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ORIGINAL PAPER

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

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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*) sampled in (1) a plant species-rich calcareous grassland, (2) a bait plant bioassay conducted directly in that grassland, and (3) a greenhouse trap experiment using soil and a transplanted whole plant from that grassland as inoculum. Roots were analyzed by AMF-specific nested polymerase chain reaction, restriction fragment length polymorphism 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, 16 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, *G. mosseae* was abundant after 3 months, but could not be detected anymore after 10 months. In contrast, other phylotypes as GLOM-A17 (*G. 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 (*G. 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 might be 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.

Keywords Arbuscular mycorrhiza · Molecular diversity · rDNA · Life history strategy · Cultivation systems

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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.lrz-muenchen.de/~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, 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: (1) direct root sampling in the field (field samples, FS); (2) cultivation of target plants in compartment systems (CS) in the greenhouse using the field soil and a transplanted field plant as inoculum; (3) 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 to detect seven genera of the Glomeromycota, which is the largest possible portion of AMF taxon diversity recognized so far.

Materials and methods

Field site

The study site was a low-input species-rich grassland close to Leymen in Alsace, France (47°29′16″N, 7°29′16″E; about 490 m above the sea level). It is mown once or twice a 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 1.3 g/100 g. Humus content was >9% (*w*/*w*; laboratory F.M. Balzer, Wetter-Amönau, Germany).

Field samples

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 analyses: the grass *B. erectus* (Poaceae), the forb *Inula salicina* (Asteraceae), the legume *Medicago sativa* (Fabaceae), and the forb *Origanum vulgare* (Lamiaceae). 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, and *M. sativa* and *O. vulgare* grew in a scattered pattern.

Greenhouse experiment with compartment systems

CS (Wyss et al. 1991; Supplementary Fig. 1a) 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 aluminum oxide, oil dry US special, Lobbe Umwelttechnik, Iserlohn, Germany), and nonautoclaved 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 pregerminated in Petri dishes with sterile sand. Two *B. erectus* plants taken directly from the field and, in addition, *P. media* and *L. corniculatus* seedlings were planted into the central compartment 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 CS (Supplementary Fig. 1b). Two CS 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 3 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 three to four 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.

Bait plants in the field site

In July 2004, an experiment with BP (Supplementary Fig. 2) was established 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 about 4 cm was cut out in the lid. A double nylon net (60 μm aperture size; Lanz-Anliker AG) was fixed 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 14 bottles were filled with the same mixture, but the soil was not autoclaved. Five milliliters of a bacterial filtrate from the nonautoclaved soil was added to all bottles.

Two seedlings of one of the target plant species were planted into each bottle (three to four repetitions per plant species per substrate, altogether 29 bottles) and cultivated for 2 months in the greenhouse to ensure initial growth of the plantlets in the bottles. In September 2004, holes were dug out in a grid with approximately 1-m distance between each other in the field site in the same area where the FS were taken. Bottles were inserted into the holes in random order and watered every 4–5 days for 3 weeks. After 3 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 of exposure in the field.

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 polymerase chain reaction (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 s at 94°C, 50 s at 51°C, and 1 min 30 s 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 (*Glomus* group B), ACAU1661/ITS4i (Acaulosporaceae), ARCH1311AB/ITS4i (Archaeosporaceae, Paraglomeraceae), 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 nonspecific 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.

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 reamplified, preferably ten positive clones of each PCR product were digested with *Hin*fI 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 reamplified, 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.

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).

Definition of sequence phylotypes

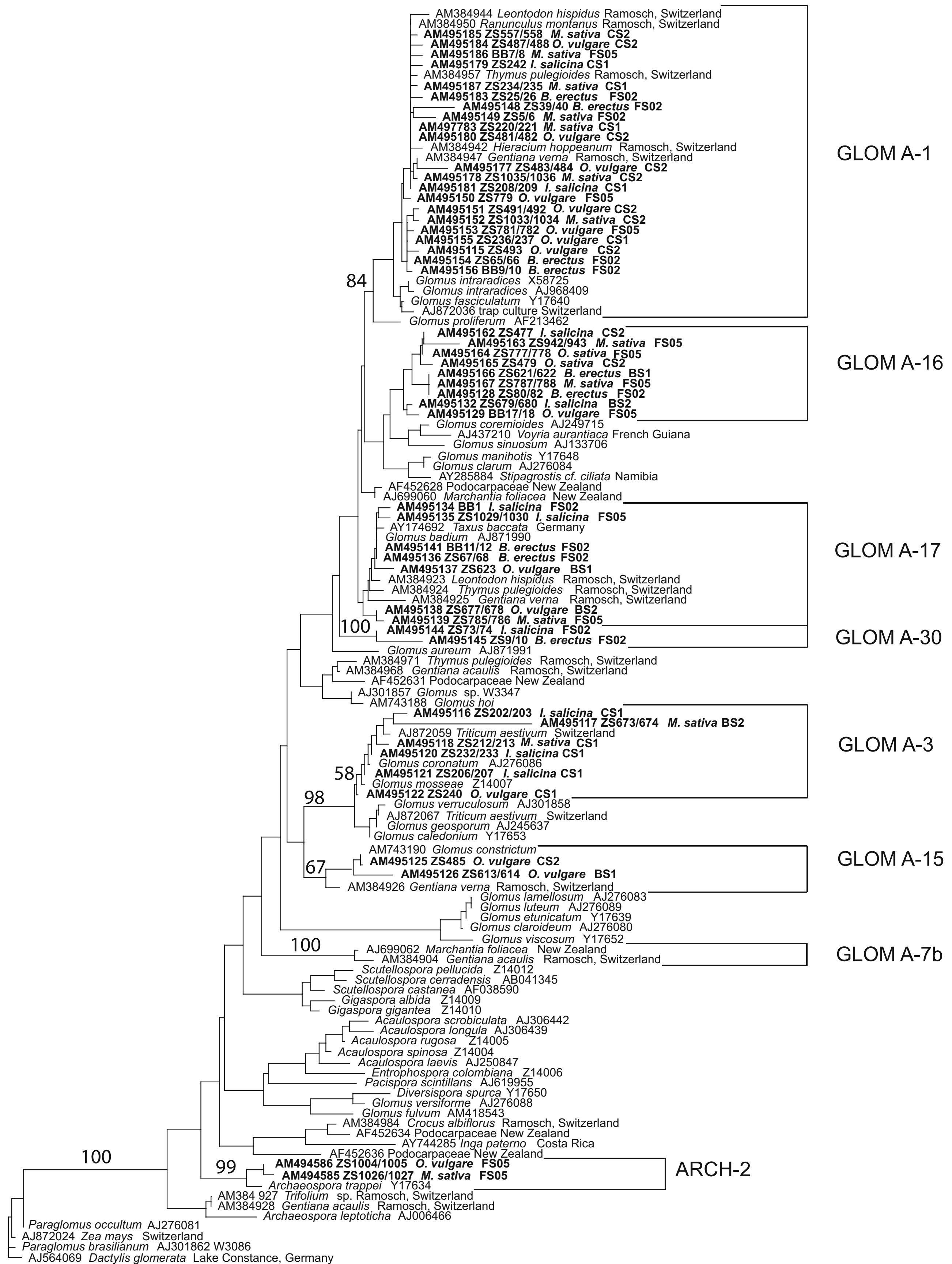
Sequence phylotypes were defined in a conservative manner as consistently separated monophyletic groups in the phylogenetic trees. Only those clades were used that were supported by neighbor-joining analysis and also present in the respective maximum likelihood tree. In case of GLOM-A and ARCH phylotypes, 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-A*x* (*Glomus* group A), GLOM-B*x* (*Glomus* group B), and ARCH-*x* (Archaeosporaceae). Representative sequences of each phylotype were checked manually for possible chimeras, which were excluded from further analyses.

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).

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). To investigate the influence of environmental factors (host plant species, experimental approach, harvest, etc.) 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 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 covariables was used to compare the influence of groups of environmental factors between each other. For example, host plants were considered as covariables 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

Fig. 1 Phylogenetic tree of the Glomeromycota obtained by neighbor-joining analysis of 311 characters of the 18S rDNA subunit. Numbers above branches denote bootstrap values from 1,000 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, first or second harvest are shown (*1 or 2*); for CS first or second harvest (*1 or 2*) are noted. The *parentheses* show the delimitation of the phylotypes ▶



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AM495180 ZS481/482 O. vulgare CS2
AM384942 Hieracium hoppeanum Ramosch, Switzerland
AM384947 Gentiana verna Ramosch, Switzerland
AM495177 ZS483/484 O. vulgare CS2
AM495178 ZS1035/1036 M. sativa CS2
AM495181 ZS208/209 I. salicina CS1
AM495150 ZS779 O. vulgare FS05
AM495151 ZS491/492 O. vulgare CS2
AM495152 ZS1033/1034 M. sativa CS2
AM495153 ZS781/782 O. vulgare FS05
AM495155 ZS236/237 O. vulgare CS1
AM495115 ZS493 O. vulgare CS2
AM495154 ZS65/66 B. erectus FS02
AM495156 BB9/10 B. erectus FS02
84
Glomus intraradices X58725
Glomus intraradices AJ968409
Glomus fasciculatum Y17640
AJ872036 trap culture Switzerland
Glomus proliferum AF213462
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AM495164 ZS777/778 O. sativa FS05
AM495165 ZS479 O. sativa CS2
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AM495167 ZS787/788 M. sativa FS05
AM495128 ZS80/82 B. erectus FS02
AM495132 ZS679/680 I. salicina BS2
AM495129 BB17/18 O. vulgare FS05
GLOM A-16
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AJ437210 Voyria aurantiaca French Guiana
Glomus sinuosum AJ133706
Glomus manihotis Y17648
Glomus clarum AJ276084
AY285884 Stipagrostis cf. ciliata Namibia
AF452628 Podocarpaceae New Zealand
AJ699060 Marchantia foliacea New Zealand
AM495134 BB1 I. salicina FS02
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AY174692 Taxus baccata Germany
Glomus badium AJ871990
AM495141 BB11/12 B. erectus FS02
AM495136 ZS67/68 B. erectus FS02
AM495137 ZS623 O. vulgare BS1
AM384923 Leontodon hispidus Ramosch, Switzerland
AM384924 Thymus pulegioides Ramosch, Switzerland
AM384925 Gentiana verna Ramosch, Switzerland
AM495138 ZS677/678 O. vulgare BS2
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GLOM A-17
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Glomus coronatum AJ276086
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AM495121 ZS206/207 I. salicina CS1
Glomus mosseae Z14007
AM495122 ZS240 O. vulgare CS1
98
Glomus verruculosum AJ301858
AJ872067 Triticum aestivum Switzerland
Glomus geosporum AJ245637
Glomus caledonium Y17653
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Glomus etunicatum Y17639
Glomus claroideum AJ276080
Glomus viscosum Y17652
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Gigaspora gigantea Z14010
Acaulospora scrobiculata AJ306442
Acaulospora longula AJ306439
Acaulospora rugosa Z14005
Acaulospora spinosa Z14004
Acaulospora laevis AJ250847
Entrophospora colombiana Z14006
Pacispora scintillans AJ619955
Diversispora spurca Y17650
Glomus versiforme AJ276088
Glomus fulvum AM418543
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AF452636 Podocarpaceae New Zealand
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ARCH-2
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Archaeospora leptoticha AJ006466
Paraglomus occultum AJ276081
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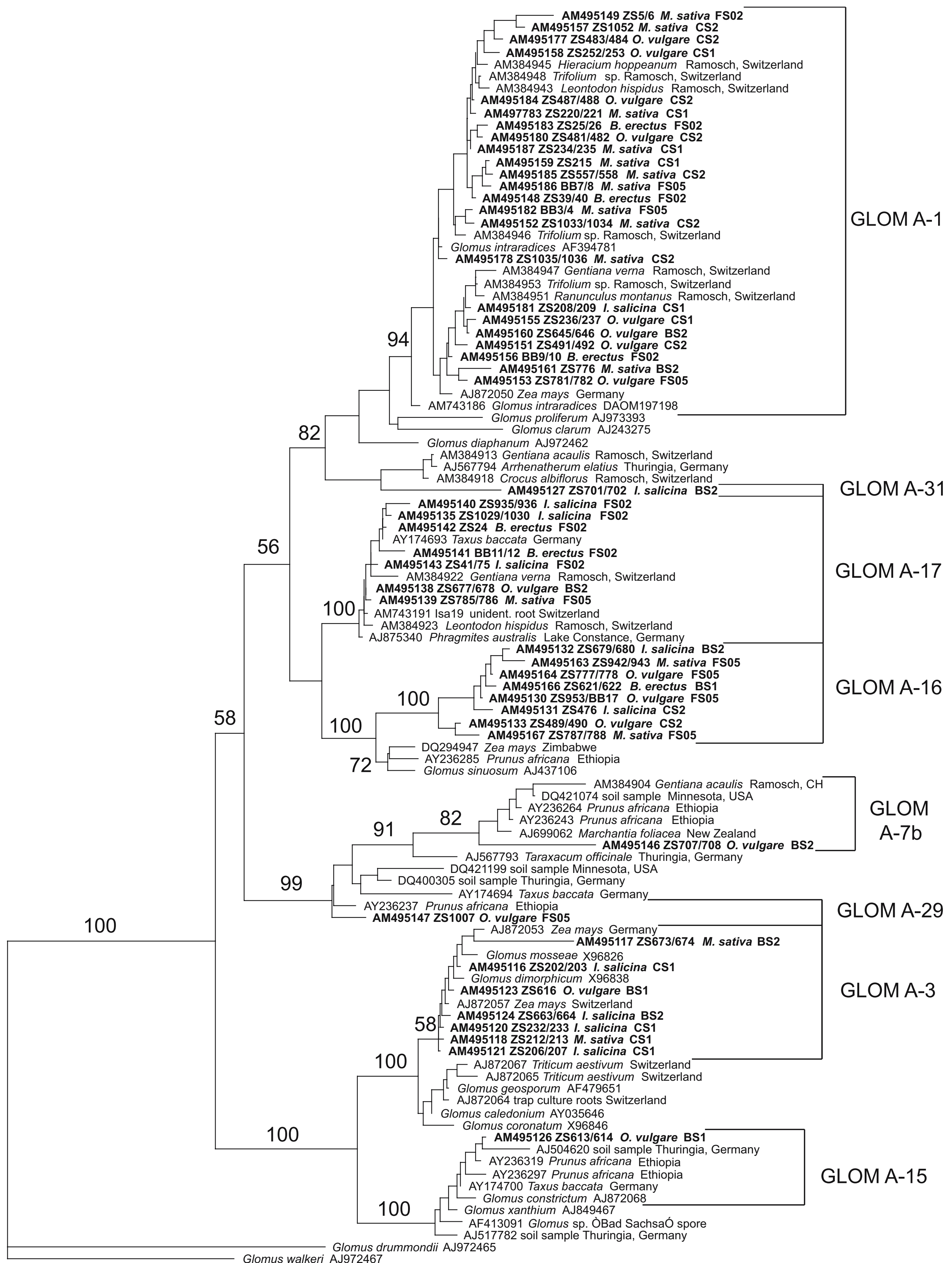
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AM384946 Trifolium sp. Ramosch, Switzerland
Glomus intraradices AF394781
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AM384953 Trifolium sp. Ramosch, Switzerland
AM384951 Ranunculus montanus Ramosch, Switzerland
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AM495155 ZS236/237 O. vulgare CS1
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AM495156 BB9/10 B. erectus FS02
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AM495121 ZS206/207 I. salicina CS1
AJ872067 Triticum aestivum Switzerland
AJ872065 Triticum aestivum Switzerland
Glomus geosporum AF479651
AJ872064 trap culture roots Switzerland
Glomus caledonium AY035646
Glomus coronatum X96846
AM495126 ZS613/614 O. vulgare BS1
AJ504620 soil sample Thuringia, Germany
AY236319 Prunus africana Ethiopia
AY236297 Prunus africana Ethiopia
AY174700 Taxus baccata Germany
Glomus constrictum AJ872068
Glomus xanthium AJ849467
AF413091 Glomus sp. ÒBad SachsaÓ spore
AJ517782 soil sample Thuringia, Germany
Glomus drummondii AJ972465
Glomus walkeri AJ972467
GLOM A-1
GLOM A-31
GLOM A-17
GLOM A-16
GLOM A-7b
GLOM A-29
GLOM A-3
GLOM A-15
94
82
56
100
100
100
72
58
91
82
99
100
58
100
100
100
0.01 substitutions/site

the environmental variables according to their importance and significance for the distribution of the phylotypes.

Results

Polymerase chain reaction yields and phylotypes detected in the root samples

An overview of sampling and phylotypes occurring is presented in Supplementary Table 1. Using our PCR approach with five nested primer sets, 74 of the 97 extracted root samples (76%) yielded 173 PCR products, resulting in 1,182 clones after cloning. A total of 130 PCR products (75%; 938 clones) could be assigned to AMF phylotypes. *I. salicina* root samples from the field and from the second harvests of the CS and BP turned out to be the most problematic—only 25% of these DNA extracts yielded PCR amplicons. Eventually, 19 root samples from the field (four to five replicates/plant species), 20 samples from the BP (three replicates/plant species/harvest), and 31 samples from the CS (five replicates/plant species/CS/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. 1, 2, and 3; Supplementary Fig. 3; Supplementary Table 1). By far, the most abundant phylotype, which was found in 58 root samples, was GLOM A-1 (Figs. 1 and 2). It corresponds to the morphologically defined species *G. intraradices*. The second and third most frequent phylotypes were GLOM B-4 and GLOM B-1 (Fig. 3), which could not be assigned to any morphologically described species, and GLOM A-3 (Figs. 1 and 2), which corresponds to *G. mosseae*. Interestingly, 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 (Supplementary Table 2).

◀ **Fig. 2** 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 1,000 replications. The tree was rooted with *G. walkeri*. Sequences obtained in the present study are shown in *boldface* and are labeled like in Fig. 1. The *parentheses* show the delimitation of the phylotypes

AMF richness and diversity

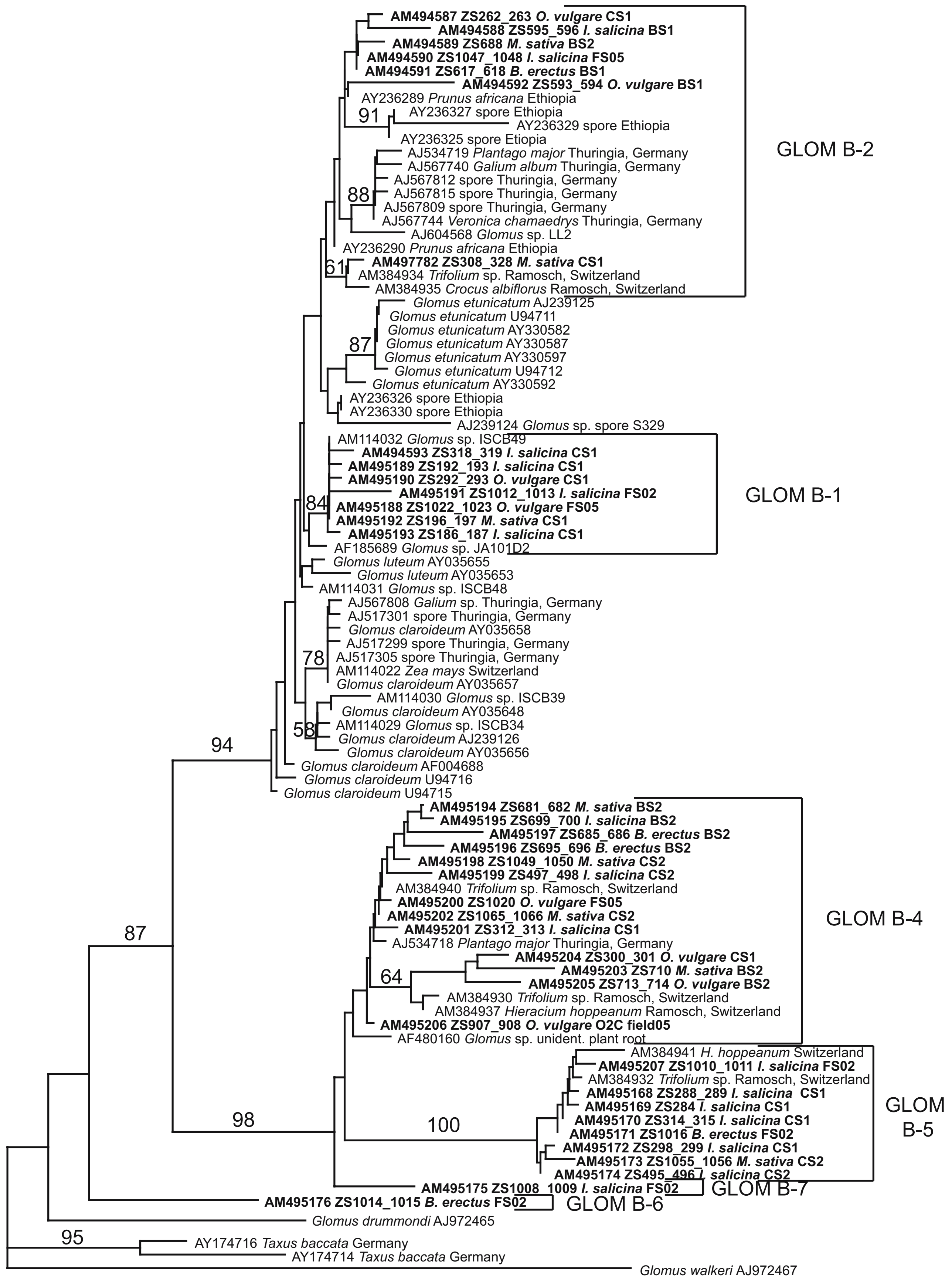
The sampling effort curves (Fig. 4a) showed that, for the CS, the number of analyzed root samples was sufficient to characterize almost exhaustively the phylotypes present in the roots, as the curve clearly approaches saturation. In contrast, the curves for FS and BP are not as clearly saturated but 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 CS (31 samples). To detect one more new phylotype, the analysis of additional four to five field or BP samples would have been necessary. Species accumulation curves calculated for each plant species across all experimental approaches (Fig. 4b) 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 analysis of variance. 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 BP approach and 2.5 in the compartments.

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 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: FS (P=0.002), CS samples (P=0.012), and *O. vulgare* (P=0.028). These results indicate that the root samples originating from field and CS differed significantly from each other as well as from the BP root samples, and that *O. vulgare* differed from all other host plants. The influence of other host plant species on the distribution of the phylotypes was not significant. Variance partitioning showed that the experimental approach accounted for 63% of the variance explained by all environmental factors,


AM494587 ZS262_263 O. vulgare CS1
AM494588 ZS595_596 I. salicina BS1
AM494589 ZS688 M. sativa BS2
AM494590 ZS1047_1048 I. salicina FS05
AM494591 ZS617_618 B. erectus BS1
AM494592 ZS593_594 O. vulgare BS1
AY236289 Prunus africana Ethiopia
91
AY236327 spore Ethiopia
AY236329 spore Ethiopia
AY236325 spore Etiopia
AJ534719 Plantago major Thuringia, Germany
AJ567740 Galium album Thuringia, Germany
AJ567812 spore Thuringia, Germany
AJ567815 spore Thuringia, Germany
88
AJ567809 spore Thuringia, Germany
AJ567744 Veronica chamaedrys Thuringia, Germany
AJ604568 Glomus sp. LL2
AY236290 Prunus africana Ethiopia
61
AM497782 ZS308_328 M. sativa CS1
AM384934 Trifolium sp. Ramosch, Switzerland
AM384935 Crocus albiflorus Ramosch, Switzerland
GLOM B-2
Glomus etunicatum AJ239125
Glomus etunicatum U94711
Glomus etunicatum AY330582
87
Glomus etunicatum AY330587
Glomus etunicatum AY330597
Glomus etunicatum U94712
Glomus etunicatum AY330592
AY236326 spore Ethiopia
AY236330 spore Ethiopia
AJ239124 Glomus sp. spore S329
AM114032 Glomus sp. ISCB49
AM494593 ZS318_319 I. salicina CS1
AM495189 ZS192_193 I. salicina CS1
AM495190 ZS292_293 O. vulgare CS1
AM495191 ZS1012_1013 I. salicina FS02
84
AM495188 ZS1022_1023 O. vulgare FS05
AM495192 ZS196_197 M. sativa CS1
AM495193 ZS186_187 I. salicina CS1
AF185689 Glomus sp. JA101D2
GLOM B-1
Glomus luteum AY035655
Glomus luteum AY035653
AM114031 Glomus sp. ISCB48
AJ567808 Galium sp. Thuringia, Germany
AJ517301 spore Thuringia, Germany
Glomus claroideum AY035658
AJ517299 spore Thuringia, Germany
78
AJ517305 spore Thuringia, Germany
AM114022 Zea mays Switzerland
Glomus claroideum AY035657
AM114030 Glomus sp. ISCB39
Glomus claroideum AY035648
AM114029 Glomus sp. ISCB34
58
Glomus claroideum AJ239126
Glomus claroideum AY035656
94
Glomus claroideum AF004688
Glomus claroideum U94716
Glomus claroideum U94715
AM495194 ZS681_682 M. sativa BS2
AM495195 ZS699_700 I. salicina BS2
AM495197 ZS685_686 B. erectus BS2
AM495196 ZS695_696 B. erectus BS2
AM495198 ZS1049_1050 M. sativa CS2
AM495199 ZS497_498 I. salicina CS2
AM384940 Trifolium sp. Ramosch, Switzerland
AM495200 ZS1020 O. vulgare FS05
AM495202 ZS1065_1066 M. sativa CS2
AM495201 ZS312_313 I. salicina CS1
87
AJ534718 Plantago major Thuringia, Germany
GLOM B-4
AM495204 ZS300_301 O. vulgare CS1
AM495203 ZS710 M. sativa BS2
64
AM495205 ZS713_714 O. vulgare BS2
AM384930 Trifolium sp. Ramosch, Switzerland
AM384937 Hieracium hoppeanum Ramosch, Switzerland
AM495206 ZS907_908 O. vulgare O2C field05
AF480160 Glomus sp. unident. plant root
AM384941 H. hoppeanum Switzerland
AM495207 ZS1010_1011 I. salicina FS02
AM384932 Trifolium sp. Ramosch, Switzerland
AM495168 ZS288_289 I. salicina CS1
AM495169 ZS284 I. salicina CS1
98
100
AM495170 ZS314_315 I. salicina CS1
AM495171 ZS1016 B. erectus FS02
GLOM B-5
AM495172 ZS298_299 I. salicina CS1
AM495173 ZS1055_1056 M. sativa CS2
AM495174 ZS495_496 I. salicina CS2
AM495175 ZS1008_1009 I. salicina FS02
GLOM B-7
AM495176 ZS1014_1015 B. erectus FS02
GLOM B-6
Glomus drummondi AJ972465
95
AY174716 Taxus baccata Germany
AY174714 Taxus baccata Germany
Glomus walkeri AJ972467
0.005 substitutions/site

◀ **Fig. 3** 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 1,000 replications. The tree was rooted using *G. walkeri*. Sequences obtained in the present study are shown in *boldface* and are labeled like in Fig. 1. The *parentheses* show the delimitation of the phylotypes

whereas the host plant species accounted only for 34%. The remaining 3% were explained by the correlation of both groups of factors.

The biplot diagram of this CCA (Fig. 5) 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. 5) indicates in which experimental system they were detected. The relative abundance of each phylotype (Table 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 Table 1. The only phylotype present at relatively high abundance in all three experimental approaches was GLOM A-1.

Field samples—effect of host plant species and sampling year

The CCA focused only on FS (Supplementary Fig. 4) revealed that the host plants and the two sampling years as environmental factors explained 30% of the whole vari-

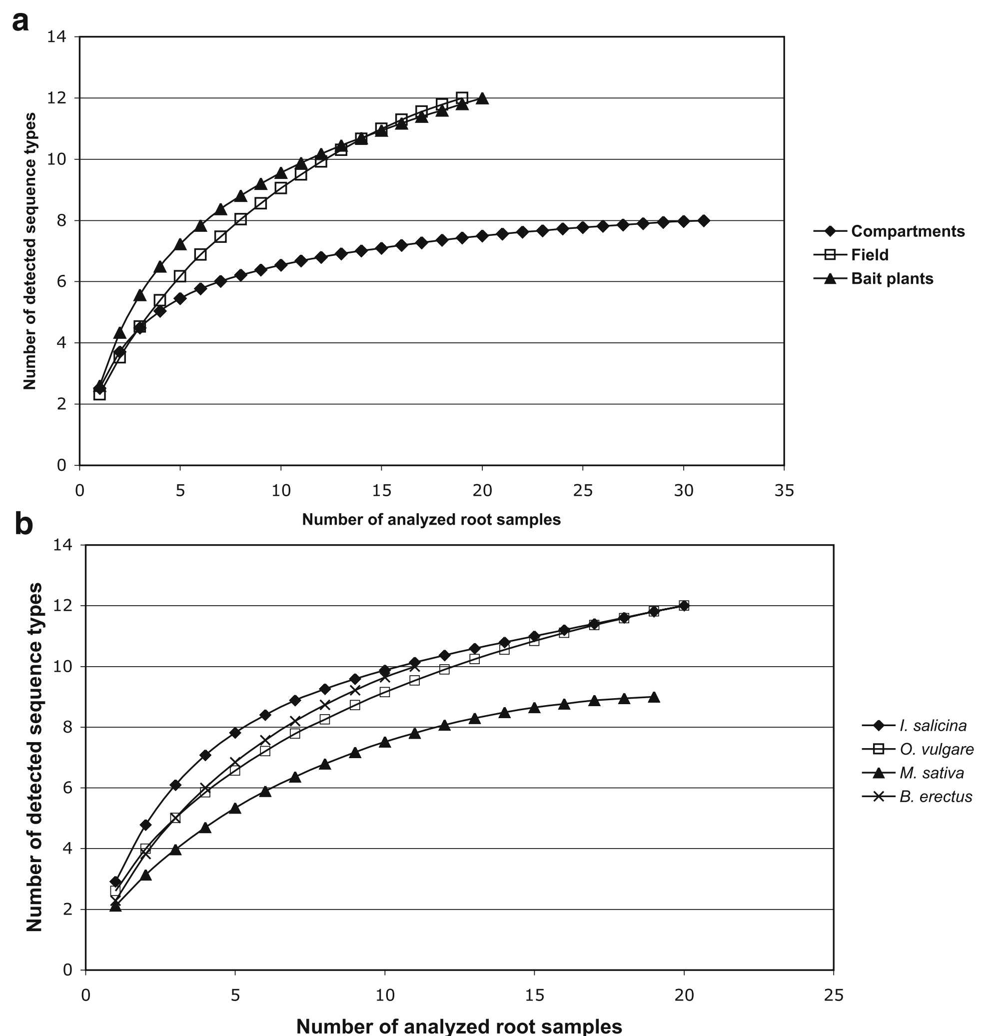


Fig. 4 Sampling effort curve for **a** CS (n=31), FS (n=19), and BP (n=20); **b** for each host plant species: *I. salicina* (n=20), *O. vulgare* (n=20), *M. sativa* (n=19), and *B. erectus* (n=11). The curves were computed analytically in EstimateS 8.0 (Colwell 2005)

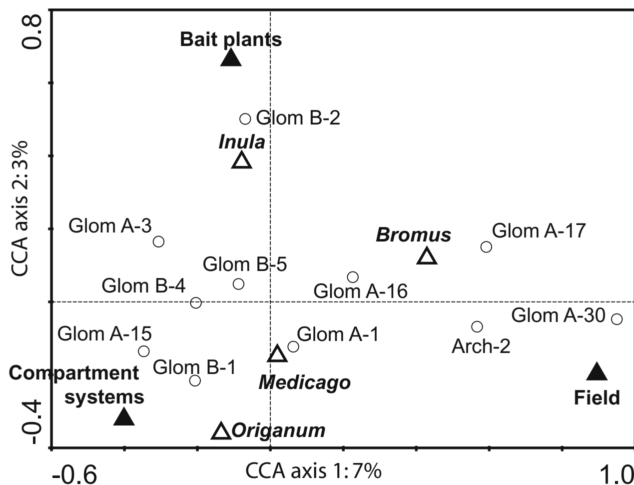


Fig. 5 CCA biplot of the phylotypes and environmental factors (using Hill's scaling focused on inter-species distances) of all samples from all three experimental approaches. Only phylotypes that occurred more than once in the whole study were included in the analysis. The three experimental approaches are represented by *filled triangles*, host plant species by *empty triangles*, and phylotypes by *circles*. The first axis accounted for 42.9% of the variability explained by all canonical axes and was significant (P=0.002). The percentages shown by first and second axis correspond to the percentage of variance of AMF phylotypes data explained by the particular axis

ance, 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 Supplementary Fig. 4 and Supplementary Table 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.

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

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 with the P value closest to 0.05 was *I. salicina* (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. Supplementary Fig. 5 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)

Table 1 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 CS (% of samples)		Relative abundance in the BP (% of samples)		Relative abundance in the field (% of samples)
	First harvest	Second harvest	First harvest	Second 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

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 from 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 FS and BP. 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.

Bait plants—influence of host plant species, duration of cultivation, and substrate treatment

The initial growth of the plantlets was more vigorous in the nonautoclaved 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 BP were generally thriving, except for *M. sativa*, which showed just limited growth with few roots; three plants even died until the second harvest.

The CCA focused on the BP (for biplot, see Supplementary Fig. 6) 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 nonautoclaved 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 vs 3.0). Overall, the samples from the second harvest were slightly higher in species richness (ten phylotypes detected) than samples from the first one (nine phylotypes detected; Supplementary Table 1). The overall number of phylotypes in the autoclaved substrate (ten) did not differ from the nonautoclaved soil.

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 12 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 currently 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, plowing, 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, plowing was identified as a 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 Table 1). Most strikingly, GLOM A-3 (*G. mosseae*) was never detected in the 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 (*G. 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, although 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 Table 1). 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, preferably 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.

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