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ORIGINAL ARTICLE

Impact of a soil sampling strategy on the spatial distribution and diversity of arbuscular mycorrhizal communities at a small scale in two winter cover crop rotational systems

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Abstract It is vital to evaluate how soil sampling affects the spatial distribution and diversity of arbuscular mycorrhizal fungi (AMF) in agricultural ecosystems. Here, the impact of soil sampling on the spatial distribution and diversity of AMF in agricultural ecosystems was evaluated using field experiments. A molecular approach was taken to assess the spatial distribution of the AMF community from 1 m^2 plots from fields cropped with soybeans [*Glycine max* (L.) Merr.], in rotation with wheat (*Triticum aestivum* L.) and winter fallow. Phylogenetic analysis revealed 18 AMF phylotypes, including five *Glomus*, three *Gigaspora*, two each from *Racocetra*, *Acaulospora*, and *Funneliformis* and one each from *Claroideoglomus*, *Rhizophagus*, *Diversispora*, and *Paraglomus* at different sampling points in this study. Our results showed that the molecular diversity and composition of AMF communities in soil cropped with wheat, or left fallow did not change at scales of<1 m^2. Canonical correspondence analysis (CCA) demonstrated that AMF communities by soil sampling point within each rotation were not significantly different. Thus, random soil sampling did not show any difference in AMF communities in soil under winter cover crop rotational systems, no matter where soil samples were collected from at a small scale (< 1 m^2) in agricultural fields. Our results suggest that agricultural management can affect the diversity and composition of AMF communities more than soil sampling strategies.

Keywords Arbuscular mycorrhizal fungi · Agricultural management · AMF communities · Crop rotation · Soil sampling strategy

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Introduction

Arbuscular mycorrhizal fungi (AMF) are among the soil microorganisms that benefit the agroecosystem enormously. AMF are widespread and can be found in most terrestrial ecosystems, where they form mutualistic relationships with a wide variety of plants, which facilitates nutrient uptake from the soil via extensive extraradical mycelium (Smith and Read 2008). This symbiotic relationship involves most agricultural crops and leads to increased crop productivity (Lekberg and Koide 2005), improved soil structure (Piotrowski et al. 2004) and increased pathogen resistance (Sikes et al. 2009). The abundant nature of AMF is crucial to the overall productivity and stability of both agricultural and natural ecosystems (van der Heijden et al. 1998). Due to the important role they play in natural and agricultural ecosystems, AMF communities have been surveyed in both natural (Lumini et al. 2010; Higo et al. 2011; Lekberg et al. 2012) and agricultural (Borriello et al. 2012; Gosling et al. 2013; Higo et al. 2014) ecosystems using molecular techniques. Factors such as soil disturbance (Brito et al. 2012), and soil type (Balestrini et al. 2010) play a role in the distribution of AMF in the environment. The majority of studies on the composition of AMF communities in agroecological fields have not considered the impact of soil sampling strategies, instead focusing predominantly on agricultural management (Alguacil et al. 2008; Borriello et al. 2012; Higo et al. 2013). Thus, knowledge of sampling strategies is essential to fully understand the spatial distribution and diversity of AMF communities in agricultural fields.

The decreasing availability of rock phosphorus (P) is causing global concern and will inhibit food production in the future. The effective utilization of available P in the soil is essential to counteract this decreasing availability. One potential strategy to maintain crop productivity is to utilize AMF to improve phosphate uptake and therefore plant growth.

Although the diversity of AMF communities is imperative for improving crop growth and P uptake, data used to investigate the numbers of AMF in agricultural fields is still collected randomly from soil (Isobe et al. 2007, 2008; Higo et al. 2010; Murray et al. 2010; Jie et al. 2013). In addition, soil sampling points from the fields have not been taken into consideration when determining the spatial distribution of AMF. There is strong evidence that AMF communities differ between crop species, habitats and agricultural management schemes (Öpik et al. 2009, 2013; Gosling et al. 2013; Holland et al. 2014). However, little attention has been paid to the spatial distribution of AMF and the small scale variability in crop species, habitat and agricultural practices. Chifflot et al. (2009) have shown that the spatial distribution of AMF spores varies significantly depending on the distance from the agricultural crop. Furthermore, development of the root system can be diverse depending on the crop species (Tsuji et al. 2002). The effects of this spatial proliferation of the root system on crop yield have been studied under field conditions (Thorup-Kristensen et al. 2012). So far, there is not much information about the difference in spatial distribution of AMF communities in agricultural field conditions, such as cover crop rotational systems. It is important to clarify the spatial distribution of AMF communities to further understand the mechanism of P uptake and the promotion of crop growth by AMF in cover crop rotational systems.

We hypothesize that the composition of the AMF community in the soil could be altered by the presence or absence of crop roots. The extent of the shift in the number of AMF found in the soil is influenced by the extent of root development in different crop types and could be different depending on the agricultural field. To test this hypothesis, we compared spatial distribution, diversity and composition of AMF communities in soil cropped from wheat and fallow in rotation with soybean on a small scale (< 1 m^2).

Materials and methods

Site description

The field experiment was conducted at Nihon University, in Kanagawa, Japan (35°22′ N and 139°27′ E) on volcanic ash soil (allophanic Andosol, sandy loam texture). At the field site, the soil pH ranged from 5.86 to 5.88 and available P content (Bray P) ranged between 10.0 to 13.5 mg/kg dry soil. Total carbon (C) ranged from 5.47 to 5.60 % and nitrogen (N) from 0.40 to 0.45 %, respectively. Plots of 4.5 m × 2.0 m were established, with three replicates and cropped to wheat (*Triticum aestivum* L. cv: Bandowase) or fallow. Soybean [*Glycine max* (L.) Merr., cv: Enrei] as the preceding crop was sown with no-tillage into each plot on 11 June 2008. The parts of the soybean plants that were above ground were removed on 30 October 2008, leaving just the roots behind in the soil. On 31 October 2008, 200 kg ha^{-1} wheat seed was sown and the amount of N (ammonium sulfate) was 100 kg ha^{-1} and K (potassium chloride) 90 kg ha^{-1}. The wheat seeds were sown manually with no-tillage into rows with 30-cm spaces in the cropped treatment. At grain-filling, the tops of the wheat were cut close to ground on 2 June 2009. In fallow, weeds were removed regularly during the winter period.

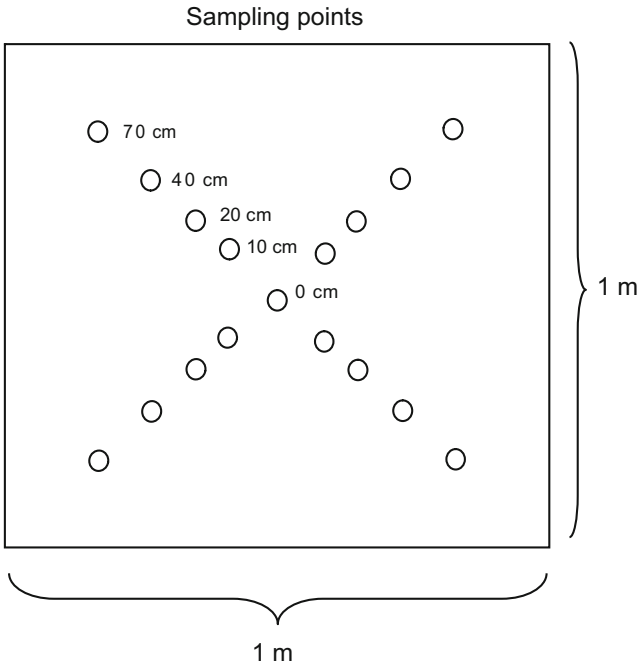
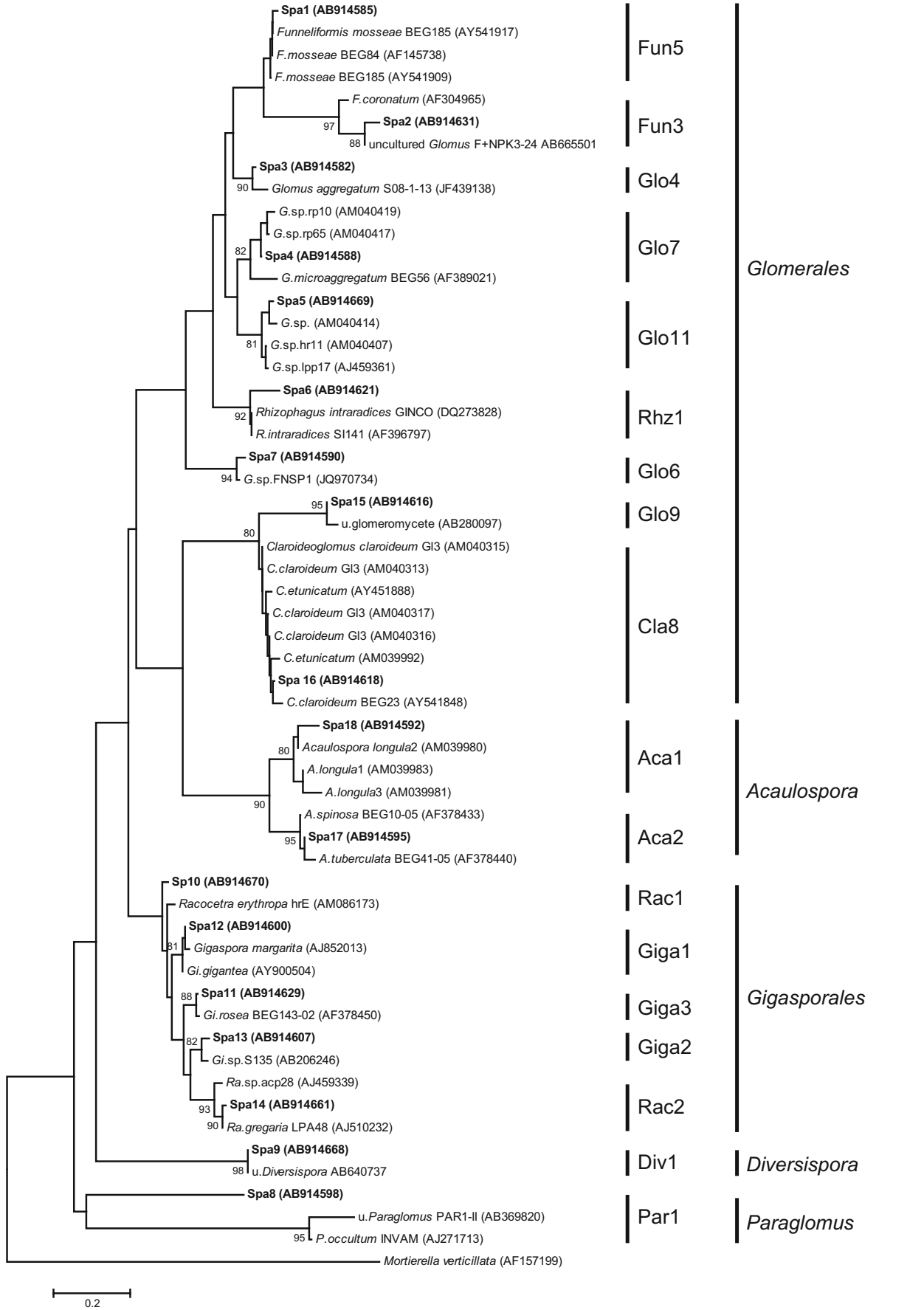


Fig. 1 Sampling strategy in plots of the wheat and fallow rotational system with soybean

Soil sampling

On each site, a 1 m x 1 m frame was placed in 4.5 m x 2.0 m plot. Soil samples (2 cm diameter, 5 cm depth) were collected from each plot at coordinates 0, 10, 20, 40, and 70 cm situated to facilitate spatial analyses, on 2 June 2009 (Fig. 1). The soil cores were collected at the four points from each distance. Following collection, the soil samples were pooled into one composite sample. Composite soil samples for each point were sieved through a 2 mm mesh and stored at −30 °C for DNA extraction.

Fig. 2 Neighbor-joining (NJ) tree of partial large subunit ribosomal ▶ DNA (LSU rDNA) sequences obtained from rhizosphere soils in the wheat and fallow plots based on maximum composite likelihood model, rooted tree with *Mortierella verticillata* as an outgroup. Bootstrap values (only values>80 are shown) were estimated from 1,000 replicates. Representative sequences from soils are incorporated. Each individual representative sequenced sample is labeled as *Spa* followed by the sample number. GenBank accessions indicate arbuscular mycorrhizal fungi (AMF) sequences obtained from soils


Spa1 (AB914585)
Funneliformis mosseae BEG185 (AY541917)
F.mosseae BEG84 (AF145738)
F.mosseae BEG185 (AY541909)
F.coronatum (AF304965)
97
Spa2 (AB914631)
88
uncultured Glomus F+NPK3-24 AB665501
Spa3 (AB914582)
90
Glomus aggregatum S08-1-13 (JF439138)
G.sp.rp10 (AM040419)
G.sp.rp65 (AM040417)
82
Spa4 (AB914588)
G.microaggregatum BEG56 (AF389021)
Spa5 (AB914669)
G.sp. (AM040414)
81
G.sp.hr11 (AM040407)
G.sp.lpp17 (AJ459361)
Spa6 (AB914621)
92
Rhizophagus intraradices GINCO (DQ273828)
R.intraradices SI141 (AF396797)
Spa7 (AB914590)
94
G.sp.FNSP1 (JQ970734)
95
Spa15 (AB914616)
u.glomeromycete (AB280097)
80
Claroideoglomus claroideum GI3 (AM040315)
C.claroideum GI3 (AM040313)
C.etunicatum (AY451888)
C.claroideum GI3 (AM040317)
C.claroideum GI3 (AM040316)
C.etunicatum (AM039992)
Spa 16 (AB914618)
C.claroideum BEG23 (AY541848)
Spa18 (AB914592)
80
Acaulospora longula2 (AM039980)
A.longula1 (AM039983)
A.longula3 (AM039981)
90
A.spinosa BEG10-05 (AF378433)
95
Spa17 (AB914595)
A.tuberculata BEG41-05 (AF378440)
Sp10 (AB914670)
Racocetra erythropa hrE (AM086173)
Spa12 (AB914600)
81
Gigaspora margarita (AJ852013)
Gi.gigantea (AY900504)
88
Spa11 (AB914629)
Gi.rosea BEG143-02 (AF378450)
82
Spa13 (AB914607)
Gi.sp.S135 (AB206246)
Ra.sp.acp28 (AJ459339)
93
Spa14 (AB914661)
90
Ra.gregaria LPA48 (AJ510232)
Spa9 (AB914668)
98
u.Diversispora AB640737
Spa8 (AB914598)
u.Paraglomus PAR1-II (AB369820)
95
P.occultum INVAM (AJ271713)
Mortierella verticillata (AF157199)
0.2
Fun5
Fun3
Glo4
Glo7
Glo11
Rhz1
Glo6
Glo9
Cla8
Aca1
Aca2
Rac1
Giga1
Giga3
Giga2
Rac2
Div1
Par1
Glomerales
Acaulospora
Gigasporales
Diversispora
Paraglomus

DNA extraction from soil

Four independent DNA extractions from soil were made using an ISOIL for Beads Beating kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The extracts were prepared using 0.5 g soil for each sample per replicate. The DNA pellet was washed once with 80 % ethanol, air dried, then resuspended in 100 μL Tris-EDTA (TE) buffer [10 mM Tris–HCl (pH 8.0) and 1 mM EDTA (pH 8.0)], and stored at −30 °C.

Nested PCR

The DNA samples for each soil replicate were diluted 20-fold prior to PCR. Amplification of the D2 region of the fungal 25S large subunit ribosomal DNA (LSU rDNA) was performed using the protocol of Higo et al. (2014). The fungus-specific primers LR1 (5′-GCA TAT CAA TAA GCG GAG GA-3′) (van Tuinen et al. 1998) and FLR2 (5′-GTC GTT TAA AGC CAT TAC GTC-3′) (Trouvelot et al. 1999) were used in the first PCR to amplify the 5′ end of the LSU rDNA region. PCR was performed in a 10-μL reaction volume, containing 1-μL template DNA, 1 μL 10 x PCR buffer, 0.2 mM of each deoxyribonucleotide triphosphate, 0.3 μM of each primer, and 0.25 U TaKaRa *Taq* DNA polymerase (Takara Shuzo, Tokyo, Japan), using the Mastercycler Ep gradient thermal cycler (Eppendorf, Hamburg, Germany). The first PCR products were diluted 100-fold and used as template for the second PCR using the nested primers FLR3 (5′-TTG AAA GGG AAA CGA TTG AAG T-3′) and FLR4 (5′-TAC GTC AAC ATC CTT AAC GAA-3′) (Gollotte et al. 2004) using the same PCR conditions (Higo et al. 2014).

Cloning, sequencing and construction of LSU rDNA libraries

The four independent second PCR products per replicate were pooled to one representative sample combined prior to cloning. This approach has been shown to detect similar levels of AMF diversity compared with cloning and sequencing of individual replicates (Renker et al. 2006; Higo et al. 2013, 2014). For nested PCR yielding amplicons, 1 μL of the reaction was cloned into the pT7 Blue plasmid, using the Perfectly Blunt cloning kit (Novagen, Madison, WI) following the manufacturer's instructions. One clone library per replicate from each sample point was constructed, resulting in 30 clone libraries. The plasmids positive for the insert were sequenced in both directions using M13 forward and reverse primers, using a DNA sequencer (ABI 3130xl, Applied Biosystems, Tokyo, Japan) and the ABI BigDye terminator v3.1 cycle Sequencing Kit (Applied Biosystems).

Reconstruction of phylogenetic trees, rarefaction curves

Multiple alignments were performed using the CLUSTAL W algorithm. Neighbor-joining (NJ) phylogenic trees were constructed using MEGA5 (Tamura et al. 2011), *Mortierella verticillata* (accession no. AF157199) was used as an outgroup and bootstrap values were estimated from 1,000 replicates. The AMF community was characterized on the basis of the LSU rDNA, allowing for the identification of phylotypes. Nucleotide sequences with a greater than 97 % identity were assigned to the same AMF phylotype group. Representative phylotype sequences, defined as groups of closely related sequences, with a high level of bootstrap support in the phylogenetic analysis, were selected. These representative sequences were combined with high similarity sequences, obtained from NCBI and used to build a smaller tree using NJ methods.

Rarefaction curves were constructed with the freely available software Analytic Rarefaction Program version 1.3 (http://www.uga.edu/strata/software/Software.hyml/) in order to determine whether the number of sequenced samples tested, sufficiently represented AMF phylotype. In this study, approximately 100 sequence samples were analyzed from each clone library.

The sequences obtained in this study have been deposited in the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/) database and assigned accession numbers from

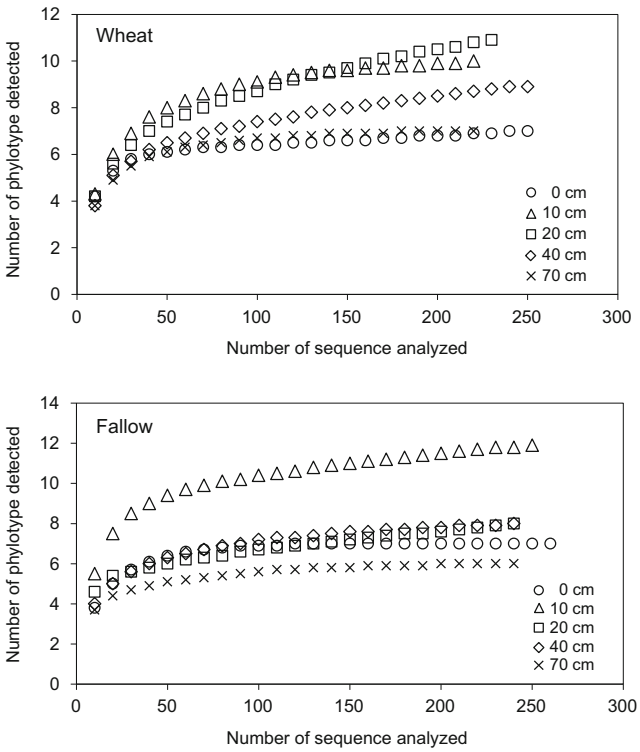


Fig. 3 Rarefaction curve of the phylotypes obtained from the soil samples of each sampling point. Nucleotide sequences with greater than 97 % identity are referred to as the same phylotype group

Table 1 Relative abundance (%) of detected phylotypes in wheat and fallow soybean rotations at different soil sampling points. Numbers denote means of $n=3$

Phylotype	Wheat					Fallow				
	0 cm	10 cm	20 cm	40 cm	70 cm	0 cm	10 cm	20 cm	40 cm	70 cm
Glo4	7.4	7.8	29.9	15.9	24.7	54.0	14.9	19.2	21.9	55.2
Glo6	13.2	2.5				8.0	10.8			
Glo7			4.0	6.4	5.3	3.7		14.1	2.6	
Glo9		10.4	6.0	3.0		1.9		1.4	0.4	0.8
Glo11			0.8							
Rhz1	11.5		7.9	11.8	4.9		0.8	0.4		
Fun3			0.4							
Fun5	11.0	4.5			9.3		9.4			
Cla8	51.9	55.4	42.8	56.1	51.5		0.4		13.5	12.4
Rac1				5.2				0.4		
Rac2		1.8	1.1	0.8	3.3	5.0	7.6	13.7		14.6
Giga1	0.4	6.9	6.1			22.6	33.3	22.3	46.6	2.4
Giga2	4.7	10.3	0.4	0.4	1.0	4.7	9.5	28.6	11.3	14.6
Giga3		0.5								
Aca1							3.6		2.5	
Aca2							4.4		1.3	
Div1				0.5	0.4		0.4			
Par1							4.8			

AB914578 to AB914670. Only representative sequences were deposited.

Statistical analysis

Canonical correspondence analysis (CCA) as multivariate analysis was performed using the vegan package version 2.0-10 in R 3.0.2 (http://www.r-project.org/). The species data matrix was composed from the abundant AMF phylotypes and winter cover crop management or sampling distance. During the CCA procedure, the Monte Carlo 999 permutation test for significance at the $P<0.05$ was used.

Results

A total of 2,449 clones was sequenced in this study, with all sequences having a high similarity (97–99 %) to the AMF LSU rRNA gene and belonging to members of phylum Glomeromycota. Phylogenetic analysis revealed 18 AMF phylotypes, including five *Glomus*, three *Gigaspora*, two each from *Racocetra*, *Acaulospora*, and *Funneliformis* and one each from *Claroideoglomus*, *Rhizophagus*, *Diversispora*, and *Paraglomus* at different sampling points in this study (Fig. 2). Rarefaction analysis revealed that cloning and sequencing of the pooled DNA extracts was an effective method for capturing the majority of the AMF community in soil samples (Fig. 3). The rarefaction curves by analyzed sequence numbers in each clone library almost reached a plateau.

The distribution of each AMF phylotype was different between the two plots (Table 1), although treatment-specific phylotypes were not detected in this study. In general, *Glomerales* was dominant and detected at a much higher frequency, *Glomerales* (Glo4, Glo6, Glo7, Rhz1 and Fun5) were detected in both treatments in fairly high abundance. Phylotype Cla8 was detected in high abundance under wheat, but not fallow. The frequency of *Gigasporales* (*Gigaspora* and *Racocetra*) was much higher during fallow than in the presence of wheat. In addition, *Gigasporales* were detected in wheat plot, albeit at very low relative abundance.

Furthermore, in each sampling point, the distribution of each AMF phylotype was different in the two plots. Among all sampling points, *Glomerales* was dominant and detected at a much higher frequency, although the distribution of phylotypes differed in wheat and fallow plots. In addition, Rac2, Giga1 and Giga2 were found at the each sampling points in the fallow plot, albeit at a higher frequency.

Canonical correspondence analysis (CCA) was used to identify relationships among AMF communities with sampling point (Fig. 4) and winter cover crop management (Fig. 5). CCA showed that the composition of AMF

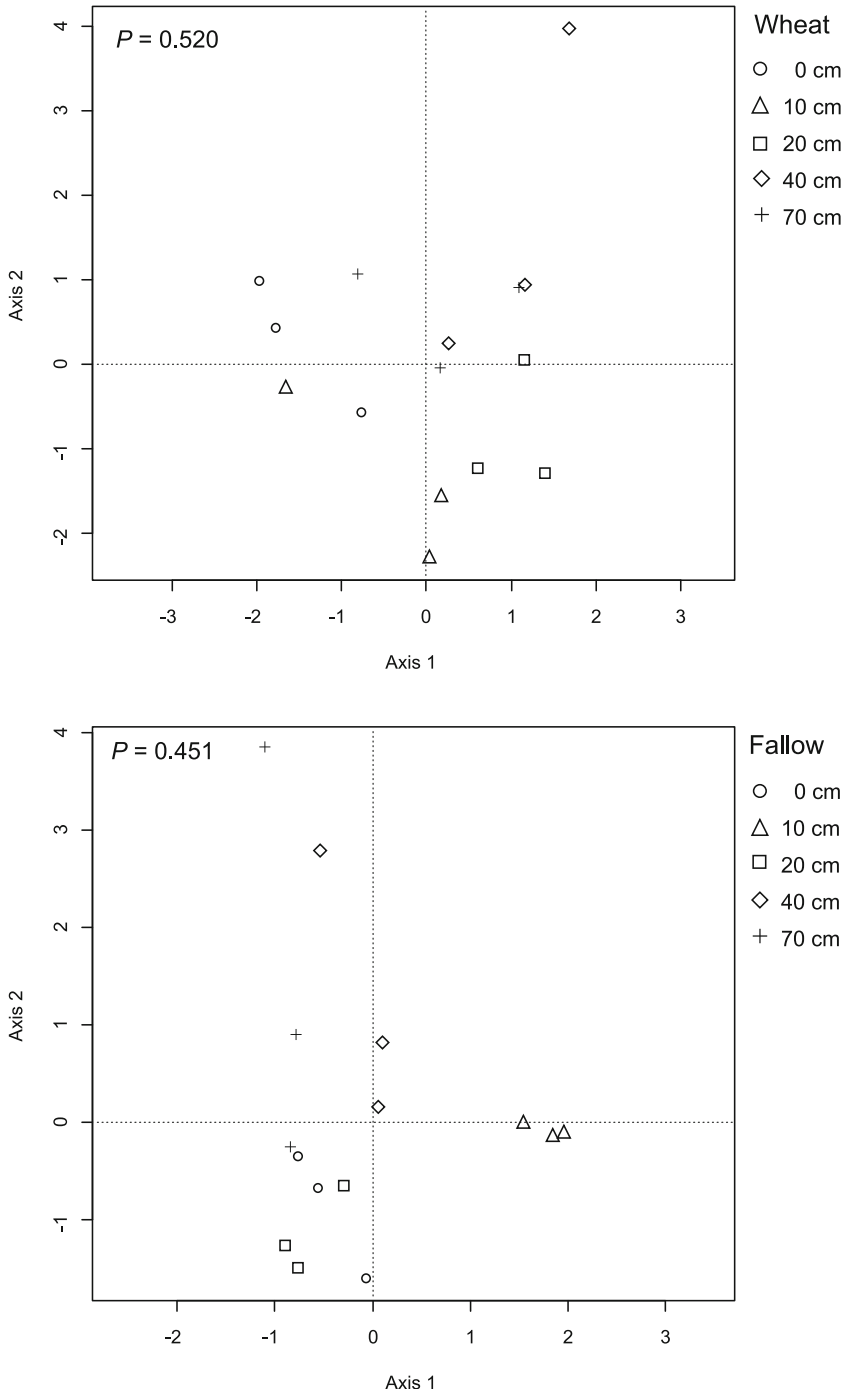


Fig. 4 Canonical correspondence analysis (CCA) biplot showing the relationship between the detected phylotypes and sampling point in wheat and fallow. In wheat, the eigenvalues of the first and second axes were 0.231 and 0.133, respectively. In fallow, the eigenvalues of the first and second axes were 0.264 and 0.138, respectively

communities did not differ significantly by sampling point (wheat: F=0.960, P=0.520, fallow: F=1.041, P=0.451). However, the composition of AMF communities were influenced significantly by winter cover crop management (F=3.187, P=0.001).

The AMF phylotype richness per soil sample was not significantly different across soil sampling points by two-way ANOVA (Table 2). No apparent differences in the mean number of AMF phylotypes, and diversity index were found with the soil sampling point in both rotations.

Discussion

Recently, there is increasing evidence that spatial distribution and variability of AMF communities in soil at small scales has been only partially revealed using molecular techniques. Wolfe et al. (2007) reported spatial variation of AMF communities in soil found within 2 m × 2 m scale, and Mummey and Rillig (2008) also indicated spatial autocorrelation in AMF communities at a small (1 m × 1 m) scale in a

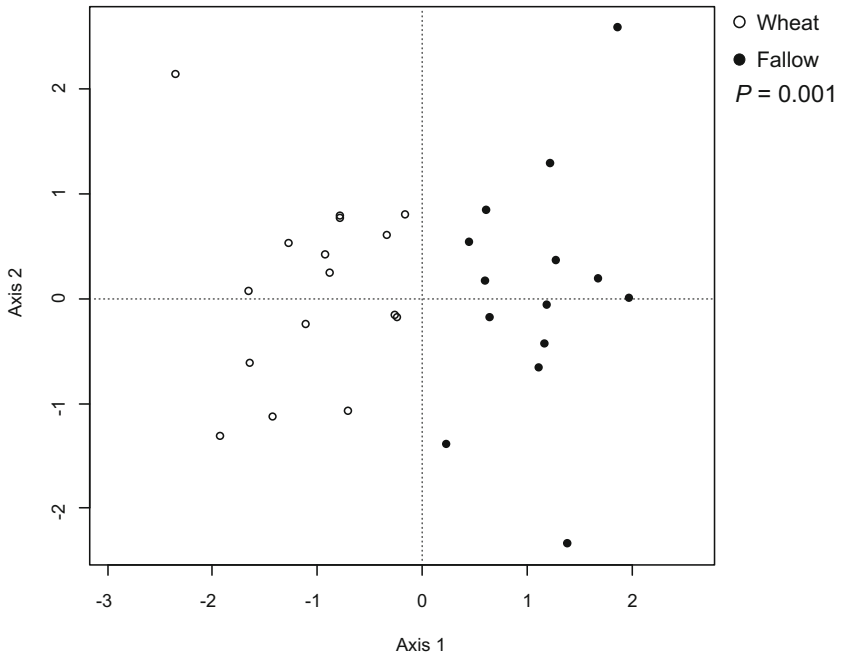


Fig. 5 CCA biplot showing the relationship between detected phylotypes and winter cover crop management. The eigenvalues of the first and second axes were 0.355 and 0.345, respectively

temperate grassland. Furthermore, Davison et al. (2012) have shown that AMF communities in soil were spatially heterogeneous at 10 m × 10 m scale in a forest ecosystem. In the present study, no difference was found in the composition of AMF communities among different sampling points in both wheat and fallow plots (Fig. 4). Our results corresponded with those of a study by Öpik et al. (2009), who reported no difference in AMF communities between sampling plots in the same forest, although this did not correspond to the studies of Mummey and Rillig (2008) and Davison et al. (2012), who have shown AMF communities to be distributed patchily in different plant vegetation at multiple scales. Our study indicates that the spatial distribution of specific AMF communities responded more to the presence of the crop in the winter cover crop rotational system than to the soil sampling point.

It is well known that *Glomerales* is the most prevalent AMF found in agricultural fields (Sasvári et al. 2011; Borriello et al. 2012; Holland et al. 2014), in agreement with this study (Table 1). *Gigasporales* propagate via sporal dispersal or infection by an intact mycelium (Biermann and Linderman 1983; Daniell et al. 2001). In contrast, *Glomerales* are prevail particularly in arable crop fields, as their ability to sporulate allows a quicker recovery in disturbed environmental conditions (Oehl et al. 2003). In addition, *Glomerales* typically form a symbiotic relationship with plant roots via fragments of hyphae or mycorrhizal root, immediately forming hyphal anastomosis (Giovannetti et al. 1999, 2001), and thus can more quickly reestablish a hyphal

Table 2 Molecular diversity of AMF communities in wheat and fallow soybean rotations at different soil sampling points. Numbers denote means of $n = 3$ and SE

Sampling point (cm)	Phylotype richness		Diversity index (H)	
	Wheat	Fallow	Wheat	Fallow
0 cm	4.33±0.67	3.33±0.33	0.99±0.43	0.83±0.13
10 cm	4.67±0.33	5.33±1.33	1.09±0.22	1.29±0.23
20 cm	6.67±0.88	5.00±1.15	1.24±0.17	1.33±0.16
40 cm	4.00±1.00	4.67±0.88	0.97±0.27	1.07±0.12
70 cm	3.67±0.33	4.00±1.15	1.08±0.14	0.93±0.16
Distance (A)	ns*		ns	
Rotation (B)	ns		ns	
A×B	ns		ns	

*Not significant

network after mechanical disruption. These variations support the dominance of *Glomerales* phylotypes over *Gigasporales* phylotypes in the soil regardless of winter cover crop rotational systems.

In previous studies, many researchers have shown that plant communities affect the diversity and composition of AMF communities. Host preference of AMF is likely to be one of the important factors in AMF community composition (Vandenkoornhuyse et al. 2002, 2003). The relative abundance of *Gigasporales* phylotypes in the fallow plot was significantly much higher compared to that in the wheat plot (Table 1). *Gigasporales* phylotypes have been found in the roots of soybean under different agricultural managements (Chifflot et al. 2009; Isobe et al. 2011; Higo et al. 2014). Higo et al. (2014) have also reported finding *Gigasporales* in subsequent soybean roots under the same rotational system used in this study, with a high relative abundance in the roots. Furthermore, An et al. (1993) have indicated that *Gigaspora* spp. increased and became dominant under continuous soybean cultivation in a western Kentucky field in the US. Scheublin et al. (2004) have also found that AMF communities clearly differed among legume and non-legume plants. Thus, it could be possible that the relative abundance of *Gigasporales* phylotypes in the fallow plot was higher than that in the wheat plot due to host preference between *Gigasporales* and leguminous crops. On the contrary, we did not investigate whether the soybean roots as a preceding crop in this rotational system were completely degraded at the end of the wheat growing season. The proliferation of AMF communities in soil could be affected if the soybean roots were not completely degraded. This study was based on DNA only from soil, not from roots or rhizosphere. Further research would be needed to better understand this point to capture the impact of the soil sampling strategy on shaping the composition of AMF communities in agricultural soil.

In conclusion, we revealed that the spatial distribution of AMF communities in soil was not affected within a 1 m × 1 m scale under both rotations examined in an agricultural soil. The diversity of AMF communities was similar regardless of soil sampling point, but the composition of AMF communities was significantly different between wheat and fallow rotations. This indicates that the soil sampling strategy, i.e., random sampling, could be an effective way to better characterize AMF communities in agricultural soil, because random soil sampling did not show any difference in AMF communities in soil under wheat and fallow rotational systems regardless of where we collected soil samples at small scales (< 1 m^2) in the cover crop rotational field. This result means that the diversity and composition of AMF communities associated to agricultural fields at a small scale (< 1 m^2) are not biased if the soil is sampled randomly. Furthermore, our results indicate that the impact of crop management on AMF communities at small scales in agricultural fields may be less than that on AMF communities in natural ecosystems, as the diversity and communities of plant vegetation in agricultural fields is generally lower than those of natural ecosystems. In addition, our results show clearly that knowledge of how AMF are dispersed in the agricultural environment is important for determining spatial distribution, and to the understanding of relationships among AMF communities, crop rotations and agricultural ecosystem processes. This knowledge would also be fundamental to understanding the underground communications that drive deviation in crop rotational systems and for determining the sampling scales that would be most applicable for reflecting changes in AMF community composition. Further research into functioning aspects of AMF would be needed for greater understanding of spatial variability and distribution of AMF in agricultural fields at small or semi-large scales.

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