae in both the alpine and Landskron site and Acaulosporaceae in the Landskron samples.

The Acaulosporaceae were present only in the Ramosch site. One of the sequence types detected there (ACAU-5) was closely related to the species *Acaulospora alpina*, which we

newly described in mountainous sites close to Ramosch (Oehl et al. 2006), however, it is clearly forming its own clade. The other sequence type, ACAU-1, clustered closely with sequences found by Hijri et al. (2006) in a long-term farming experiment in the Swiss lowland. In other grassland studies, the Acaulosporaceae were relatively common (e.g. Gollotte et al. 2004; Ahulu et al. 2006), with some phylotypes showing strong seasonality with predominant root colonization in winter and spring (Hijri et al. 2006). Interestingly, in the study of Scheublin et al. (2004), one *Acaulospora* phylotype showed strong preference for legume nodules. In the Landskron meadow, neither spores of Acaulosporaceae (Oehl et al. 2003) nor root colonizing phylotypes from this family were found. This might be due to higher pH and calcium content in comparison to Ramosch and other studies where this AMF family was detected (e.g. Gollotte et al. 2004; Börstler et al. 2006). However, as other authors do not always provide pH data for their field sites, we can only speculate on this topic. Oehl et al. (2006) reported a much higher spore abundance of species belonging to the genus *Acaulospora* in high mountainous areas in Switzerland compared to lowlands. From all the findings mentioned above we can draw conclusions about some level of ecological specialization in this family - some species and phylotypes might occur in higher mountainous ecosystems, others might show a trend for seasonality in the root colonization. As we did not conduct any sampling in spring, we cannot test this hypothesis.

Glomus group C (Diversisporaceae) was not detected in any investigated site. This is due to the fact, that our primers do not amplify this group, a specific primer for the *Glomus* group C is being constructed (Redecker et al. 2007). The "*G. fulvum* subgroup" of this family forming large sporocarps seems to occur mainly in tropical ecosystems. However, as AMF belonging to the *G. versiforme/Diversispora spurca* subgroup were detected in several studies targeting temperate grasslands using the primer combination AM1/NS31 or SSU-Glom1/LSU-Glom1 (Wirsel 2004; Renker et al. 2005; Börstler et al. 2006; Santos et al. 2006; Hempel et al. 2007) and their spores were detected in the Landskron field site (Oehl et al. 2003) it would have been of interest to target this group in our samples as well.

The family Pacisporaceae cannot be detected with primers used in our study. The genus *Pacispora* was described only recently and its spores were reported from high altitudes (Oehl and Sieverding 2004), but also from temperate grasslands and intensively managed fields (Oehl et al. 2005), and many other sites with different extent of human influence in Europe (http://www.agro.ar.szczecin.pl/~jblaszkowski/Pacispora%20scintillans.html). However, it has not been reported from any root samples, probably due to the fact, that the different primer combinations used by different research groups do not target this AMF family. Therefore, it would be of high importance to construct a primer set specific for the Pacisporaceae.

The Paraglomeraceae are an ancient AMF family (Morton and Redecker 2001). As it cannot be detected with the primer combination AM1/NS31, it has been overlooked in many molecular AMF diversity studies so far (Öpik et al. 2006). The primer combination ARCH1311AB/ITS4i amplifies this group (Appoloni 2006; Hijri et al. 2006), however, we never detected any of its members. Paraglomeraceae have been reported from root and soil samples from grasslands in Germany (Börstler et al. 2006; Hempel et al. 2007), thermal sites in USA (Appoloni 2006) and arable fields in Switzerland (Hijri et al. 2006), but never in such a high abundance as *Glomus* group A or B. Interestingly, the spores of *P. occultum* were found with a relatively high abundance in the Landskron field site and several other agroecosystems (Oehl et al. 2003) as well as in trap cultures (Oehl et al. 2005), their abundance increased with the increasing intensity of the land use. Therefore, we can hypothesize that members this AMF family might be preferring disturbed or ruderal sites like arable fields or trap cultures. In our sites, they might colonize plant roots to low extent while forming predominantly extraradical mycelium and spores, which would be in agreement with the findings of Hempel et al. (2007) from a grassland in Germany.

We did not detect any member of the family Gigasporaceae in any of the investigated field sites. The genus *Gigaspora* was thought to be absent from Europe (Walker 1992), however, its spores have been recently found in several European arable (Jansa et al. 2002) as well as grassland ecosystems (Börstler et al. 2006; Hempel et al. 2007). The genus *Scutellospora* was detected relatively frequently in roots in field sites with different levels of human impact (Hijri et al. 2006; Santos et al. 2006). Interestingly, some authors described their seasonality in grasslands with higher abundance in roots in winter (Helgason et al. 1999) or in winter and summer but not fall (Heinemeyer et al. 2004). In the Landskron site, the spores of AMF belonging to this family were detected only at a very low abundance (Oehl et al. 2003). From all these observations we can hypothesize that the Gigasporaceae might be rather infrequent in European species-rich nutrient-poor grasslands, with possible seasonal fluctuations in root colonization peaking in winter. As we did not conduct any root sampling in winter, we cannot draw conclusions about this hypothesis.

4.3. AMF species richness and diversity in both field sites; host plant and site preferences of AMF

In the Landskron site, 12 AMF sequence types were detected directly in the field root samples in comparison to Ramosch, where 17 sequence types were found. This difference might be caused by the different numbers of analyzed root samples. In the Ramosch study, 67 root

samples were investigated, which yielded 45 AMF-containing samples, whereas from the 27 investigated Landskron field samples, 19 contained AMF. An important factor may also be the lower number of investigated plant species - four in Landskron in comparison to ten in Ramosch. These arguments are supported by our observations from the Ramosch site, where we clearly showed a trend in increasing number of different detected AMF phylotypes with increasing number of analyzed root samples as well as plant species. However, the 12 and 17 phylotypes found in our field sites, respectively, are within the range of 10-24 phylotypes found by other authors applying molecular methods in temperate grasslands (Öpik et al. 2006).

The field root samples from Ramosch and Landskron shared just four AMF phylotypes, which indicates strong site-dependent differences. This is not surprising, as the two field sites had distinct environmental conditions as well as plant communities, they shared approximately 15 from the total number of 80 plant species growing in each site. A similarly large variation in AMF community composition among distinct grassland locations was reported by Öpik et al. (2006). As the plant communities were different between the sites, different plant species were analyzed in the two studies. This fact may have contributed to the differences in AMF community composition detected between the two sites, because in the Ramosch study clear differences were observed among the AMF phylotypes harbored within the three plant taxa studied in full detail.

The AMF communities of the two meadows located close to each other in Ramosch were compared. The two meadows had similar environmental conditions and shared approximately 50 plant species, 30 were unique to each meadow. The AMF community in both meadows was relatively similar as well, they shared 12 AMF sequence types, three were unique to one meadow, two to the other one. Similarly to the comparison between Landskron and Ramosch, the AMF sequence types unique to one of the two Ramosch meadows had low overall abundance, thus, they can be considered as rare ones.

Based on these findings we can conclude: i) different field sites inhabiting different plant communities and facing different environmental conditions may host different AMF communities; ii) different plant species co-occurring in a single field site may host different AMF communities in their roots.

4.4. Ecological strategies and global distribution of different AMF species or phylotypes

Interestingly, if the root samples from the Landskron bait plant approach are included for calculating the number of AMF sequence types shared by the Ramosch and the Landskron sites, the shared number of phylotypes increases from four to six. This comparison indicates that some AMF present in one field site in the field roots may also have been present in the other site, however, predominantly as spores representing the inoculum potential of this site. These AMF may be detected using the trap culturing or bait plant approach.

Due to technical limitations, we did not conduct a bait plant assay also in the Ramosch study. This might have been an interesting perspective enabling further comparisons of the two investigated sites. It would have been interesting to see whether we would find the same or similar AMF as those ones found in the bait plants in Landskron. Based on the results of these studies and previously published work by other authors, a similar set of rapidly-colonizing r strategists could be expected to be shared between the two sites. Some AMF species quickly appearing in trap cultures as spores have been found in a broad range of environments world wide (e.g. Jansa et al. 2002; Mathimaran et al. 2005; Oliveira et al. 2005; Gai et al. 2006) as well as in roots from arable fields with regular disturbance (Helgason et al. 1998; Daniell et al. 2001; Hijri et al. 2006).

The majority (74%) of the sequence types detected in both our studies was also found by other authors in different parts of the world, which indicates their relatively broad distribution and low site specificity from a global point of view. We could draw conclusions about this topic even though we could not directly compare our results with considerable part of other molecular studies of the root colonizing AMF communities, as their authors targeted different DNA regions. Within a single field site, however, the AMF phylotypes seem to show strong host preferences for different host plants species, as we have reported in the Ramosch study and other authors in the majority of molecular studies focused on this issue (e.g. Vandenkoornhuyse et al. 2003).

By far the most abundant sequence type in field samples from both sites was the ubiquitous *Glomus intraradices*. Indeed, this fungus was revealed to be dominant or highly abundant in most of the molecular diversity studies of root-colonizing AMF in different ecosystems around the globe (Öpik et al. 2006). The second most frequent sequence types we detected were GLOM B-4, which could not be assigned to any morphologically described species in Ramosch, and *Glomus badium* in the Landskron samples. Neither of these phylotypes showed specificity for the field site. Sequences belonging to these clusters have

also been found by other authors (Bidartondo et al. 2002; Wubet et al. 2003; Wirsel 2004; Börstler et al. 2006).

Two of the four AMF phylotypes shared between Landskron and Ramosch field samples – GLOM A-1 (*Glomus intraradices*), GLOM A-17 (*Glomus badium*) – were relatively highly abundant in both field sites. GLOM B-4 and GLOM B-5 were highly abundant preferentially in the *Trifolium* spp. samples in Ramosch, however, in the Landskron study, these phylotypes showed high abundance in the compartment systems and bait plants but not field samples. Rare phylotypes with low overall abundance were not shared between the sites at all. These results are in agreement with the hypothesis of Öpik et al. (2003) and Börstler et al. (2006), who proposed a concept of some AMF species occurring globally with high local abundance and low host specificity. The sequence types GLOM A-1 and A-17 clearly fall into this category. The sequence types GLOM B-4 and B-5 showed relatively strong host preferences for *Trifolium* spp. in the Ramosch site, however, they were also found with low frequency in other plant species in this site. In the Landkron samples, we did not observe any host specificity of these sequence types. The group GLOM B-4 was found also in other studies from different parts of the world (Bidartondo et al. 2002; Börstler et al. 2006). Thus, we can draw a conclusion that they may fall into this category as well.

Based on these findings we can conclude: i) most of the AMF sequence types seem to show a broad global distribution, however, a specific assembly of these globally occurring AMF can be established in each field site, ii) some AMF sequence types species are rarely detected and appear to occur only under specific conditions, iii) some AMF species and sequence types seem to represent r strategists – they prefer uncolonized ruderal habitats and, thus, may be found in the roots of plants growing in e.g. intensively managed agriculture sites as well as in trap cultures or bait plant assays, iv) other AMF - the K strategists - occur mainly in late successional stages of the fungal community and therefore can be found in roots in stable natural plant communities, v) one AMF species - *Glomus intraradices* - is ubiquitous and thus detectable in almost all investigated sites using trap culturing as well as direct root sampling, vi) there are some AMF species or sequence types like *Glomus intraradices* and *G. badium* occurring globally in different grasslands and showing there high local abundance and low host specificity as well.

4.5. Co-occurrence of AMF in plant roots with other endophytic fungi

Using either specific primers for the basidiomycetous order Sebacinales or occasionally also by a non-specific amplification using our AMF-primer set, these investigations revealed that most AMF-colonized roots were co-inhabited by Sebacinales. However, as we did not conduct any morphological and ultrastructural investigations, we cannot answer the question whether these non-AM fungi were predominantly present on the root surface or whether they grew intraradically. We can only hypothesize that the Sebacinales, proven to colonize root cells and intercellular spaces in ericoid plants, orchids and ectomycorrhizal trees (Weiß et al. 2004) and detected in almost all our analyzed root samples, represent probably root endophytic fungi. As we properly washed all the root samples before we prepared aliquots for later DNA extractions, it would be rather unlikely to detect root surface-colonizing fungi in such a high proportion of samples.

A large diversity of fungal endophytes has been reported from different plant tissues, e.g. Ascomycetes as *Lophodermium piceae* from conifer needles (Muller et al. 2001), *Epichloë bromicola* from grass leaves (Groppe et al. 1999) or *Curvularia* sp. from grass roots, crowns, leaves, and seed coats (Redman et al. 2002). Many of these fungi do not cause any disease symptoms of the host plants. There are numerous reports of Asco- and Basidiomycetes colonizing AMF-host plant roots (e.g. Verma et al. 1998; Redman et al. 2002; Renker et al. 2004), and, interestingly, also AMF spores (Hijri et al. 2002). However, in many cases no host specificity was detected and the nature of the relationship between these fungi, AMF and their plant hosts has remained obscure. The most-studied non-AMF root endosymbiont is the sebacinoid fungus *Piriformospora indica* (Deshmukh et al. 2006), as it significantly contributes to host plant fitness.

A relatively well-investigated group of root endophytes are the dark septate endophytes (DSE). These are conidial or sterile ascomycetous fungi colonizing plant roots (Jumpponen and Trappe 1998). The DSE have been reported from various habitats and from a wide range of hosts including species known to be non-mycorrhizal or hosts of arbuscular, ericoid, orchid or ectomycorrhiza. Fuchs and Haselwandter (2004), for instance, found regular DSE colonization in several AMF-host plants from Austrian wetlands. So far, the role of DSE in the ecosystem is not clearly understood, the relationship between host plants and DSE probably ranging from mutualistic to parasitic associations (Jumpponen 2001).

The present study adds a new aspect to the diversity of endophytes in plants and opens an interesting new field of research on the occurrence and function of these ubiquitous fungal symbionts.

4.6. Future perspectives in the field of AMF molecular ecology

In order to compare the results of morphological and molecular studies and to draw conclusions about the global distribution of AMF and their ecological preferences, DNA marker sequences for species, which have been so far described only morphologically, are necessary. This will enable to match morphospecies to sequence types, to compare AMF present as spores and those ones colonizing the roots or growing as mycelium in the soil in a single field site and thus, compare different strategies of AMF.

Furthermore, there is a strong need for a specific PCR primer pair targeting all taxa of the Glomeromycota. As the primer pairs targeting the SSU, ITS and LSU of the nuclear rDNA commonly used in the field studies turned out to miss some taxa, the description of the AMF diversity in the investigated field sites or host plants is not complete. The mitochondrial LSU rDNA (Raab et al. 2005) seems to be a promising marker for this purpose.

Based on the work presented here, it would be highly interesting to investigate more deeply the specific morphological *Paris* type of arbuscular mycorrhiza formed by plants from the family Gentianaceae. Its role in nutrient transport between the fungus and the plant remains unclear, as it often lacks arbuscules which are the places of nutrient exchange in the *Arum* type. Our previous study focused on *Gentiana verna* showed that the coexistence with neighboring plants has a key role for inducing mycorrhizal colonisation in its roots and for its integration into an existing extraradical mycelial (ERM) network (Sýkorová et al. 2003). Such hyphal links could ensure one- or bi-directional transport of assimilates and/or inorganic nutrients between plants. Thus, it would be of high interest to address the impact of AMF on nutrient acquisition by gentian plants, identify the symbionts in the roots and the place of the nutrient exchange between the symbionts and address the importance of hyphal connections for the transfer of mineral nutrients and carbon compounds between the gentians and neighboring plants. Speculations about a possible tendency to mixotrophy in gentians are tempting because some members of the Gentianaceae are achlorophyllous and apparently mycoheterotrophs, i.e. they obtain all of their nutrients via bridges of AMF mycelium from neighboring green mycorrhiza hosts (Imhof and Weber 1997; Bidartondo et al. 2002).

The influence of neighboring plants on the AMF of a given species clearly deserves further attention, also in other plant families than the Gentianaceae. An interesting subject in this context, which may be targeted using molecular methods, are invasive plant species interacting with AMF. These plants have been shown to shift the AMF community of surrounding native plant species (Mummey and Rillig 2006; Hawkes et al. 2006). However, comparisons of the AMF communities of these invasive plant species in their original and

new habitats as well as investigations of altered AMF communities as a possible key factor for the invasive success have not been conducted yet.

Molecular and ultrastructural methods should be used in combination to elucidate the nature of the interaction of AMF with other fungi in the roots. Investigations of the relationship between the AMF, host plants and non-AM fungi might provide evidence for an understanding of the ecology and physiology of the organisms involved and will have possible implications for agricultural systems.

4.7. References

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