Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity

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Abstract

Communities of arbuscular mycorrhizal fungi (AMF) in five agricultural field sites of different management intensities were studied. Variable regions of the ribosomal RNA genes were used to detect and identify AMF directly within colonized roots. Roots from a continuous maize monoculture showed low AMF diversity, in agreement with previous reports on molecular diversity of AMF in agricultural soils. In contrast, a substantially higher diversity of AMF was found throughout the long term 'DOK' field experiment, where organic and conventional agricultural practices have been compared side by side since 1978. In this experiment, a 7-year crop rotation is performed under lower levels of inorganic fertilizer input and chemical pest control. These results are in good agreement with analyses of the spore community previously conducted in these field sites. In a third site, an organically managed leek field with soil of very high phosphate content reflecting the highly intensive conventional field history and intensive tillage, we detected a low-diversity community comparable to the maize monoculture. In addition to fungi from *Glomus* group A, which have previously been reported to dominate arable soils, we regularly found members of the genera *Scutellospora*, *Paraglomus* and *Acaulospora*. The genus *Acaulospora* was shown to occur more frequently early in the growing season, suggesting that the life history strategy of AMF may influence the active community at a given time. These data show that the diversity of AMF is not always low in arable soils. Furthermore, low-input agriculture involving crop rotation may provide better conditions to preserve AMF diversity, by preventing the selection for the few AMF taxa tolerating high nutrient levels.

Keywords: arable soils, arbuscular mycorrhiza, community analysis, organic agriculture, rDNA

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Introduction

The involvement of arbuscular mycorrhiza in nutrient uptake of many plants is well established. The great majority of higher plants are hosts of this ancient root symbiosis, among them many important crops. Fossil and molecular evidence suggest that this interaction has accompanied land plants since their origin (Redecker *et al.* 2000a). It has also become clear that arbuscular mycorrhizal fungi (AMF) have a decisive influence on the composition of natural plant communities (van der Heijden *et al.* 1998).

Because of its importance for crop plants (Sieverding 1991), arbuscular mycorrhiza could potentially be used in agriculture to increase crop yields while minimizing the need to rely on chemical fertilizers. However, in spite of numerous studies claiming substantial yield increases, mycorrhizal technology is still far from being routinely applied in agricultural practice. The main reasons are predictability, problems to identify and track fungal species in the field, the poor understanding of the basic biology of AMF and the inability to grow these obligately biotrophic fungi in pure culture.

Over 150 species of the mycosymbionts have been described up to now by morphological features of their spores (Morton 1988; Walker & Trappe 1993). They were previously placed in the order Glomales of the Zygomycota but recently the phylum Glomeromycota was established (Schüßler *et al.* 2001). The production of spores by AMF is highly dependent on physiological conditions and species

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diagnosis requires considerable expertise, both facts that have seriously hampered ecological studies of arbuscular mycorrhiza in the rhizosphere. By their potential to facilitate identification directly from roots, molecular methods hold great promise to give a more realistic estimate of biodiversity and population structure of AMF.

Recent molecular studies have provided some fascinating insights into AMF communities under field settings, among which also agricultural settings were analysed. One of the major breakthroughs in this field of research was the study by Helgason *et al.* (1998). These authors showed that, compared to a woodland, agriculturally used soils were seriously depleted with regard to the species diversity of AMF. The authors attributed this depletion to the regular disturbance by ploughing in the arable soils, which only certain AMF can survive, as well as fertilizer and pesticide input. However, this study and the follow-up by Daniell *et al.* (2001) did not provide any details about soil nutrient contents or farming practice and did not address possible differences among different types of agriculture. Moreover, the molecular identification system used in these studies is known to miss certain groups of AMF (Redecker *et al.* 2000b). Therefore, these studies are very likely to have missed considerable parts of the diversity. Other studies in different types of ecosystems used polymerase chain reaction (PCR) primers that detect only a small fraction of the whole diversity in the Glomeromycota (van Tuinen *et al.* 1998; Kjøller & Rosendahl 2001; Jansa *et al.* 2003). Most of them focused on *Glomus* group A (as defined by Schwarzott *et al.* 2001). In order to study the largest possible portion of taxon diversity in the Glomeromycota in arable soils, we used the set of PCR primers designed by Redecker (2000) which allows to detect seven genera of Glomeromycota.

In contrast to molecular methods that allow to characterize the currently active symbiotic AMF community in the roots at any given time, spore-based analyses define the fungal inoculum in the soils studied. AMF communities in agricultural systems have also been analysed by these conventional nonmolecular approaches. Oehl *et al.* (2003, 2004) analysed AMF communities from a wide range of field sites on Loess soils in the three-country corner Switzerland/France/Germany by spore collection and trap culturing. Two of these field sites were also analysed in the present study. These authors reported an inverse relationship between management intensity and AMF diversity: the highest number of morphospecies in arable soils was found in organically managed fields, and even higher numbers in seminatural grasslands. They also presented evidence for the survival in lower soil layers of some AMF species that may not be adapted to disturbance by ploughing. Oehl *et al.* (2005) showed that only long-term trap culturing reveals some slowly sporulating AMF species. The number of morphospecies found even in the most intensively managed site outnumbered the sequence diversity previously reported by Helgason *et al.* (1998).

One of the locations studied here and by Oehl *et al.* (2003, 2004, 2005) is the so-called 'DOK experiment'. In this long-term field trial, organic and conventional management has been compared side by side since 1978. Chemical and biological soil parameters have been studied extensively. Mäder *et al.* (2002) reported consistently enhanced biodiversity and soil fertility in the organic plots and concluded that organic farming may constitute a sustainable alternative to conventional farming systems. This experiment offers a unique opportunity to study the influence of agricultural management practice on AMF communities under well-controlled conditions.

In the present study, we used the molecular identification method developed by Redecker (2000) to characterize the diversity of AMF communities in the DOK experiment, an intensively managed maize monoculture and an organically managed leek field. The aim of this study was to investigate the relationship between management intensity and the diversity of the symbiotically active AMF community in the crop roots. We also intended to elucidate the spatial structure of AMF communities in arable sites. Spore analyses provide a relatively indirect measure of AMF diversity by describing the inoculum potential of the soil, and the problems caused by possible sporulation bias are well known. Moreover, the spores in a field site reflect the accumulated sporulation history of the respective soil and not necessarily the current symbionts of the crop. We therefore wanted to test whether the inverse relationship between management intensity and AMF diversity indicated by the spore-based studies of Oehl *et al.* (2003, 2004, 2005) would also be found in the fungal community within roots. Our hypothesis was that low AMF diversity in arable soils is not inevitable and inversely correlated to management intensity.

Materials and methods

Field sites

Selected chemical soil parameters and management practices of the sites are shown in Table 1. Sites R, K, O and M are situated on Loess soils in the Upper Rhine valley. Site R near Rheinweiler, Germany, is a maize monoculture with no regular crop rotation. Sites K, O and M are plots in the 'DOK experiment' near Therwil, Switzerland, representing conventional 'Swiss integrated production', bio-organic, and mineral fertilization treatments, respectively. This long-term experiment has been conducted since 1978 by Agroscope FAL Reckenholz (Zurich, Switzerland) and the Research Institute of Organic Agriculture (FiBL, Frick, Switzerland). The abbreviation 'DOK' stands for the main treatments bio-dynamic, bio-organic and conventional. Two

Table 1 Chemical parameters of soils in the sites studied

| Site | Management | Fertilization | | P content (mg P/kg) | pH | Crop rotation |
		N (kg/ha)	P (kg/ha)			
U	organic	0	0	59	7.0	+
R	conventional, mineral fertilization	170*	60*	11	7.7	−
M	conventional, mineral fertilization†	124	41	7.4	4.8	+
K	conventional†	154	39	10	5.4	+
O	organic	96	27	3.9	5.2	+

*From Oehl *et al.* (2003). †According to Swiss Integrated Production rules (reduced pesticide and fertilizer input). Sites K, O and M belong to the 'DOK' long-term field experiment.

other treatments in this experiment are nonfertilized (N) and mineral fertilization (M). The whole experiment is subjected to a seven-year crop rotation; the single treatments are managed according to the respective management system. For additional details about the sites, see Oehl *et al.* (2003, 2004) and Mäder *et al.* (2002). The crop rotation scheme and other information on the DOK experiment can be found at www.fibl.org/english/research/annual-crops/dok/index.php. All field plots in the DOK experiment are numbered, and the exact plots analysed here are indicated by the number after the letter that stands for the treatment (e.g. M40).

Site U is an organically managed field in Muri, Switzerland. Its soil phosphate content is several folds higher than in any of the other sites (Table 1). The soil is a sandy loam with 5% organic matter. At the time of sampling in August 2003, *Allium porrum* cv. Prelina was grown in the field which had been preceded by a clover-grass meadow the year before. The leek was pregrown in the greenhouse and planted into the site in mid-April. The growth medium in the greenhouse had been amended with 2 kg/m^3 horn shavings in the potting substrate. As no additional fertilization took place afterwards, the overall N and P input is negligible. The field site has been used since 1933 for horticultural agricultural production. Until 1996, high amounts of mineral fertilizer and pesticides were used, before the field was converted to organic farming. Now, nutrient budgets are balanced and chemical inputs are avoided. However, tillage is still relatively intensive with yearly ploughing and several applications of a rotary harrow. The crop rotation comprises 2 years of grass/clover, 1 year of vegetables, 1 year of maize and 1 year of cereals. The extremely high P content in the soil is not a result of organic farming but reflects the high nutrient input from when it was managed conventionally. According to organic standards, it has to be covered by grass/clover at least 10–20% of the time to restore soil fertility.

Sampling

In the DOK experiment, root samples were taken in July 2002 (see Table 2 and Table S1, Supplementary material).

Soil cores were sampled with a corer of 10 cm diameter to a depth of 15 cm. In each 20 by 10 m plot, roots were sampled along a transect at three sampling points A, B, C each point separated by 5 m. Sampling point B was in the centre of the plot. Roots were washed from the soil and were blotted dry on filter paper. Aliquots of 50 mg were assembled by randomly taking pieces from different parts of the root mass, frozen in liquid nitrogen and stored at −80 °C until use. From each of the sampling points, three aliquots (designated by numbers 1, 2, 3, etc.) were extracted and analysed, with the exception of plot K64 (see Table S1). The sampled plots at that time were planted with maize and wheat, respectively. These samples were analysed for all six glomeromycotan groups by the molecular identification procedure described below. Another set of samples from the K, O and M plots was taken from these and some additional plots in July 2002 and May 2003 to analyse the seasonal variation of the genus *Acaulospora* only (Table 3). The location of the root cores within the plots followed the scheme outlined above, i.e. in plots K62, M40 and O18 the same sampling points were sampled in 2002 and 2003.

Sampling in the R field took place in July 2002. Five soil cores were taken in the maize field along a similar transect as in the DOK experiment. In the U site, samples were taken in August 2003. Five plants were dug out randomly from four plots, each 14 m by 2.25 m. AMF were analysed using two aliquots taken from the pooled roots of each plot. The field experiment contained two adjacent rows of plots along a distance of 210 m. The sampled plots U13 and U59 were separated by a distance of 140 m, U14 and U40 were located in the second row at a distance of 70 m from each other. Root aliquots were taken from the plants and processed as described above.

Additional samples taken in October 2001 from grass/clover meadows M38, N37 and K20 in the DOK trial were not analysed as thoroughly as the 2002 samples by the molecular identification procedure described below. Four interesting phylotypes from these analyses that are new to science were included in the phylogenetic tree in Fig. 1.

In the initial stages of the project in May 2001, roots from trap cultures of field sites R, K, O, M were sampled to

Table 2 AMF phylotypes found in each soil core in the field. Field sites are R, U, M40, K64 and O18 as explained in Table 1. Columns show the five subgroups of Glomeromycota detected using a specific PCR primer. The numbers within each column represent the phylotypes detected (e.g. GLOM-A1, GLOM-A2). No phylotypes of *Archaeospora* were found in any of these soil cores, therefore no column is shown for this genus. Sites K, O and M are within the DOK experiment. Samples were taken in July 2002 from R, M, K and O, and in August 2003 from U. in For detailed within-core data, see Table S1, Supplementary material

Soil core (sampling point)	Acaulosporaceae ACAU-	Paraglomus PARA-	Gigasporaceae GIGA-	Glomus group A GLOM-A	Glomus group B GLOM-B
Maize monoculture:					
R-A				1, 3	
R-B				1, 3	
R-C					
R-E				1, 3	
R-F				1, 3, 5	
Organic leek field:					
U13				1	
U14				1	
U59				1, 3	
U40				1, 3, 4	
Maize, mineral fertilization:					
M40A		1	1	1, 3	
M40B		1		1	
M40C		1	1	1, 3, 4	
Conventionally managed maize:					
K62A		1		1, 3	
K62B		1	1	3	1
K62C			1	2, 3	
Conventionally managed wheat:					
K64A	1	1			
K64B	1		1	1, 3	
K64C			1	1, 2, 3, 4	
Organically managed maize					
O18A	2	1	1	1	
O18B		1	1, 2	3	
O18C	2	1		1, 3	

obtain a preliminary overview of the AMF to be expected in the field and to optimize the methods. One root sample was analysed from a trap culture of each plot. The cultures had been set up in April 2000. Roots were washed from 1.5 cm by 10 cm cores taken from the trap cultures. The setup and maintenance of these trap cultures used to characterize the AMF spore communities of the field sites was described by Oehl *et al.* (2003).

Molecular identification

DNA extraction. Roots were ground in liquid nitrogen with pestle and mortar or with a pellet pestle within the 1.5 mL tube. DNA was extracted from roots with a DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. DNA was eluted in one step in 30 µL elution buffer. Purified DNA was used as template for the first PCR of the nested procedure. Glomeromycotan spores were obtained from soil samples or trap cultures as described by Oehl *et al.* (2003). Crude DNA extracts from spores were obtained as described by Redecker *et al.* (1997) and used as template for the PCRs.

Polymerase chain reaction. PCR was performed in a nested procedure as described by Redecker (2000) using *Taq* polymerase from Amersham or Applied Biosystems, 2 mM $MgCl_2$, 0.5 µM primers and 0.25 mM of each desoxynucleotide phosphate. The first round of amplification was performed using universal eukaryote primers NS5 and ITS4 (White *et al.* 1990). The cycling parameters were 3 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s of 51 °C and 2 min at 72 °C. The programme was concluded by a final extension phase of 10 min at 72 °C.

PCR products were diluted 1:100 in TE buffer and used as template in the second round. Five separate PCRs were performed using the following primer pairs (Redecker 2000; Redecker *et al.* 2003): ARCH1311AB/ITS4i, ACAU1661/ITS4i, LETC1670/ITS4i, GLOM1310/ITS4i, NS5/GIGA5.8R. In addition to these primers used in the standard procedure, other primers were designed for special purposes: GLOMBS1670

Table 3 Occurrence of *Acaulospora* phylotypes ACAU-1, 2, 3 in wheat and maize-grown plots of the DOK trial at two sampling times. K62, K64 are plots managed conventionally, M40 and M58 are managed conventionally with mineral fertilization, O18 and O80 are managed according to organic agriculture. A, B, C denote different soil cores within a plot, the numerical indices 1, 2, 3 indicate the root aliquots within each core. Light shading indicates data that are also presented in Table 2 and Table S1. The remaining samples in the unshaded areas were exclusively analysed for the presence of *Acaulospora*

K plots	*Triticum* spring 03 K62	*Triticum* summer 02 K64	*Zea* summer 02 K62
A1		ACAU-1	
A2	ACAU-1		
A3			
B1		ACAU-1	
B2	ACAU-1	ACAU-1	
B2			
C1	ACAU-1		
C2			
C3			

M plots	*Triticum* spring 03 M40	*Triticum* summer 02 M58	*Zea* summer 02 M40
A1	ACAU-3		
A2	ACAU-3		
A3	ACAU-3		
B1	ACAU-3		
B2	ACAU-3		
B2	ACAU-3		
C1	ACAU-3		
C2	ACAU-3		
C3	ACAU-3		

O plots	*Triticum* spring 03 O18	*Triticum* summer 02 O80	*Zea* summer 02 O18
A1			
A2			
A3			ACAU-2
B1	ACAU-3		
B2	ACAU-3		
B2	ACAU-3		
C1			ACAU-2
C2			
C3			ACAU-2

(AGCTTTAACCGGCATCTGT) for the *Glomus mosseae* subgroup within *Glomus* group A, and PARA1313 (CTAAAT-AGCCAGGCTGTTCTC) for the genus *Paraglomus*.

The PCR parameters for the second round were 3 min at 95 °C, followed by 30 cycles of 45 s at 95 °C, 50 s of 61 °C and 1.5 min at 72 °C. The programme was concluded by a final extension phase of 10 min at 72 °C. A 'hot start' at 61 °C was performed manually to prevent nonspecific amplification. In order to control the success of amplification, PCR products from the second round of nested PCR were run on agarose gels (2%:1% NuSieve/SeaKem, FMC) in Tris/Acetate buffer at 90V for 45 min.

Cloning and sequencing

PCR products were cloned into a pGEM-T vector (Promega/Catalys). Inserts were re-amplified, digested with *Hin*fI and *Mbo*I and run on agarose gels. Restriction fragment patterns were compared to a database based on the spreadsheet developed by Dickie *et al.* (2003). Representative PCR products of new restriction types were purified using a High Pure Kit from Hoffmann LaRoche and sequenced in both directions. A BigDye Terminator Cycle Sequencing Kit (ABI) was used for labelling. Samples were run on an ABI 310 capillary sequencer. Sequences were deposited in the EMBL database under the accession numbers shown in the phylogenetic trees.

Sequence analysis

Sequences were aligned to previously published sequences in PAUP*4b10 (Swofford 2001). The glomeromycotan origin of the sequences was verified by BLAST (Altschul *et al.* 1997) and by phylogenetic analysis of an alignment of 5.8S subunit sequences (Redecker *et al.* 1999). ITS alignments were made for each of the target groups of the specific primers LETC1670, ACAU1661 and GLOM1310. *Archaeospora* and *Paraglomus*-like sequences amplified with ARCH1311 were placed in two separate alignments. In addition, an alignment of the partial 3' end of the 18S rDNA small subunit was compiled (Bidartondo *et al.* 2002) for the 18S sequences amplified with GIGA5.8R, GLOM1310 and ARCH1311. Representative sequences of each sequence type were checked manually for possible chimaeras, which were excluded from the analysis. As 12 out of the 13 sequence phylotypes shown in Table 2 and Table S1 were recovered from several root samples independently, the influence of undetected chimaeric sequences on our conclusions would be negligible.

Phylogenetic trees were primarily obtained by distance analysis using the neighbour-joining algorithm in PAUP* using the Kimura 2-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

Single morphospecies and even individual spores of Glomeromycota contain multiple slightly differing variants of rDNA

sequences. Thus, it is not possible to assign a single sequence to a certain species or a fungal isolate. Sequence phylotypes were therefore defined in a conservative manner as consistently separated monophyletic groups in the phylogenetic trees. Only clades that were supported by neighbour joining bootstrap analyses and were present in the respective maximum-likelihood tree, were used. We avoided splitting of lineages unless there was positive evidence for doing so. Therefore we expected to underestimate the true AMF diversity rather than overestimating it. The sequence phylotypes were designated after the major AMF clade they were placed in, followed by a numerical index (x in the following examples) identifying the type: GLOM-Ax (*Glomus* group A), GLOM-Bx (*Glomus* group B), ACAU-x (Acaulosporaceae), GIGA-x (Gigasporaceae), PARA-x (*Paraglomus*).

Statistical analyses

Shannon diversity indices were calculated for each plot from the presence/absence data for each root aliquot. To ensure an approximately equal number of root samples across field sites, only three of the sampling points in field R were included, comprising nine aliquots. Jaccard similarity indices were calculated among all three sampling points within plots in the DOK experiment. Indices for adjacent and distant plots were then pooled and subjected to ANOVA to test for significant differences between the two pools of distant and adjacent sampling points. For chi-squared tests and ANOVA, the program NCSS was used (NCSS). The sampling effort was assessed using ESTIMATES 7.5 (Colwell 2005) to randomize sample order in 100 replications. Ordination analyses were conducted in CANOCO for Windows version 4.5 (ter Braak & Smilauer 2004). Phylotype abundance was coded in the species matrix as the frequency of occurrence in the three root aliquots of each core (0 = absence, 1 = presence in all three aliquots). Abundances were weighed previous to ordination by a factor derived from the average frequency of the respective phylotype in the whole study. This method was used to reduce possible artifacts by rare phylotypes that occurred in only one site. Canonical Correspondence Analysis (CCA) was run although the gradient lengths of previous Detrended Correspondence Analysis (DCA) reached only 2.9. CCA sorts out subtle differences among relative contributions of species better than Redundancy Analysis (RDA) that stresses absolute species abundance. Forward selection tests were conducted using 500 permutations.

Results

Phylotypes detected in the field sites

The AMF community was analysed in detail from 60 root samples, yielding approximately 1130 clones of the PCR products. After restriction fragment length polymorphism (RFLP) screening, approximately 400 clones were sequenced and analysed phylogenetically. Representative sequences of each sequence phylotype were submitted to the EMBL database and are included in the phylogenetic trees shown in Figs 1 and 2 and Figs S1 to S4 of the Supplementary material.

Overall, five genera of AMF were detected. Only a subset of the sequence types could be assigned to named species because they were nested within a clade of spore-derived sequences. By far the most abundant and diverse group in the field sites was *Glomus* group A (as defined by Schwarzott *et al.* 2001), with five sequence types (Fig. 1, see also Figs S1 and S2) A representative of this group was found in 95% of root samples that yielded a glomeromycotan PCR product (Table S1). The most frequent phylotypes were GLOM-A1 (*Glomus intraradices*), with an overall frequency of 100% in the R field and 70% in the DOK trial, and GLOM-A3 (*Glomus mosseae*) which occurred in 70% of all positive samples in the R field, and 77% in the DOK experiment plots. GLOM-A4 was closely related to *Glomus geosporum*. GLOM-A2 was related to *Glomus intraradices*, but was consistently distinguished from this species (Fig. 1, supplementary material), in particular by unique sequence motifs in ITS1. GLOM-A5 and GLOM-A6 are not closely related to any known species. *Glomus* group B was detected much less frequently, and only one sequence type related to *Glomus claroideum* was defined (Fig. S3, Table S1).

Three phylotypes of *Acaulospora* were found (Fig. 2). One of them (ACAU-2) could be identified as *Acaulospora paulinae* because sequences of the same type were obtained from spores grown in trap cultures (Oehl *et al.* 2003, 2004). A similar phylotype (ACAU-3) was found in the same field site. It corresponds to an undescribed species with spores similar to *A. paulinae* (Fritz Oehl, unpublished). The two sequence types form separate clades in the phylogenetic tree, and were clearly distinguishable by sequence motifs. They could never be obtained from the same spore, suggesting genetically different taxa. The morphospecies corresponding to ACAU-1, the most frequently found phylotype of that genus, is not known. A *Scutellospora* phylotype (GIGA-1) related to *Scutellospora gilmorei* (Fig. 1) was detected frequently. The rarely found GIGA-2 most likely was a *Gigaspora* species. A *Paraglomus* phylotype was regularly found which was clearly distinct from all other *Paraglomus* ITS sequences and may represent a third species of this genus (Fig. S4). Spores of an undescribed *Paraglomus* species (C. Walker, personal communication) found in a calcareous grassland in the Basel area in Switzerland contained sequences of the same phylotype (not shown).

Additional phylotypes were found in samples from other field plots, which were not as systematically sampled as the ones listed in Table 2, and therefore not included for
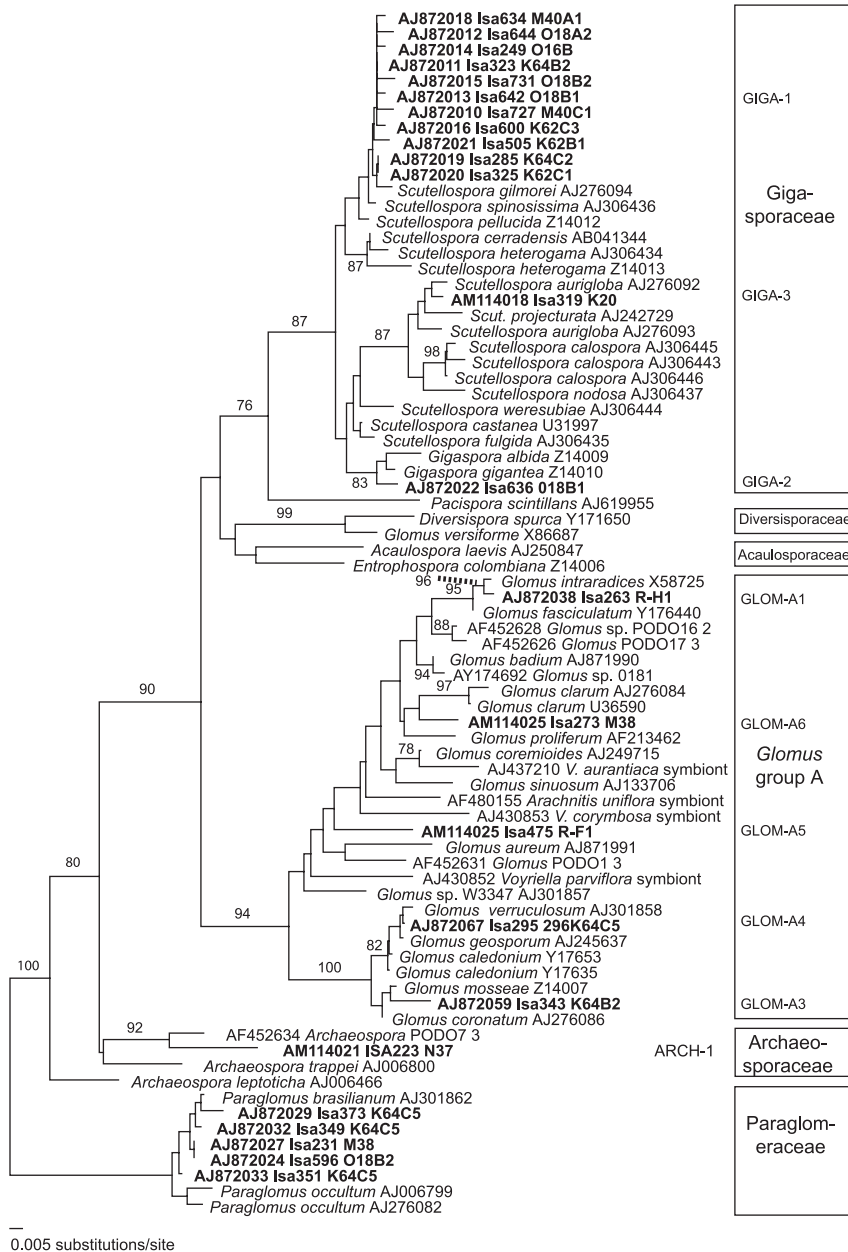
Fig. 1 Phylogenetic tree of the Glomeromycota based on partial small subunit sequences. The tree was obtained by neighbour joining of 301 characters. Numbers above branches denote bootstrap values from 1000 replications. Sequences obtained in the present study are shown in boldface. They are labelled with the database accession number (e.g. AJ872018), the internal identification number (e.g. Isa634), and the root sample they were obtained from (e.g. plot M40, core A, root aliquot 1). Sequences obtained from spores are labelled with the species name, the accession number and the isolate code, if applicable. The boxes to the right show the glomeromycotan subgroups and the delimitations of the phylotypes. Note that GLOM-A phylotypes with the exception of GLOM-A5 and GLOM-A6 were defined based on ITS sequences and only one representative sequence each is shown here.

0.005 substitutions/site

further analysis of the data. One of them was a rare phylotype of *Archaeospora*, which is only distantly related to the three known *Archaeospora* species (Fig. 1). It was found in a clover-grass meadow in a nonfertilized plot of the DOK experiment (N37). No sequence of *Archaeospora* could be obtained from any arable wheat or maize field. Furthermore, an additional *Scutellospora* type (GIGA-3), was detected in wheat roots in the conventionally managed plot K20.

The root aliquots and samples that did not yield any PCR products were most probably not colonized. This is consistent with the relatively low level of colonization in the R field, where many 'negative' samples were found (Table S1).

Trap cultures

In addition to the field-collected root samples, roots from trap cultures were analysed which had previously been set up from the field sites to study the spore communities. The studied trap cultures originated from the arable fields of the DOK experiment (K, O, M treatments) and the R field. These roots were not studied as systematically as the field-collected roots, but nevertheless showed some interesting differences with respect to the AMF phylotypes found. Among the phylotypes detected were GLOM-A1 (*Glomus intraradices*) and ACAU-1, which were also found directly
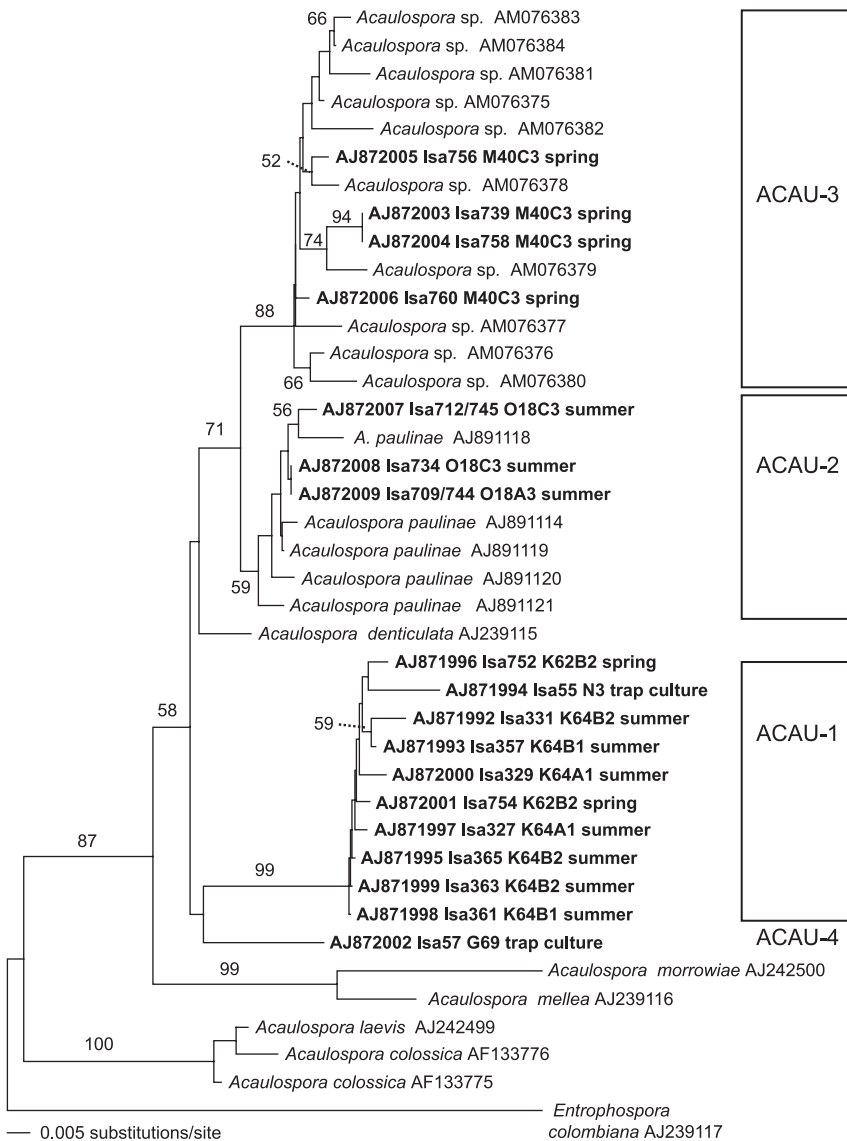
Fig. 2 Phylogenetic tree of the Acaulosporaceae based on ITS2 and 5.8S rDNA sequences. The tree was obtained by neighbour joining of 315 characters. Numbers above branches denote bootstrap values from 1000 replications. Sequences obtained from roots in the present study are shown in boldface. They are labelled with the database accession number (e.g. AJ872018), the internal identification number (e.g. Isa634), and the root sample they were obtained from (e.g. plot M40, core A, root aliquot 1). Sequences obtained from spores are labelled with the species name and the database accession number. Sequences of *Acaulospora paulinae* are from (Oehl *et al.* in press), sequences of *Acaulospora* sp. are new in the present study. The boxes to the right show the delimitations of the phylotypes.

in the DOK experiment. *Archaeospora trappei*, however, was only detected in the trap cultures and never in the field-collected roots. On the other hand, GLOM-A3 (*Glomus mosseae*), PARA-1 and the *Scutellospora* types were never detected in any trap culture. In order to exclude the possibility that those taxa were 'obscured' by others amplified with the same primer, specific primers were designed for the *G. mosseae* group (GLOM-BS1670) and *Paraglomus* (PARA1313). Even with these primers, none of the two taxa could be detected in the trap cultures. Interestingly, spores of *G. mosseae* and *Scutellospora* spp. were in fact found in the trap cultures, the latter only in fall (Oehl *et al.* 2004).

Sampling issues and spatial structure

We explored the possible influence of sampling effort on the diversity of AMF communities by constructing a sampling effort curve (Fig. 3). For field sites R and U, there is a clear leveling-off of the curve and it is very unlikely that more intensive sampling would have revealed more phylotypes. Furthermore, the AMF diversity in R and U remains considerably lower than in K, O and M, the treatments in the DOK experiment. For the field sites in the DOK experiment, the number of phylotypes also approaches saturation and the curve indicates that in order to find one more phylotype, the number of samples would have to be approximately doubled. These data clearly show that the sampling effort per plot was sufficient to allow the detection of the majority of phylotypes. The intention behind our sampling design was to intensively explore the spatial structure of diversity of AMF within the field plots, rather than to compare the different treatments in the DOK experiment. Interestingly, Table S1 shows that although the same taxa were encountered again and again all over
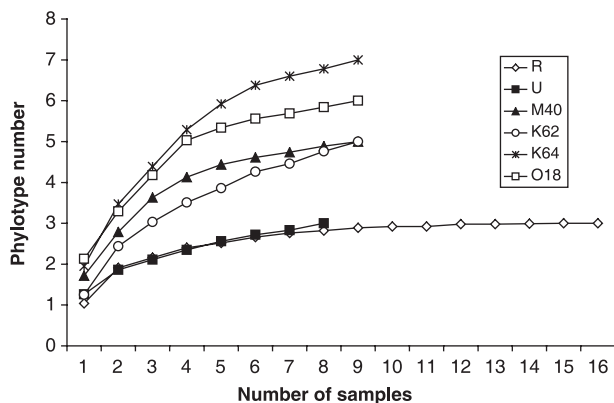
Fig. 3 Sampling effort curve for each of the plots studied. Sample order was randomized by 100 replications in ESTIMATES 7.5 (Colwell 2005). The codes R, U, M40, K62, K64 and O18 stand for field sites and field plots (see also Table 1): R, conventional/mineral fertilization, maize; U, organic, leek; M40, conventional/mineral fertilization, maize; K62, conventional, maize; K64, conventional, wheat; O18, organic, maize.

Fig. 4 Shannon diversity index H′ of AMF in the different treatments. Error bars show standard deviation among three to five sampling points, respectively. Letters denote significant differences at the $P = 0.05$ level in Fisher's LSD multiple comparison test. The codes R, U, M40, K62, K64 and O18 stand for field sites and field plots (see Table 1 and Materials and methods).

the DOK trial, two root aliquots from the same soil core never contained the identical set of AMF phylotypes. In the DOK experiment, the average Jaccard similiarity coefficient of aliquots within cores (average 0.407, standard deviation 0.222) was similar to the coefficient among cores of the same plot (average 0.458, standard deviation 0.112). There was a trend among cores to be more divergent if they were distant (10 m apart, average Jaccard coefficient 0.333 ± 0.209) than if they were adjacent (5 m apart, average Jaccard coefficient 0.463 ± 0.094). However, it was significant only in K62, K64 and M40 (0.244 ± 0.135 vs. 0.479 ± 0.092, $P = 0.016$ in ANOVA), because O18 showed the opposite trend.

AMF diversity in the different plots and field sites

The intensively managed field sites R and U clearly showed a considerably lower AMF diversity than K, O and M both in terms of quantity as well as quality: several families of AMF (Acaulosporaceae, Gigasporaceae, Paraglomeraceae) are completely absent from R and U, but consistently present in K, O and M. The AMF community in R and U exclusively consisted of members of *Glomus* group A (Table 2). With the exception of the rare type GLOM-A5, the AMF community in U and R was a subset of the phylotypes found in K, O and M. The most frequent types GLOM-A1 and GLOM-A3 were detected in all field sites. The Shannon diversity index showed a clear difference between sites R/U and the DOK experiment. The diversity in O was significantly higher than in R and U, and U is significantly different from all DOK plots (Fig. 4). Phylotype richness in U and R was significantly different from K64 and O by Fisher's LSD multiple comparison test (not shown).
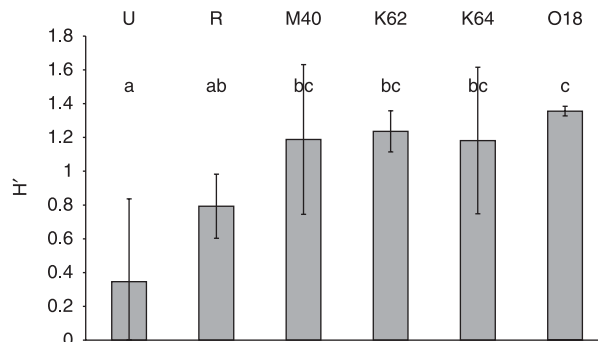
CCA results showed that AMF communities in sites R and U were clearly different from those in the DOK experiment (Fig. 5), even though there is also some variation among the communities in the different plots. The differences most strongly correspond with the environmental factors soil P content and pH along the first CCA axis. The vectors representing fertilization appeared to point in a different direction, which is due to the presently low fertilization in field site U, which had the highest P content and pH. When excluding this field site from the analysis, all vectors pointed in a similar direction (not shown).

The forward selection procedure revealed the ranking of the environmental variables in their importance for determining the phylotype data, which corresponds to the part of variance each environmental variable explained at the time it was included into the model. Variables with conditional effects (constrained variables, which contributed to the model), were pH, K64, K62, O18 and the N-fertilization. Only pH had a significant ($P = 0.008$) influence on the variance. The field plots K64, K62 and O18 were the most species-rich plots, while the plots R and U hosted mainly a subset of the phylotypes present in these fields.

In an alternative analysis excluding all soil and fertilization parameters, forward selection indicated that AMF communities in R and U were significantly different from each other and the DOK treatments (U: $P = 0.02$; R: $P = 0.03$).

Seasonal occurrence of Acaulospora

In order to study in further detail the temporal variability of AMF communities, additional root samples were assessed for the genus *Acaulospora*. In addition to the samples from July 2002, roots samples from May 2003 were analysed (Table 3). Based on hints from previous studies (Daniell *et al.* 2001), we hypothesized that *Acaulospora* preferentially colonizes roots in the spring (when winter wheat is far ahead of maize in
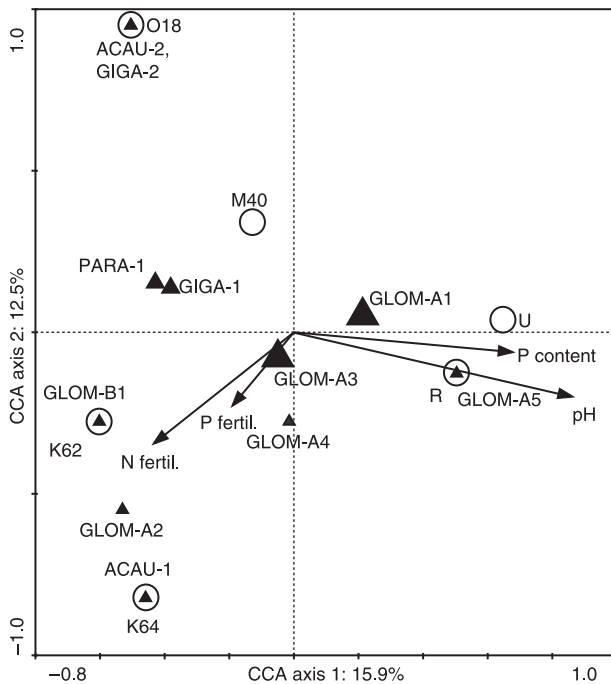
Fig. 5 CCA-biplot of the phylotype data: AMF phylotypes as triangles; environmental factors as arrows (soil parameters) and circles (nominal variables: plots). Average frequencies of the phylotypes are displayed in three triangle sizes corresponding with the classes 0.05–0.2, 0.21–0.5, > 0.5, respectively. An overlay of circles (plot) and triangles points to the fact that these phylotypes uniquely occurred in the respective plot. All environmental variables explained 41.3% of the total variance. The significance (according to the Monte Carlo permutation test) of all canonical axes was $P = 0.032$, which means that the environmental factors have a significant influence on the distribution of the phylotypes. The first canonical axis (which is strongly correlated with the variable 'P content') was marginally nonsignificant ($P = 0.058$).

growth) and may persist in winter wheat at higher frequency throughout the summer as a result of this early spring activity. Indeed *Acaulospora* phylotypes occurred significantly more frequently in spring than in summer ($P < 0.001$), but there was a strong heterogeneity among fields. The ACAU-3 phylotype was exclusively found in plots M40 and O18 in spring 2003, but not in samples from the same plots taken in July 2002. Likewise, ACAU-1 was found in K62 in spring 2003, but not in summer 2002. These data suggest a strong seasonal turnover of AMF symbionts in the arable fields. On the other hand, the data did not lend support to the hypothesis that *Acaulospora* occurs more frequently in wheat than in maize in the summer: it was not detected in M58 and O80 and overall was not detected more frequently in wheat.

Discussion

To our knowledge, this is the most extensive molecular analysis of the diversity of AMF communities in arable

soils conducted so far. Previous studies on the topic used PCR primers that are known to exclude several deeply divergent lineages of the Glomeromycota (Helgason *et al.* 1998; Daniell *et al.* 2001) or assessed only a small subset of known taxa (Kjøller & Rosendahl 2001; Jansa *et al.* 2003). Neither of these studies demonstrated that their sampling effort was sufficient.

With the exception of site U, the AMF spore communities in the field sites had been analysed previously (Oehl *et al.* 2003, 2004, 2005). In agreement with those results, this study revealed an inverse relationship between management intensity and morphospecies richness, as well as a relatively high species richness throughout the DOK experiment.

These data do not merely reflect a lower overall colonization level in R and U as a result of higher soil phosphate content in those sites. Under that scenario, GLOM-A1 and GLOM-A3 might be the most abundant types in all sites and the only ones detectable in R and U because the other phylotypes do not exceed the detection threshold. If that was true, the two most common phylotypes ought to have been in higher abundance in K, O and M than in R and U, but that was clearly not the case.

The overall numbers of morphospecies reported by Oehl *et al.* (2003, 2004, 2005) were generally higher than the numbers of phylotypes shown here. However, the two methods used are not directly comparable. Whereas the procedure of collecting, propagating and enumerating spores measures the inoculum potential of a soil, molecular detection of fungi within roots determines the symbiotically active community at a given time. Discrepancies between those two methods are therefore to be expected and have been reported previously (Renker *et al.* 2003). It is quite plausible that diversity of AMF present in the spore bank is higher than the diversity within the roots. In fact, it may be easier to recover rare species by spore collection, as this method allows to screen relatively large amounts of soil and spores at one time, whereas molecular identification has a stochastic problem to encounter those taxa in the small amounts of root tissue that can be analysed and the limited number of clones that can be screened from each PCR product. The finding that *Glomus mosseae* (GLOM-A3) and *Scutellospora* were found in the trap cultures as spores but were never detected within the roots of the trap culture plants may be due to a discrepancy between prolific sporulation and relatively low root colonization, or seasonality. *Glomus constrictum* was detected occasionally in some samples in site U using a primer specifically designed for this species (results not shown). This finding indicates that a certain amount of taxa may be missed using group-specific primers as it is not feasible to use a separate primer for every species-level taxon. However, compared to using a single primer for all Glomeromycota, using multiple group-specific PCR primers decreases the risk of missing less abundant phylotypes in the presence of a more abundant type that is amplified with the same primer.

The data we present on AMF in roots of trap culture indicate that (i) symbiont communities in the roots of trap cultures can differ strongly from the situation in the respective field site and (ii) not all taxa present as spores are detected by PCR of the roots, either because they are not symbiotically active at the respective time or colonize the roots at levels below the detection threshold. Discrepancies between trap culture roots and field-collected roots are likely to be the result of the conditions in the pot cultures, which may favour certain AMF taxa. As the trap culture samples were taken in May and the field samples in July, there may also be some influence of seasonality. For instance, *Paraglomus* was never detected in trap cultures, although it occurred frequently in the DOK experiment. On the other side, *Archaeospora trappei* was frequent in the trap cultures, but never found in the arable soils. These findings suggest that trap cultures follow their own dynamics and their roots may provide a different picture of AMF communities than analysis of field-collected roots.

Season-dependent fluctuations in AMF communities were previously reported from a tropical forest (Husband *et al*. 2002). In arable soils, Acaulosporaceae preferentially sporulate in spring, which may indicate that these fungi may be more active in the early year (Fritz Oehl, unpublished). This notion was also discussed by Daniell *et al*. (2001). However, the molecular data presented in that article show that this AMF family was most frequently detected in September in relatively low frequencies, making it difficult to draw conclusions. We provide molecular evidence supporting preferential early season activity of Acaulosporaceae and possible life history strategy preferences of an AMF taxon in arable soils. Our results suggest that seasonality of AMF in arable soils has to be considered for a better understanding of the processes involved in community dynamics of AMF and therefore deserves further study. Our data unequivocally show that some arable soils show an unexpectedly high diversity of AMF, which is in strong contrast to the studies by Daniell *et al*. (2001) and Helgason *et al*. (1998). These authors reported a strongly reduced number of AMF taxa in roots from all agriculturally used soils they tested. The fungi detected by these authors most frequently were also members of *Glomus* group A and partly correspond to the *Glomus* phylotypes we detected in sites R and U. Whereas *Glomus intraradices* was most frequent in our analyses, followed by *G. mosseae*, the most frequently found phylotypes detected by Daniell *et al*. (2001) were related to *Glomus geosporum/caledonium*, followed by *G. mosseae*. Over 2 years, these authors detected 10 fungal types in 32 samplings from four arable fields. In their June and July samples, which may be most appropriate to compare to ours, they detected zero to four phylotypes per field (average: 2.4). Although *Acaulospora* and *Scutellospora* were occasionally found, they were relatively rare: only 12.5%

and 6.25% of all samplings, respectively, contained fungi of these genera, none of them though in June and July. In contrast, in the DOK experiment, *Acaulospora* was detected in 4 of the 12 sampled soil cores and *Scutellospora* in eight (33% and 66%, respectively). *Paraglomus* was not detected at all in the previous study, as the PCR primer AM1 does not amplify members of this genus. We frequently found a *Paraglomus* phylotype in the DOK experiment, but it was never detected in U and R. Members of *Glomus* group C, which are not detectable with our primers, were rarely found by Daniell *et al*. (2001).

Even the conventionally managed treatments in the DOK experiment showed a higher diversity than the more intensively used field R. The different agricultural treatments within the DOK experiment appeared to have a less pronounced impact on the AMF community than on other soil biota that have been analysed previously in this long-term trial (Mäder *et al*. 2002). However, even the K and M treatments in the DOK experiment are managed according to the rules of Swiss Integrated Management. This kind of management is less intensive than other 'conventional' management schemes (e.g. in site R), and requires a balanced nutrient budget, proper crop rotation and integrated plant protection. The phosphate content of the soil, the fertilization and the pesticide treatment are all higher in R, and, probably most significantly, there is no crop rotation. These factors may explain the striking difference between a conventionally managed field like R on one side and M/K on the other side. AMF taxa like *Paraglomus*, *Acaulospora* and *Scutellospora* appear to be sensitive to one or a combination of these parameters. Another potentially important factor is the soil pH, but the data of Oehl *et al*. (2003, 2004) do not provide evidence for general relationship between AMF diversity and lower soil pH, as the highest diversity these authors found was in a calcareous grassland. As all the sites studied here are ploughed, soil disturbance alone cannot be the sole factor responsible for reduced diversity. However, the intensity of tillage differs among the sites: sites K and M are ploughed shallowly (15–18 cm) every second year on average, whereas site R is ploughed deeper (down to 30 cm) every year. Therefore, tillage intensity may also influence AMF diversity, which is agreement with the studies by Jansa *et al*. (2002, 2003). Organic management and crop rotation also involves shorter periods of time during which the soil is not covered by plants and a more diverse accompanying weed flora, both factors that may potentially contribute to higher AMF diversity.

The low AMF diversity in the organically managed site U may appear surprising at first sight. However, in spite of its present organic management including low fertilization and crop rotation, the U field has very high levels of phosphate in the soil due to earlier high-input management. As high phosphate concentrations are known to reduce AMF root colonization, it is very likely that AMF diversity is

concurrently reduced. It may therefore take decades of low-input management in this site to reduce phosphate to levels that allow establishment of a diverse AM fungal community.

Finally, there appears to be no simple correlation between organic management and high AMF diversity, and in order to predict AMF diversity, knowledge regarding environmental conditions and site history is needed. However, our results also suggest that low input management including crop rotation is more likely to provide the conditions necessary to preserve AMF diversity in the long term. High-input agriculture has a profound effect on AMF diversity and selects for a certain reduced subset of AMF taxa. Our results suggest that this deprivation can be avoided to maintain the diversity of AMF in arable soils as a possible agricultural resource of the future.

Acknowledgements

Supplementary material

The supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2921/MEC2921sm.htm

Fig. S1 Phylogenetic tree of the *Glomus intraradices* subgroup of *Glomus* group A.

Fig. S2 Phylogenetic tree of the *Glomus mosseae* subgroup of *Glomus* group A.

Fig. S3 Phylogenetic tree of *Glomus* group B.

Fig. S4 Phylogenetic tree of the genus *Paraglomus*.

Table S1 AMF phylotypes found in each root sample.

References

Altschul SF, Madden TL, Schaeffer AA *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389–3402.

Bidartondo MI, Redecker D, Hijri I *et al.* (2002) Epiparasitic plants specialized on arbuscular mycorrhizal fungi. *Nature*, **419**, 389–393.

ter Braak CJF, Smilauer P (2004) CANOCO reference manual and CANO-DRAW for Windows user's guide: software for canonical community ordination (version 4.5). Biometris, Wageningen, Netherlands; Ceské Budejovice, Czech Republic.

Colwell RC (2005) ESTIMATES: Statistical estimation of species richness and shared species from samples. *Version 7.5*. http://purl.oclc.org/estimates

Daniell TJ, Husband R, Fitter AH, Young JPW (2001) Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. *FEMS Microbiology Ecology*, **36**, 203–209.

Dickie IA, Avis PG, McLaughlin DJ, Reich PB (2003) Good-Enough RFLP Matcher (GERM) program. *Mycorrhiza*, **13**, 171–172.

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? *Nature*, **394**, 431.

Husband R, Herre EA, Turner SL, Gallery R, Young JPW (2002) Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. *Molecular Ecology*, **11**, 2669–2678.

Jansa J, Mozafar A, Anken T *et al.* (2002) Diversity and structure of AMF communities as affected by tillage in a temperate soil. *Mycorrhiza*, **12**, 225–234.

Jansa J, Mozafar A, Kuhn G *et al.* (2003) Soil tillage affects the community structure of mycorrhizal fungi in maize roots. *Ecological Applications*, **13**, 1164–1176.

Kjøller R, Rosendahl S (2001) Molecular diversity of Glomalean (arbuscular mycorrhizal) fungi determined as distinct *Glomus* specific DNA sequences from roots of field grown peas. *Mycological Research*, **105**, 1027–1032.

Mäder P, Fliessbach A, Dubois D *et al.* (2002) Soil fertility and biodiversity in organic farming. *Science*, **296**, 1694–1697.

Morton JB (1988) Taxonomy of VA mycorrhizal fungi: classification, nomenclature, and identification. *Mycotaxon*, **32**, 267–324.

Oehl F, Sieverding E, Ineichen K *et al.* (2003) Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of Central Europe. *Applied and Environmental Microbiology*, **69**, 2816–2824.

Oehl F, Sieverding E, Mäder P *et al.* (2004) Impact of long-term conventional and organic farming on the diversity of arbuscular mycorrhizal fungi. *Oecologia*, **138**, 574–583.

Oehl F, Sieverding E, Ineichen K *et al.* (2005) Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. *New Phytologist*, **165**, 273–283.

Oehl F, Sykorová Z, Redecker D, Wiemken A, Sieverding E (in press) *Acaulospora alpina*, a new arbuscular mycorrhizal fungal species characteristic for high mountainous and alpine regions of the Swiss Alps, Mycologia, in press.

Posada D (2004) MODELTEST 3.5. Facultad de Biologia, Universidad de Vigo, Vigo, Spain.

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. *Mycorrhiza*, **10**, 73–80.

Redecker D, Thierfelder H, Walker C, Werner D (1997) Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. *Applied and Environmental Microbiology*, **63**, 1756–1761.

Redecker D, Hijri M, Dulieu H, Sanders IR (1999) Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are of Ascomycete origin. *Fungal Genetics and Biology*, **28**, 238–244.

Redecker D, Kodner R, Graham LE (2000a) Glomalean fungi from the Ordovician. *Science*, **289**, 1920–1921.

Redecker D, Morton JB, Bruns TD (2000b) Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). *Molecular Phylogenetics and Evolution*, **14**, 276–284.

Redecker D, Hijri I, Wiemken A (2003) Molecular identification of arbuscular mycorrhizal fungi in roots: perspectives and problems. *Folia Geobotanica*, **38**, 113–124.

Renker C, Heinrichs J, Kaldorf M, Buscot F (2003) Combining nested PCR and restriction digest of the internal transcribed spacer region to characterize arbuscular mycorrhizal fungi on roots from the field. *Mycorrhiza*, **13**, 191–198.

Schüßler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research*, **105**, 1413–1421.

Schwarzott D, Walker C, Schüßler A (2001) *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is non-monophyletic. *Molecular Phylogenetics and Evolution*, **21**, 190–197.

Sieverding E (1991) *Vesicular-arbuscular Mycorrhiza Management*. Gesellschaft für technische Zusammenarbeit, Eschborn, Germany.

Swofford DL (2001) Paup*. *Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sinauer Associates, Sunderland, Massachusetts.

van der Heijden MGA, Klironomos JN, Ursic M *et al.* (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature*, **396**, 69–72.

van Tuinen D, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25 s rDNA-targeted nested PCR. *Molecular Ecology*, **7**, 879–887.

Walker C, Trappe JM (1993) Names and epithets in the Glomales and Endogonales. *Mycological Research*, **97**, 339–344.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, A Guide to Methods and Applications* (ed. White TJ), pp. 315–322. Academic Press.