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Diversity of arbuscular mycorrhizal fungi in grassland spontaneously developed on area polluted by a fertilizer plant

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Almost all plant species were mycorrhizal.

Abstract

Mycorrhizal colonization and diversity of arbuscular mycorrhizal fungi (AMF) were analyzed in a calcareous grassland with residual phosphate contamination 10 years after the closure of a pollutant fertilizer plant in Thuringia (Germany). AMF were detected in 21 of 22 plant species analyzed. Mean mycorrhization levels reached up to 74.5% root length colonized. AMF diversity was analyzed based on 104 sequences of the internal transcribed spacer (ITS) of the ribosomal DNA. Phylogenetic analyses revealed a total of 6 species all belonging to the genus *Glomus*. There was no overlap between species detected as active mycorrhizas on roots (2 taxa) or as spores (4 taxa). Compared to the regional context, the diversity of AMF at our field site was reduced, which may reflect a residual disturbance effect. However, none of the detected species was exclusive to the polluted site as they are commonly found in the region.

Keywords: Arbuscular mycorrhizal fungi; Diversity; Internal transcribed spacer; Phosphate pollution; Root colonization

1. Introduction

Arbuscular mycorrhiza (AM) is one of the most common symbioses worldwide and about 80% of the known plant species form AM (Smith and Read, 1997). The influence of AM on plant community structure was shown in microcosm experiments (van der Heijden et al., 1998a), which led to the conclusion that the occurrence and abundance of a vascular plant species in a particular community may depend on the presence of one to several specific arbuscular mycorrhizal fungi (AMF) (Read, 1998; van der Heijden et al., 1998b). Vice versa, Johnson et al. (2003) demonstrated also in microcosms that the floristic composition of vegetation significantly affects the AMF diversity. Besides these direct interactions between the fungus and its host plant, recent studies indicate that even more complex interactions involving soil bacteria (Hildebrandt et al., 2002) and other soil fungi (e.g. yeasts; Renker et al., 2004; Sampedro et al., 2004) might occur. Moreover, Helgason et al. (1998) showed a decreased AMF diversity in a disturbed arable field, suggesting that other environmental factors than the sole interactions between plants and their fungal partners, in this case the soil disturbance by ploughing, may affect the AMF diversity. These

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lines of evidence illustrate the complexity of the ecological involvement of AMF for functioning of ecosystems and justify the actual trend to investigate AMF diversity in field studies.

More than 150 AMF species are described based on their spore morphology (Walker and Trappe, 1993), but spore morphotyping requires considerable experience (Clapp et al., 2001) and spore counts may not reflect the in planta composition of AMF communities (Clapp et al., 1995; Merryweather and Fitter, 1998; Turnau et al., 2001), due to taxon-specific differences between sporulation and root colonization rates. Therefore, information regarding the active AMF in roots is crucial in ecological field studies. Morphotyping on roots at best allows discriminating AMF at the family or genus level (Merryweather and Fitter, 1998). Progress in analyzing AMF diversity at species level in planta has recently been made by sequence analysis of the small subunit of the nuclear ribosomal DNA (18S rDNA) (Helgason et al., 1998, 1999, 2002; Vandenkoornhuyse et al., 2002). The internal transcribed spacer (ITS) region of the rDNA also allows species composition analysis (Wubet et al., 2003). In this context, we have developed a method combining a nested PCR with an intermediate restriction digest to amplify the ITS of AMF from roots (Renker et al., 2003). This technique was assessed at different field sites and allowed monitoring of a broad spectrum of taxa within the Glomeromycota with a unique set of primers, whilst also reducing amplification of ITS from contaminating fungi.

It is well established that AM symbiosis improves phosphorus nutrition, but since the work of Sanders and Tinker (1973), numerous experiments have also shown that increasing phosphate availability decreases the mycorrhization level, suggesting that AMF might play a minor role in natural ecosystems or agriculture fields with high P availability (e.g. Ryan and Graham, 2002). Taking the opportunity offered by a regeneration survey on a field site that was heavily polluted by large quantities of phosphate dusts for more than two decades, the present paper addresses two questions in this regard: (i) does AM formation occur at the site despite the high phosphorus concentration? (ii) which AMF species are involved and do they or their diversity structure reflect the particular ecological context at the field site?

2. Materials and methods

2.1. Field sites

The field site in the focus of this study is situated in Steudnitz (Thuringia, Germany), in the central Saale Valley, 13 km north of Jena (11°40′51″E/51°00′46″N) near a former phosphorus fertilizer plant. It consists of a calcareous grassland of roughly 1000 ha on an east-facing slope of the valley with altitudes ranging from 150 to 180 m above sea level and a soil pH of about 8. The mean annual rainfall is 587 mm and the average annual air temperature is 9.4 °C. In the early 1970s, during fertilizer production, a monthly input of dust up to 35 g m^{-2} was measured (Heinrich, 1984). The plant was closed in 1990, but the phosphorus content of the soil is still high (Table 1) and appears to be constant, while other components of the deposition such as NaCl have been almost completely washed out. As further after-effect of the fertilizer production, the cadmium concentration in the soil remains over the admitted toxicity levels (Table 1), but due to the high pH cadmium is not plant available. N content in soil is on the contrary low (Table 1). The vegetation recovery on the area started directly after the closure of the fertilizer plant with a two-species vegetation of *Puccinellia distans* and *Atriplex sagittata*. During the last 10 years, species richness and evenness of the field site increased dramatically, and about 70 plant species with a complex distribution pattern along the slope are now present (for details see Heinrich et al., 2001). Our survey of the arbuscular mycorrhizal fungi (AMF) started in 2001.

Some additional sequences obtained from AM spores or roots were included in the analyses. This reference material was gathered within a radius of 80 km around the site, from a calcareous forest near the small stream Erdengraben in the central Saale Valley 10 km north of Jena (11°39′25″E/50°59′31″N, elevation 150 m above sea level); one intensively farmed meadow near Schlegel (11°37′31″E/50°24′32″N, elevation 640 m above sea level), one extensively farmed meadow near Grumbach (11°30′48″E/50°25′22″N, elevation 710 m above sea level), and a fresh meadow near a stream in Friedmannsdorf (12°13′33″E/50°45′25″N, elevation 300 m above sea level).

Table 1
Soil properties, total element contents (extracted by aqua regia) and extractable phosphorus (extracted with calcium acetate/calcium lactate) for soil samples collected at the field site in Steudnitz

Cd (mg kg^{-1})	9.20–33.67
F (mg kg^{-1})	1500–3350
P (g kg^{-1})	43.00–121.00
P_{CAL} (g kg^{-1})	6.3–12.2
pH ($CaCl_2$)	7.06–7.42
C_{org} (%)	3.8–8.1
N_{tot} (%)	0.30–0.72
WHC (%)	74–160
Texture (%)	
Sand	53–85
Silt	13–44
Clay	2–8

Data adopted from Langer and Günther (2001) and Held (unpublished data). For further descriptions of soil conditions refer to the above mentioned references (WHC – water holding capacity).

2.2. Soil parameters

Analyses of C_{org}, N_{tot} and element concentrations were conducted by the Agrar- u. Umweltanalytik GmbH Jena according to the appropriate EN ISO, DIN, VDI and VDLUFA standards. Cd and P were extracted by aqua regia and the total concentration of P was determined by inductively coupled plasma emission spectrometry and in the case of Cd by atomic absorption spectrometry. Plant available phosphorus (P_{cal}) was extracted with the method of Schüller (1969). F was extracted by alkaline extraction and determined by a F-ion-selective electrode (96-09, Orion). The total amount of C_{org} was determined by dry combustion in an elementar analyzer (Leco CHN 600). Total soil N was measured after Kjeldahl digestion. Soil acidity was determined using a glass electrode and a 1:2.5 soil to 0.01 M $CaCl_2$ ratio.

2.3. Root and spore sampling from the field and trap cultures

Arbuscular mycorrhizal (AM) roots or spores from the field used in this work all originated from fresh soil samples. Sampling was done in Steudnitz at seven dates between February 2001 and October 2002. To get an insight into the community structure of AMF in planta, roots of the 22 most common plant species reported by Heinrich et al. (2001) on the polluted field site were examined (Table 2). Total plant root systems were removed, and fine roots of a single plant were chosen randomly for determination of the mycorrhization level or for molecular analysis of the mycobiont. For the AM roots from the four reference plots, the host-plant species are specified in Figs. 3–5.

Some trap cultures were additionally established with maize seedlings (*Zea mays* L.) inoculated with roots from the polluted field site. Plants were watered daily and supplied with Hoagland solution weekly without phosphorus and once a month with 1 mM K_2HPO_4. After 250 days of growth, plants were not watered any more for 2 weeks. Then the shoots were cut off and the pots were stored in the greenhouse without watering (usually 4–10 weeks). Such procedure has been described to drastically enhance spore formation. AM spores were isolated from soils following the protocol of Esch et al. (1994).

2.4. Mycorrhization levels

Roots were washed carefully, fixed in formaldehyde–acetic-acid (FAA: 6.0% formaldehyde, 2.3% glacial acetic acid, 45.8% ethanol and 45.9% H_2O (v/v)) and stained with lactophenol blue (Phillips and Hayman, 1970). Fixed roots were heated at 90 °C for about 1 h (depending on the thickness and colour of the roots) in 10% KOH. Afterwards roots were rinsed in tap water and acidified with 3.7% HCl for 10 min. They were stained for 90 min in lactophenol blue solution for staining fungi (Merck, Darmstadt) and the excess stain was removed in 50% lactic acid for at least 12 h. Total mycorrhization levels (percentage of the examined root segments with mycorrhizal structures) were determined with a Zeiss Axioplan light microscope using the gridline intersect method (Ambler and Young, 1977 modified after Schmitz et al., 1991) at 100× magnification. A minimum of 300 root segments per plant were counted.

2.5. DNA extraction and nested PCR with restriction digest

DNA extraction from single 2 cm long root fragments per plant was performed as described by Redecker (2000). To amplify DNA from single spores, these were separated in a drop of sterile water. The water was removed before spores were crushed, pipetted with 8 µl of the PCR-Mix and used directly for PCR.

The technique for the nested PCR has been described by Renker et al. (2003). The first step of the nested PCR was performed with the primer pair SSU-Glom1 (ATT ACG TCC CTG CCC TTT GTA CA) and LSU-Glom1 (CTT CAA TCG TTT CCC TTT CA). In order to avoid further amplification of ITS regions containing an *Alu*I restriction site and mainly belonging to contaminants (Renker et al., 2003), 5 µl of the products of the first PCR were digested for 1 h in a total volume of 20 µl with 5 U of *Alu*I (MBI Fermentas, St. Leon-Rot, Germany). After this digest, the DNA was precipitated with 50 µl of 100% ethanol and incubated on ice for at least 1 h. The samples were centrifuged at 20 000 × g for 10 min and after discarding the supernatant, the pellets were dried for 30 min at 50 °C and solved in 10 µl demineralized water. One microliter of this DNA template was used for the second reaction of the nested PCR, performed with the primers ITS5/ITS4 (White et al., 1990).

2.6. Cloning, sequencing and sequence analyses

The final PCR products were cloned into the pCR4-Topo Vector following the manufacturer's protocol of the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into TOP10 Chemically Competent *Escherichia coli*. Sequencing was done using a LI-COR DNA Sequencer Long Reader 4200 and the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, UK). We sequenced only one clone for each PCR product after preliminary sequencing attempts of a number of clones had revealed each PCR product to contain only one phylotype based on sequence analysis (see also Section 3).

Table 2
Host plants analyzed for colonization by AMF at a polluted field site in the central Saale Valley near Steudnitz (Thuringia, Germany), based on sequence data of the ITS region or light microscopic survey of roots

Plant species	Arbuscular mycorrhizal fungi based on sequence data of plant roots			Arbuscular mycorrhiza revealed by light microscopic survey			
	Glomus sp.	*Glomus intraradices*		*n*	Total AM	Arbuscules	Vesicles
	'Steudnitz'	Type A	Type B				
Apiaceae:							
Torilis japonica (Houtt.) DC.	+	–	–	2	27.5	1.7	6.7
Asteraceae:							
Artemisia vulgaris L.	+	–	–	8	40.9 ± 2.4	9.5 ± 10.1	35.5 ± 25.1
Cirsium vulgare (Savi) Ten.	–	–	–	1	74.5	68.6	10.8
Inula conyzae (Griess.) Meikle	+	–	–			n.d.	
Lactuca serriola L.	–	–	–	2	57.5	21.0	57.5
Picris hieracioides L.	+	–	–	4	66.8 ± 19.7	19.9 ± 21.5	61.9 ± 19.5
Senecio vernalis Waldst. & Kit.	–	–	–	1	27.1	24.6	2.0
Tragopogon pratensis L.	+	+	–			n.d.	
Caryophyllaceae:							
Cerastium glutinosum Fr.	–	–	+	1	0.0	0.0	0.0
Chenopodiaceae:							
Atriplex sagittata Borkh.	+	–	–	5	24.2 ± 16.7	3.0 ± 4.4	22.4 ± 15.6
Fabaceae:							
Vicia hirsuta (L.) Gray	+	+	–			n.d.	
Vicia sativa L. s. l.	+	+	–	1	14.5	0.9	12.7
Vicia tetrasperma (L.) Schreb.	+	–	–			n.d.	
Onagraceae:							
Epilobium ciliatum Raf.	+	–	–			n.d.	
Poaceae:							
Arrhenatherum elatius (L.) P. Beauv. ex J. Presl & C. Presl	+	+	–	2	33.0	17.8	17.1
Elymus repens (L.) Gould	+	–	–	20	24.0 ± 10.5	15.9 ± 13.0	8.2 ± 6.9
Festuca rupicola Heuff.	+	–	–			n.d.	
Poa pratensis L. s. l.	+	–	–			n.d.	
Puccinellia distans (Jacq.) Parl.	+	–	–	6	17.7 ± 6.9	0.6 ± 0.8	12.8 ± 7.2
Ranunculaceae:							
Clematis vitalba L.	+	–	–			n.d.	
Rubiaceae:							
Galium aparine L.	+	+	–			n.d.	
Urticaceae:							
Urtica dioica L.	n.d.	n.d.	n.d.	1	0.0	0.0	0.0

Mycorrhization levels [% root length colonized] were determined according to Ambler and Young (1977) and Schmitz et al. (1991). Standard deviations are given if more than 3 plants (n = number of plant root systems) were sampled (n.d. – not determined).

DNA sequences of the complete ITS were submitted to the EMBL database under the accession numbers shown in Figs. 1–5 in bold type. Reference sequences to analyze the systematic position of the new sequences were taken from GenBank. In a first step, only the 5.8S subunit genes embedded between the ITS1 and ITS2 regions were aligned by hand to allow an analysis of the AMF community structure at the field site. In a second step, full-length ITS sequences of single taxa were aligned to assess and precise the separation found at the 5.8 level. An alignment of the full-length ITS sequences of all data was not possible due to the high variation within the data set.

Phylogenetic trees (Figs. 1–5) were inferred using distance criteria as implemented in PAUP* 4.0b8 (Swofford, 2000). The confidence of branching was assessed using 1000 bootstrap resamplings in distance analysis (neighbor joining method).

3. Results

3.1. Mycorrhizal status of the polluted site

The mycorrhizal status was assessed on 22 of the most common host plant species growing at the

phosphate-polluted field site (Table 2). In a first step, 12 species belonging to typical (Asteraceae, Fabaceae, Poaceae) or facultative (Chenopodiaceae, Caryophyllaceae, Urticaceae) AM host plant families were analyzed microscopically. AM structures, i.e. arbuscules or vesicles, were observed in all plants except *Cerastium glutinosum* and *Urtica dioica* (Table 2), which belong to the Caryophyllaceae and Urticaceae, respectively. In all other plant taxa, mean mycorrhization levels ranging from 14.5 to 74.5% were determined.

The molecular analyses of plant roots were performed on 21 species including all 12 microscopically investigated plant species except *U. dioica* (Table 2). In *Cirsium vulgare*, *Lactuca serriola*, and *Senecio vernalis*, AM structures had been observed but the molecular analyses did not allow detection of any AMF partner. In contrast, one AMF was detected by nested PCR in roots of *C. glutinosum*, although no AM structures had been observed under the microscope. In 16 of the 21 plant species analyzed by PCR, *Glomus* sp. 'Steudnitz' (see iv in next paragraph) was identified as the AMF partner. *Glomus intraradices* Type A (see ii in next paragraph) was detected as a second possible mycobiont in only 5 plant species (Table 2). In one case, *G. intraradices* Type B was detected as mycobiont (see Section 4).

In addition to the direct analyses on field roots, PCR was also performed on single spores isolated from the site and on roots from trap cultures with maize. Altogether, 91 new sequences were obtained in this study, 44 from spores and 37 from roots of the polluted field, and 10 from maize roots in trap cultures. Based on the neighbor joining analysis of the 5.8S region six AMF phylotypes (''species'') could be clearly differentiated on the field site: (i) *Glomus* sp. 'Bad Sachsa', which has sequence similarities with *Glomus geosporum* and *Glomus coronatum* and was only found as spores; (ii & iii) two different types of *G. intraradices* separated by high bootstrap support and classified as *G. intraradices* Types A and B, one type on roots and the latter mainly on spores; (iv) a *Glomus* species not closely related to any of the Glomeromycota sequenced so far, and which was classified as *Glomus* sp. 'Steudnitz' (71% identity with *Glomus clarum* AJ243275), this was the most common species on roots; (v) *Glomus claroideum*, detected on the basis of a single spore; (vi) *Glomus versiforme*, only found as spores.

Five of them were investigated by sequence analysis of the whole ITS region. Depending on the species, the analyzed materials were AM roots, AM spores, or in the case of *G. intraradices* Type B both. Besides samples from the polluted site, this part of the study included samples from the four additional sites mentioned in Section 2.

3.2. Diversity analysis within Glomus sp. 'Bad Sachsa'

The ITS neighbor joining analysis within this taxon included 13 sequences obtained from 6 spores collected at the polluted site, one sequence from a spore isolated at the extensively farmed meadow near Grumbach in the Thüringer Schiefergebirge, and the sequence available in GenBank of the original material from Bad Sachsa (see Landwehr et al., 2002). Two GenBank reference sequences of *G. coronatum* and *Glomus mosseae* were used as outgroups (Fig. 2). The data support the relationship between the *Glomus* sp. 'Bad Sachsa' found at the polluted site in Steudnitz and the two additional findings of this taxon in Bad Sachsa and in the Thüringer Schiefergebirge (Fig. 2). Their delimitation to the closest related taxon, *G. coronatum*, was also supported and confirms the distinction obtained by consideration of the sole 5.8S region (see Fig. 1). All 13 sequences obtained in Steudnitz clustered separately from the two other sequences. Interestingly, sequences from Steudnitz additionally clustered in two microclades, but the sequence polymorphism within one spore was often found to be higher than the one between certain clones issued from different spores. Hence, sequences from one spore were found on both microclades.

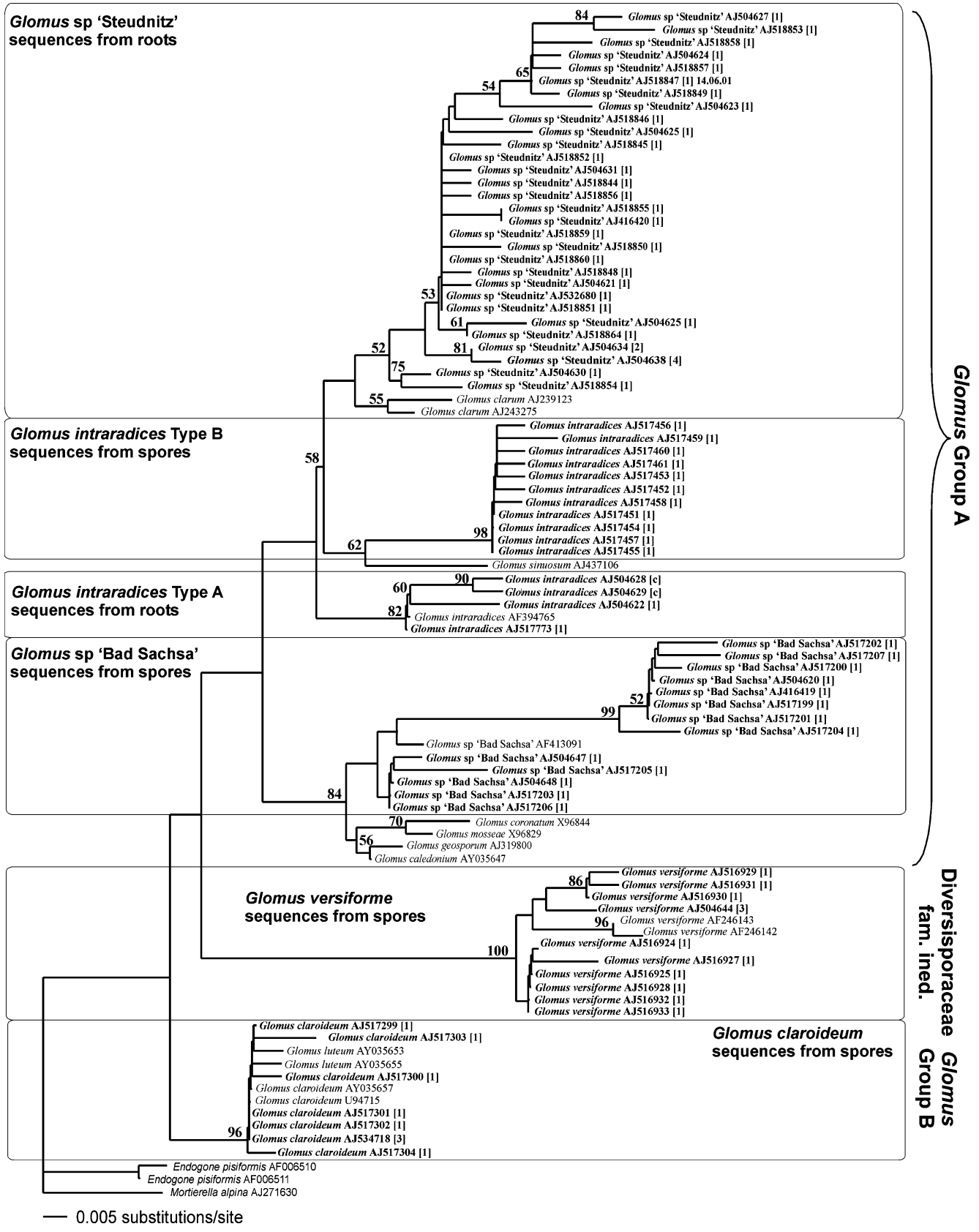
3.3. Diversity analysis within G. intraradices

The ITS neighbor joining analysis of *G. intraradices* included sequences obtained from 3 spores, AM-colonized roots from *Arrhenatherum elatius* and *Tragopogon pratensis* collected at the polluted field site and also roots from trap cultures inoculated either with field material or with a reference strain BEG 140 (Fig. 3). Separation into two clades was found confirming the analysis of the sole 5.8 region (Fig. 1). *Glomus sinuosum* which, in this tree, is close to *G. intraradices* was not alignable with the whole ITS region of the *G. intraradices* sequences and is therefore not included in Fig. 3.

Remarkably, all sequences from roots fall into Type A, while the ones from spores all clustered in Type B. The sole apparent exception was the sequence obtained from roots of *C. glutinosum* (see Table 2 and Section 4). Sequences from field roots were clearly separated from sequences available in GenBank and from the cultures of *G. intraradices* BEG 140, whereas sequences from trap cultures inoculated with roots from the field clustered together with sequences directly gathered from field roots (Fig. 3).

3.4. Diversity analysis within Glomus sp. 'Steudnitz'

The neighbor joining analysis of the whole ITS region of *Glomus* sp. 'Steudnitz' included 28 sequences from


Glomus sp 'Steudnitz' sequences from roots
Glomus intraradices Type B sequences from spores
Glomus intraradices Type A sequences from roots
Glomus sp 'Bad Sachsa' sequences from spores
Glomus versiforme sequences from spores
Glomus claroideum sequences from spores
Glomus Group A
Diversisporaceae fam. ined. Glomus Group B
Glomus sp 'Steudnitz' AJ504627 [1]
Glomus sp 'Steudnitz' AJ518853 [1]
Glomus sp 'Steudnitz' AJ518858 [1]
Glomus sp 'Steudnitz' AJ504624 [1]
Glomus sp 'Steudnitz' AJ518857 [1]
Glomus sp 'Steudnitz' AJ518847 [1] 14.06.01
Glomus sp 'Steudnitz' AJ518849 [1]
Glomus sp 'Steudnitz' AJ504623 [1]
Glomus sp 'Steudnitz' AJ518846 [1]
Glomus sp 'Steudnitz' AJ504625 [1]
Glomus sp 'Steudnitz' AJ518845 [1]
Glomus sp 'Steudnitz' AJ518852 [1]
Glomus sp 'Steudnitz' AJ504631 [1]
Glomus sp 'Steudnitz' AJ518844 [1]
Glomus sp 'Steudnitz' AJ518856 [1]
Glomus sp 'Steudnitz' AJ518855 [1]
Glomus sp 'Steudnitz' AJ416420 [1]
Glomus sp 'Steudnitz' AJ518859 [1]
Glomus sp 'Steudnitz' AJ518850 [1]
Glomus sp 'Steudnitz' AJ518860 [1]
Glomus sp 'Steudnitz' AJ518848 [1]
Glomus sp 'Steudnitz' AJ504621 [1]
Glomus sp 'Steudnitz' AJ532680 [1]
Glomus sp 'Steudnitz' AJ518851 [1]
Glomus sp 'Steudnitz' AJ504625 [1]
Glomus sp 'Steudnitz' AJ518864 [1]
Glomus sp 'Steudnitz' AJ504634 [2]
Glomus sp 'Steudnitz' AJ504638 [4]
Glomus sp 'Steudnitz' AJ504630 [1]
Glomus sp 'Steudnitz' AJ518854 [1]
Glomus clarum AJ239123
Glomus clarum AJ243275
Glomus intraradices AJ517456 [1]
Glomus intraradices AJ517459 [1]
Glomus intraradices AJ517460 [1]
Glomus intraradices AJ517461 [1]
Glomus intraradices AJ517453 [1]
Glomus intraradices AJ517452 [1]
Glomus intraradices AJ517458 [1]
Glomus intraradices AJ517451 [1]
Glomus intraradices AJ517454 [1]
Glomus intraradices AJ517457 [1]
Glomus intraradices AJ517455 [1]
Glomus sinuosum AJ437106
Glomus intraradices AJ504628 [c]
Glomus intraradices AJ504629 [c]
Glomus intraradices AJ504622 [1]
Glomus intraradices AF394765
Glomus intraradices AJ517773 [1]
Glomus sp 'Bad Sachsa' AJ517202 [1]
Glomus sp 'Bad Sachsa' AJ517207 [1]
Glomus sp 'Bad Sachsa' AJ517200 [1]
Glomus sp 'Bad Sachsa' AJ504620 [1]
Glomus sp 'Bad Sachsa' AJ416419 [1]
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Glomus sp 'Bad Sachsa' AJ517204 [1]
Glomus sp 'Bad Sachsa' AF413091
Glomus sp 'Bad Sachsa' AJ504647 [1]
Glomus sp 'Bad Sachsa' AJ517205 [1]
Glomus sp 'Bad Sachsa' AJ504648 [1]
Glomus sp 'Bad Sachsa' AJ517203 [1]
Glomus sp 'Bad Sachsa' AJ517206 [1]
Glomus coronatum X96844
Glomus mosseae X96829
Glomus geosporum AJ319800
Glomus caledonium AY035647
Glomus versiforme AJ516929 [1]
Glomus versiforme AJ516931 [1]
Glomus versiforme AJ516930 [1]
Glomus versiforme AJ504644 [3]
Glomus versiforme AF246143
Glomus versiforme AF246142
Glomus versiforme AJ516924 [1]
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Glomus versiforme AJ516925 [1]
Glomus versiforme AJ516928 [1]
Glomus versiforme AJ516932 [1]
Glomus versiforme AJ516933 [1]
Glomus claroideum AJ517299 [1]
Glomus claroideum AJ517303 [1]
Glomus luteum AY035653
Glomus luteum AY035655
Glomus claroideum AJ517300 [1]
Glomus claroideum AY035657
Glomus claroideum U94715
Glomus claroideum AJ517301 [1]
Glomus claroideum AJ517302 [1]
Glomus claroideum AJ534718 [3]
Glomus claroideum AJ517304 [1]
Endogone pisiformis AF006510
Endogone pisiformis AF006511
Mortierella alpina AJ271630
0.005 substitutions/site

AM roots of 16 plants species growing at the polluted field site. Additionally, *Glomus* sp. 'Steudnitz' has been detected on three other field sites in Thuringia (i.e. Erdengraben, Grumbach, and Schlegel). Three of these sequences were included in the analysis (Fig. 4). On this material a rather reduced intraspecific polymorphism was revealed, which did not appear to be related to any host plant or plot specificity.

3.5. Diversity analysis within G. versiforme

G. versiforme was only found as spores on our field site, and 11 sequences from 3 spores were included in the ITS neighbor joining analysis together with sequences from roots of three plant species from other field sites in Thuringia. Based on the analysis, the small population studied appeared to display a high polymorphism, which was reflected by the presence of different clades (Fig. 5). Clones obtained from the same spore clustered either closely or very distantly, and so did the clones from root materials of the other field sites.

4. Discussion

4.1. Mycorrhization levels

In the context of the huge P contamination, the value of available P (6.3–12.2 g kg^{-1} soil) in Steudnitz was at an extremely high level. However, most plants were found to be mycorrhizal (Table 2), which seems to contradict the hypothesis of Treseder and Allen (2002) and the observations by Sanders and Tinker (1973) according to which AM should not form at this level of available P. A possible explanation for the mycorrhization could be N-limitation and a resulting unbalanced N/P ratio at the site (Blanke et al., in press). AMF play an important role for N budgets of plants (Johansen et al., 1992; Bago et al., 1996).

Mycorrhization of typically non-mycorrhizal plants may be interpreted as an additional indication of stress conditions at the site, making mycorrhization necessary despite high P availability. *A. sagittata*, a member of the normally non-mycorrhizal Chenopodiaceae, was fairly well colonized (24.2 ± 16.7% mean ± SD). Landwehr et al. (2002) found *Salicornia europaea* (another member of the Chenopodiaceae) to be mycorrhizal in German inland salt marshes, but non-mycorrhizal on other sites. The authors conclude that AMF might ameliorate drought stress, which is pronounced at inland salt marshes, and which might also play a role at our field site, due to the sandy texture of the soils (Table 1). A further hint for this interpretation can be seen in the weak formation of arbuscules in the root of *A. sagittata*, which would be necessary for an efficient nutrient exchange, but not for the amelioration of drought stress. The same might apply for *P. distans*, a plant species whose mycorrhizal status is also still under discussion (Harley and Harley, 1987). Besides *A. sagittata* we found the plant species *Torilis japonica*, *L. serriola*, *S. vernalis*, *Festuca rupicola* not included in the list of Harley and Harley (1987) to be mycorrhizal.

For three of 12 plant species, we however failed to confirm microscopic observation of AM by molecular detection. The discrepancy was observed in Asteraceae (i.e., *L. serriola*, *C. vulgare*) the roots of which produce milky substances that could have hampered the PCR. In *C. glutinosum*, no mycorrhizas were found by microscopic investigation, but an AM fungus was detected by PCR. This could reflect a spatially restricted infection (even by pure mycelium without any mycorrhizal structures like arbuscules) or even the presence of spores or hyphae on the root surface of this supposedly non-mycorrhizal member of the Caryophyllaceae.

4.2. Approach to study the AMF diversity

Difficulties with molecular field studies in particular result from the large coenocity with high level of genetic diversity in spores and hyphae of AMF (see Clapp et al., 2001 and citations therein). Besides the ongoing discussion on the heterokaryotic nature of spores (see Kuhn et al., 2001; Pawlowska and Taylor, 2004) there is evidence of nuclear exchange mediated by anastomosis at least within strains of the same species (Giovannetti et al., 1999). PCR products from spore or root samples contain a diversity of amplified DNA fragments, the separation of which is time and money consuming.

In this context, field studies are often restricted to the AMF of one or two plant species (see for example Helgason et al., 1999; Vandenkoornhuyse et al., 2002; Johnson et al., 2003; Wubet et al., 2003). Such

Fig. 1. Phylogenetic tree of arbuscular mycorrhizal fungi from different field sites based on 5.8S rDNA sequences obtained by distance analysis with the neighbor joining method. Sequences of *Endogone pisiformis* and *Mortierella alpina* were used as outgroup. Bootstrap values (>50%) were determined for neighbor joining (1000 resamplings). Sequences obtained in this study are shown in bold face. The numbers in brackets refer to the field sites, which are all located within Thuringia (Germany). [1] Phosphate polluted grassland in the central Saale Valley near Steudnitz 13 km north of Jena; [2] calcareous forest in the central Saale Valley, 10 km north of Jena; [3] intensively farmed mountain meadow in the Thüringer Schiefergebirge (near Schlegel); [4] extensively farmed mountain meadow in the Thüringer Schiefergebirge (near Grumbach); [c] material taken from reference cultures. Sequences of *Glomus clarum* (AJ239123, AJ243275), *Glomus sinuosum* (AJ437106), *Glomus intraradices* (AF394765), *Glomus* sp. 'Bad Sachsa' (AF413091), *Glomus coronatum* (X96844), *Glomus mosseae* (X96829), *Glomus geosporum* (AJ319800), *Glomus caledonium* (AY035647), *Glomus versiforme* (AF246143, AF246142), *Glomus luteum* (AY035653, AY035655) and *Glomus claroideum* (AY035657, U94715) from GenBank were used as references.

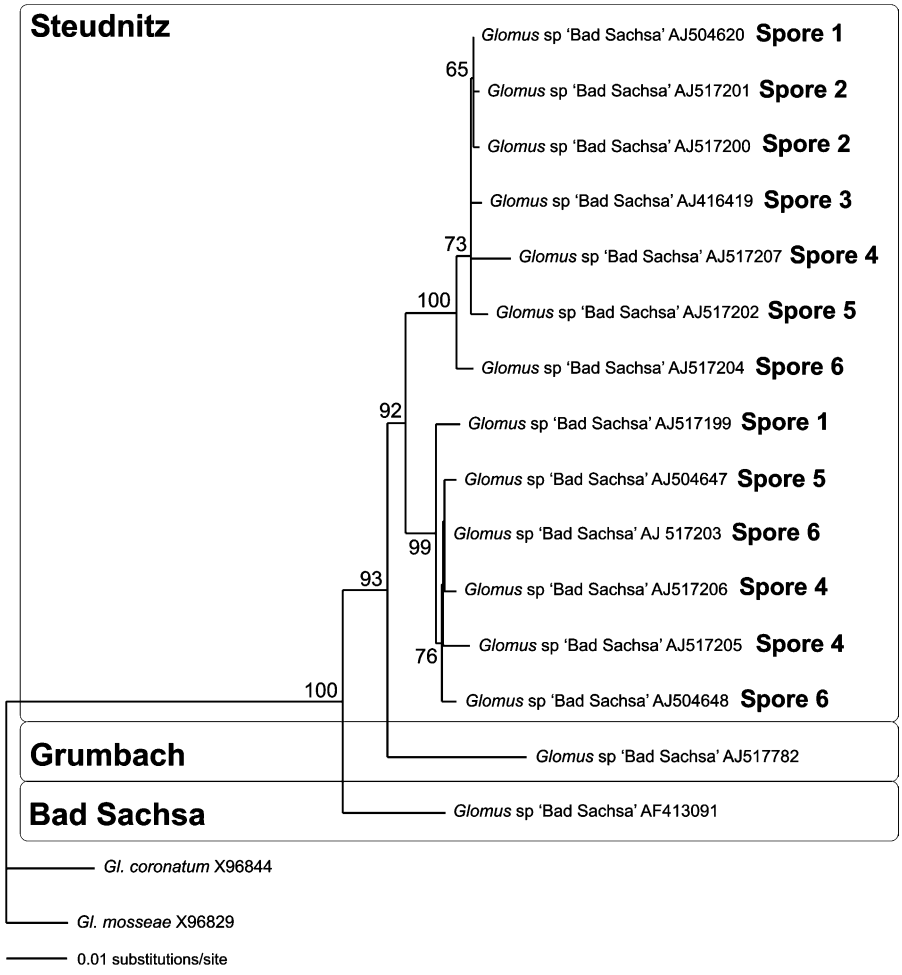

Fig. 2. Phylogenetic tree of *Glomus* sp. 'Bad Sachsa' obtained with the neighbor joining method from full-length ITS rDNA sequences by distance analysis. Sequences refer to clones from different single spores collected at three locations in central Germany. Sequences of *Glomus coronatum* and *Glomus mosseae* were used as outgroup. Bootstrap values (>50%) were determined for neighbor joining (1000 resamplings).

restrictions cannot warrant an exhaustive description of the whole AMF diversity in species rich plant communities as there is increasing evidence that fungus host plant preference exists in AMF (Helgason et al., 2002; Vandenkoornhuyse et al., 2003). In addition, field molecular analyses usually consider a reduced number of root samples in the range of 12–89 (see Johnson et al., 2003; Vandenkoornhuyse et al., 2002, 2003). Such low probe numbers are however often compensated by the fact that each sample consists in several roots (100 mg fresh weight), leading to amplification of DNA from a diversity of AMF phylotypes within each single PCR.

We analyzed roots from 22 plant species, field spores and materials from trap cultures to warrant detection of a wide AMF diversity if present at the site. To avoid the hazardous amplification from mixtures of target regions in too complex DNA extracts, we restricted our probes to single spores or short fragments of single roots. As such probes contain minute quantities of AMF DNA, we used the ITS nested PCR technique we recently optimized (Renker et al., 2003). Normally, we sequenced only one clone per PCR product after preliminary sequencing of several clones from one PCR product had shown that such sequences did differ but constantly fall in common clades in phylogenetic studies. A remaining critical point is that only the number of samples in the same low range as in all other studies could be examined. This design was insufficient to allow an exhaustive analysis of the AMF diversity at our field, and our results must be discussed keeping this major restriction in mind. However, by considering many plant species in addition to spores, we should not have failed to detect a high number of phylotypes if they would have been present. Using a similar approach with a comparable number of analyzed probes, Wubet et al. (2003) found 21 AMF taxa in Ethiopian forests despite working on one plant.

4.3. Identified AMF species

Within the ITS, the less variable 5.8S allows a separation in the Glomeromycota that is conform to

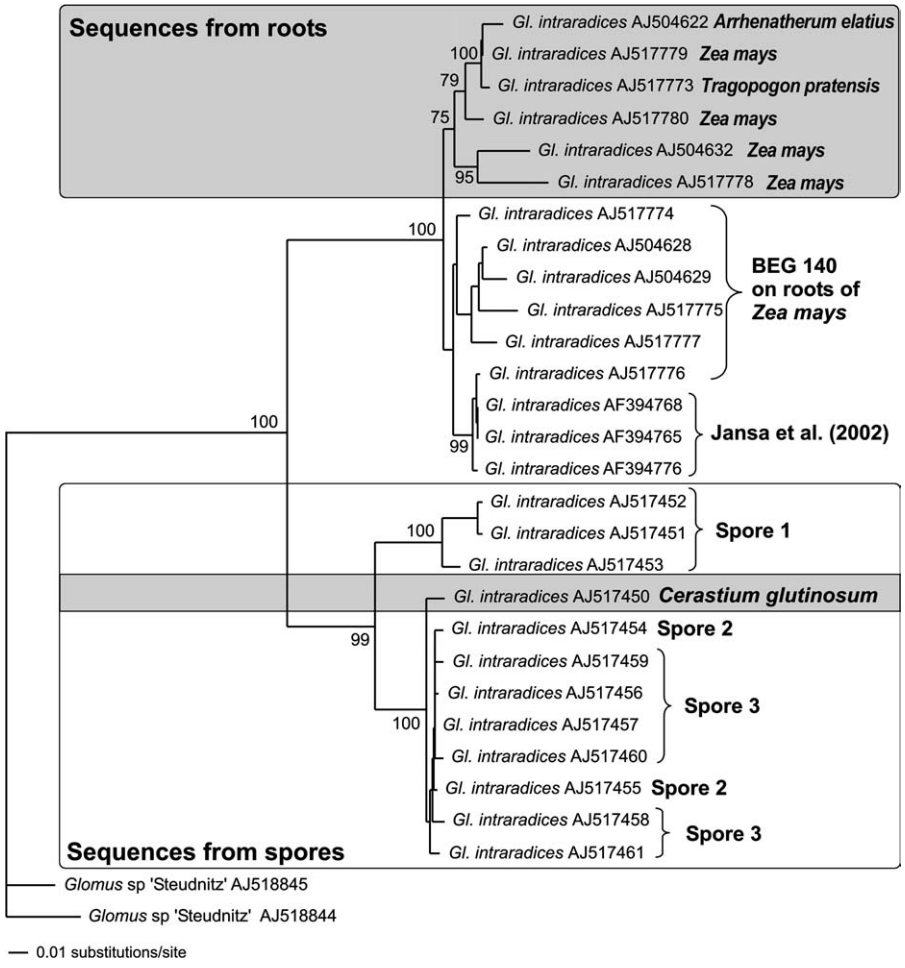

Fig. 3. Phylogenetic tree of *Glomus intraradices* obtained with the neighbor joining method from full-length ITS rDNA sequences by distance analysis. Sequences in boxes were obtained directly from the phosphate-polluted field site in Steudnitz (Germany) or from roots of *Zea mays* inoculated with mycorrhizal roots from the site (as indicated in the figure). Sequences recently published by Jansa et al. (2002) and sequences of *G. intraradices* BEG 140 were used as references. Sequences of *Glomus* sp. 'Steudnitz' were used as outgroup. Bootstrap values (>50%) were determined for neighbor joining (1000 resamplings). Sequences which were only found on roots are referred to as *G. intraradices* Type A. Sequences which were, with one exception, only detected with spores are referred as *G. intraradices* Type B.

the one given by the SSU (Cullings and Vogler, 1998; Redecker et al., 1999). The separation can be improved by analyzing the spacers of the ITS (Wubet et al., 2003). We attended the major clades of the phylogenetic tree in Fig. 1 as "species". As *G. coronatum*, *G. mosseae*, *G. geosporum* and *G. caledonium* fall in a common cluster, this broad concept might appear questionable. However, the vicinity of the four taxa in Fig. 1 should not be overestimated as only one sequence per taxon was considered. The intraspecific variability found in the 5.8S sequence of *G. versiforme* and *Glomus* sp. 'Bad Sachsa', two taxa for which different spores were examined, justifies the adopted species concept.

On our phosphate-polluted grassland, we found 6 AMF taxa, with members of *Glomus* Group A within the Glomeraceae sensu Schüßler et al. (2001) (i.e. *G. intraradices* Type A and Type B, *Glomus* sp. 'Steudnitz' and *Glomus* sp. 'Bad Sachsa'), one species falling into *Glomus* Group B (*G. claroideum*), and one member of the Diversisporaceae fam. ined. (former *Glomus* Group C, *G. versiforme*). None of the detected species is uncommon in the region. *Glomus* sp. 'Bad Sachsa' was first detected 100 km northwest of our field site (Landwehr et al., 2002) and again found 60 km south of Steudnitz (B. Börstler pers. communication). *Glomus* sp. 'Steudnitz' was also found in the region around our field site. Its closest relative seems to be *G. clarum* (AJ243275) with a sequence identity of roughly 71%. Even closer is a recently detected AMF (Bidartondo et al., 2002), which is specialized on epiparasitic plants and displays about 94% sequence identity with the ITS1 and the 5.8S region of our type, while the ITS2 region shows a weak identity. The study of *Glomus* sp. 'Steudnitz' indicates no preference of subpopulations for plant species and the revealed ITS polymorphism is rather reflecting the intraspecific variability of this region in AMF that Wubet et al. (2003) estimates to 7%.

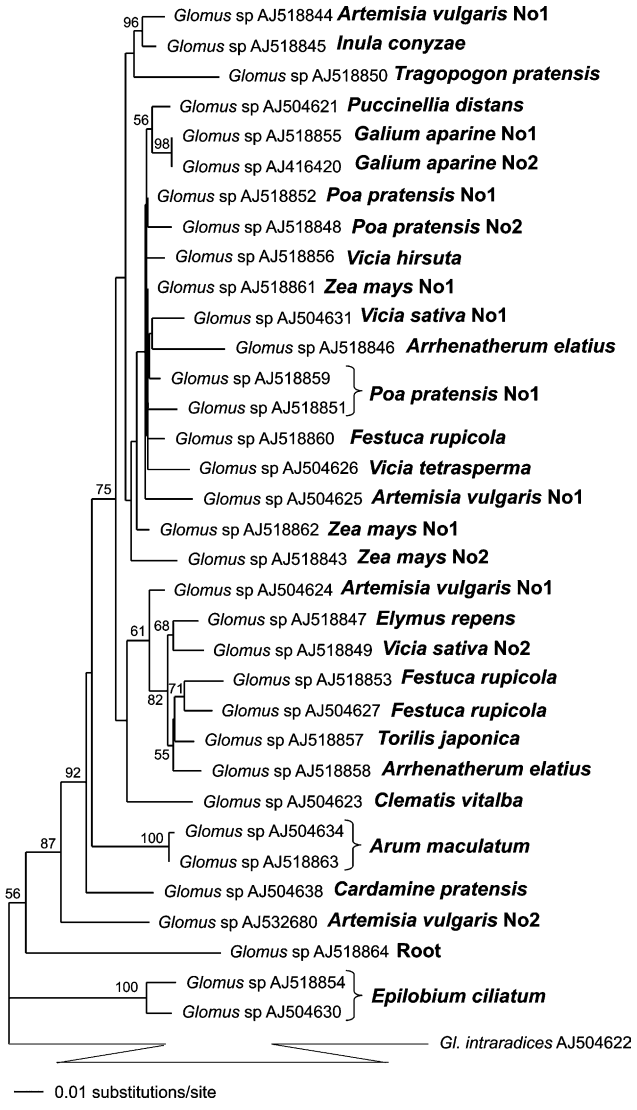

Fig. 4. Phylogenetic tree of *Glomus* sp. 'Steudnitz' obtained with the neighbor joining method from full-length ITS rDNA sequences by distance analysis. Sequences were exclusively obtained from roots that were mainly collected at a phosphate-polluted field site in Steudnitz (Germany) or from roots of *Zea mays* inoculated with mycorrhizal roots from the site (as indicated in the figure). If more than one specimen of a plant species was considered, different plant root systems are indicated by numbers. Sequences AJ504634 and AJ518863 from *Arum maculatum* were found in a calcareous forest in the central Saale Valley 10 km north of Jena. Sequence AJ504638 from *Cardamine pratensis* was revealed from an extensively farmed mountain meadow in the Thüringer Schiefergebirge (near Grumbach). *Glomus intraradices* was used as outgroup. Bootstrap values (>50%) were determined for neighbor joining (1000 resamplings).

Concerning *G. intraradices*, our analyses both at the 5.8S and full ITS level is consistent with the data set in GenBank and supports that this taxon should be treated as a species group. The fact that all sequences from spores nearly perfectly clustered separately from sequences from roots may indicate that we detected distinct species, one on spores and the other on roots.

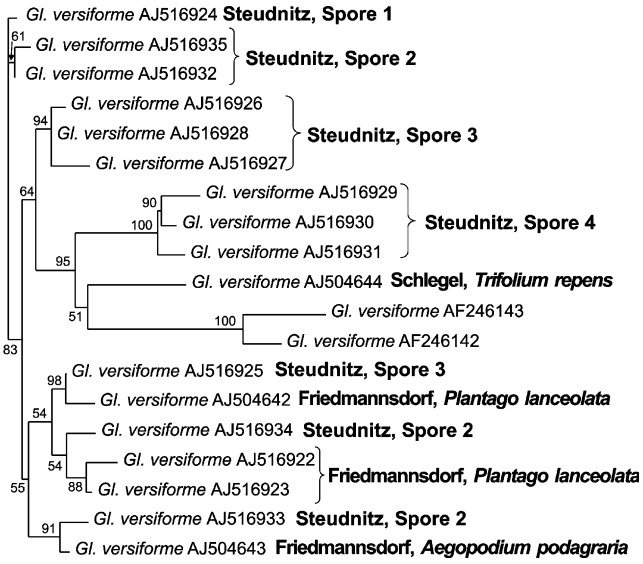

Fig. 5. Unrooted Phylogenetic tree of *Glomus versiforme* obtained with the neighbor joining method from full-length ITS rDNA sequences by distance analysis. Sequences from a phosphate-polluted field site in Steudnitz (Germany) were all obtained from spore material, while reference sequences from two additional localities, one intensively farmed mountain meadow in the Thüringer Schiefergebirge (near Schlegel) and one fresh meadow near a stream in Friedmannsdorf, were all based on root material. Bootstrap values (>50%) were determined for neighbor joining (1000 resamplings).

None of the detected taxa was exclusively found under the highly phosphate conditions of our field site.

4.4. Relation of the found AMF diversity to the ecological context

Especially when considering that only two species were found on roots, it can be concluded from the study that the AMF diversity at the site was reduced. With the same technique and analogous sampling designs, we currently detect 10 AMF species per site representing all families within the Glomeromycota in non-disturbed grasslands of the region (see Renker et al., 2003), a diversity in the same range as mentioned in many other field studies on AMF (e.g. Clapp et al., 1995; Stutz et al., 2000; Hildebrandt et al., 2001; Franke-Snyder et al., 2001). Wubet et al. (2003) quoted 20 species in east African forests, while some authors reported up to 40 species for sites in the USA (Allen et al., 1995; Egerton-Warburton and Allen, 2000; Bever et al., 2001). The reduced AMF diversity at our site was found although a species rich plant cover has been restored by succession (Heinrich et al., 2001). It can be interpreted as indicating a residual soil disturbance at the site 10 years after closure of the fertilizer plant. This interpretation is supported by the finding of Langer and Günther (2001) that the structure and function of the soil microbial community at the field site are still disturbed. It is consistent with the finding by Helgason

et al. (1998) of a decreased diversity of AMF at disturbed arable field sites.

The fact that AMF diversity at our field site was low pleads for a constraining influence of environmental disturbances. Our results support the idea that such disturbances may be of higher hierarchical importance for ruling the diversity structure of AMF than the vegetation factor may be.

5. Conclusions

Despite high soil phosphate contents at our field site we were able to reveal almost all investigated plant species to be mycorrhizal, while plants in greenhouse experiments supplied with even lower amounts of phosphate are known to lose their ability to form AM. We hypothesize that certain stress factors (e.g. unbalanced N/P ratio or drought) might be an important factor for sustaining a functional AM.

Diversity of AMF found at our site was reduced in comparison to other field studies, indicating that soil disturbance is a factor ruling AMF. However, all Glomeromycota detected at our field site were also found at other localities in the surrounding.

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