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Effects of different management practices on arbuscular mycorrhizal fungal diversity in maize fields by a molecular approach

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Abstract

As obligate mutualistic symbionts, arbuscular mycorrhizal fungi (AMF) colonize the roots of many agricultural crops, and it is often claimed that agricultural practices are detrimental to AMF. As a result, agroecosystems impoverished in AMF may not get the fully expected range of benefits from these fungi. Using molecular markers on DNA extracted directly from soil and roots, we studied the effects of different management practices (tillage and N fertilization) on the AMF communities colonizing an experimental maize field in Central Italy. Our molecular analysis based on three different nuclear rRNA regions (18S, 28S and ITS) allowed us to assess AMF biodiversity. Glomeraceae members were the main colonizer, and they co-occurred with Gigasporaceae and *Paraglomus* regardless of the management practices applied. Diversisporaceae and Entrophosporaceae members were instead detected in the N-fertilized soils and in the untreated soil, respectively. The results obtained indicated that the general AMF assemblages structure and composition in the maize field plots appear to be primarily influenced by N fertilization and, to a lesser extent, by tillage. This study also validates the usefulness of multiple molecular markers to consolidate and refine the assessment of the environmental AMF diversity.

Introduction

The symbiotic fungi that form arbuscular mycorrhizae (all belonging to the phylum Glomeromycota) are among the most important soil microorganisms (Schüßler et al. *2001*). They are thought to be the oldest group of organisms living in symbiosis with land plants (Redecker et al. *2000*). Arbuscular mycorrhizal fungi (AMF) are generalist and colonize the roots of most land plants, where they facilitate mineral nutrient uptake from the soil in exchange of plant-assimilated C (Smith and Read *2008*). In addition to improved plant nutrition, AM fungi protect their hosts from pathogens (Pozo and Azcón-Aguilar *2007*) and affect plant growth traits (Artursson et al. *2006*; Barret et al. *2011*). Although their spores can germinate in the absence of host plants, AMF are obligate biotrophs, and they depend on a living photoautotrophic partner to complete their life cycle.

About 240 AMF species have been described so far (Oehl et al. *2011*), primarily on the basis of spore morphology, but increasingly combined with DNA sequence analyses (Öpik et al. *2010*). The use of DNA sequencing methods and high-throughput molecular tools has improved our understanding of the diversity of AMF, in particular because they have allowed AMF to be directly detected from environmental samples (Öpik et al. *2009*; Lumini et al. *2010*; Dumbrell et al. *2011*). Apparently, natural communities of AM fungi are biologically diverse, with large numbers of undescribed taxa. Large numbers of plant–fungus combinations occur naturally, but not all fungus–plant combinations behave similarly, implying that the cost/benefit ratio differs with different fungal–plant cohorts (Helgason and Fitter *2009*). There is evidence that experimental (van der Heijden et al. *1998*) and natural (Moora et al. *2004*) AMF communities, with different taxa compositions, may induce a different growth response in plants and may thus influence the structure and composition of vascular plant communities. Like natural host plants, many important crop plants (sorghum, maize, wheat, rice) belonging to worldwide agroecosystems establish symbioses with AMF (Öpik et al.*2006*), having the possibility to exploit the entire range of benefit granted by this interaction.

However, modern intensive farming practices seem to be a threat to AMF, affecting and shaping AMF communities to a great extent. Many studies indicated that AMF abundance and effectiveness, with respect to root colonisation and plant growth promotion, are declining as agricultural practices are intensified (Jansa et al. *2003*; Oehl et al. *2005*; Alguacil et al. *2008*; Verbruggen et al. *2010*).

Until now, most surveys described AMF communities present in natural or in agricultural fields using molecular and morphological approaches, on either mycorrhizal roots (Alguacil et al. *2009*; Verbruggen et al.*2010*) or spores (Moreira-Souza et al. *2003*; Oehl et al. *2003*), but just a handful of studies have considered the entire soil, or at least the entire topsoil (Hempel et al. *2007*; Pivato et al. *2007*; Cesaro et al. *2008*).

In this work, we have undertaken a study of the impact of different management practices on AMF diversity in a maize crop agroecosystem located in the coastal hills of the Marche region (Central Italy) mainly using a molecular approach based on "environmental" DNA directly extracted from soil and roots. Samples were collected during the maize-growing season in conventional and no-tillage systems in fertilized and unfertilized soils, and the composition of the arbuscular mycorrhizal fungal community was then characterized.

The innovative aspect of this work lies in the application of three different primer sets to molecularly assess the AMF biodiversity. The simultaneous analysis of three different rRNA gene regions allowed obtaining a wide coverage of the AMF taxa, either as spores, extraradical and intraradical mycelia, present in maize fields subjected to different agricultural practices.

Our final goal was to identify which of the aforementioned agricultural practices may have impacted AMF communities and therefore potentially affected soil quality and the sustainability of this agricultural ecosystem.

Materials and methods

Experimental sites

The "P. Rosati" Experimental Agriculture Station, established in 1994 and located in Agugliano (43°32′20.62″ N, 13°22′07.51″ E, 100 m a.s.l.), Ancona, Italy, was selected for this study as a sampling site. The mean annual temperature is 15.3°C and the mean annual precipitation 700 mm. The field experiment was set up on a hillside (20 % slope) where the soil is classified as Calcaric Gleyic Cambisol (FAO, 2006), which in the first 30 cm has an Ap horizon, silt–clay texture (sand 10 %, clay 47 %, silt 43 %), 7.9 g kg^{-1} organic C, 33 % carbonates and a pH of 8.3. Between 30 and 50 cm, the soil has a Bw horizon, pH of 8.5, 6.40 g kg^{-1}organic C, as well as a texture and carbonate percentages similar to those of the upper layers. The site is managed under a *Triticum durum* and *Zea mays* rotation. *Z. mays* (hybrid DK440) was planted on 20th April and harvested on 24th September 2007. The experimental field was organized as two main plots to compare two tillage techniques (T, conventional 40-cm-deep ploughing; S, no-till with chemical desiccation and chopping) with two N fertilizer treatments as the subplots (1, 90 Kg N ha^{-1} year^{-1} as ammonium nitrate; 0, no fertilization—320 m^2 each). The T plots were ploughed every year by moldboard at 40-cm depth in autumn (October) for wheat and at the end of summer (from 30th August until 30th September) for maize. The seedbed was prepared with double harrowing before the sowing date. The S plots were left undisturbed, except for sod seeding, crop residuals and weed chopping, and total herbicide spraying prior to seeding. Tilling at 40-cm depth represents the traditional agronomic practice adopted by local farmers (Marche region) for agricultural fields placed on a hillside and characterized by heavy textured soil. Ammonium nitrate or urea was applied at a rate of

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90 kg N ha^{-1}, split equally in February and April to wheat; a single application of 90 kg N ha^{-1} was applied to maize. Triple superphosphate was always applied before sowing at a rate of 50 kg ha^{-1} of P_2O_5 per year. The four considered treatments were abbreviated as follows for ease of labelling: tilled with N fertilizer (T1), tilled without N fertilizer (T0), no-tilled with N fertilizer (S1) and no-tilled without N fertilizer (S0). In winter, no cover crops were present in the tilled plots (T0, T1), whilst the no-tilled plots (S0, S1) were covered by weeds (*Setaria* sp.).

Soil and root sampling

Soil samples (three replicates) were collected on 11th June 2007, during maize rotation, in the four considered treatments, for a total of 12 samples. The soil cores, measuring 5 cm in diameter and 20 cm in length, were taken at 10- to 30-cm depth. The soil samples were sieved immediately at 2 mm, frozen and stored until analysed.

Two full maize plants were collected during the anthesis development stage from each treatment plot and taken to the laboratory (a total of eight plants). Root fragments from each plant (0.5 g each sample) were washed free of soil, air-dried at room temperature and immediately used for morphological analyses. The roots were stored at −20°C until used for molecular analyses.

Assessment of root colonisation by AMF

Maize roots were stained with 0.01 % cotton blue in lactic acid for about 20 h and then destained four times with lactic acid. Roots were cut into small fragments (1 cm each) and mounted onto microscope slides with lactic acid. Forty-five fragments were observed for each root apparatus, for a total of 360 root fragments. AMF colonisation intensity in the root cortex and arbuscule presence were determined, as described in Trouvelot et al. (*1986*). All the root fragments analysed showed a good mycorrhization level with a mean value of 78.5 %.

DNA extractions from soils and roots

Two independent DNA extractions (0.5 g of soil each) from the 12 soil samples were performed using a FastDNA Kit (MP Biomedicals) according to the manufacturer's recommendations. An additional step, consisting of six washes with guanidine thiocyanate (6 M), was introduced after the addition of the binding matrix suspension due to the high levels of clay in the soil samples. DNA extractions from the eight root samples were performed using a DNeasy Plant Mini Kit (Qiagen, Crawley, UK), according to the protocol for frozen samples.

PCR, cloning and sequencing of the fungal ribosomal (rRNA) genes

Three sets of primers were independently used to amplify three different regions of Glomeromycota ribosomal DNA. The universal eukaryotic primer NS31 (Simon et al. *1992*) was used as the forward primer for the first primer set. A mixture of three different primers (AMmix) was used as the reverse primer—AM1 (Helgason et al. *1998*), AM2 and AM3 (Santos-González et al. *2007*) primers—designed to amplify ~550-bp fragments of partial SSU rDNA. The second primer set (28G1–28G2) was designed to amplify a ~600-bp fragment of partial LSU rDNA (da Silva et al. *2006*). In addition, a nested PCR approach (Millner et al. *2001*) was applied to both the soil and root DNA first with the primer set LSU-Glom1/SSUGlom1 (Renker et al.*2003*) and then a PCR round with the Paraglomeraceae family-specific primer pair GOCC56/GOCC427 designed to amplify a fragment of rDNA gene including part of the ITS1, the complete 5.8S and part of ITS2 regions. The annealing temperature was raised to 58°C when these primers were used to increase specificity, as suggested by Hempel et al. (*2007*).

Amplification of genomic DNA from soil and root

PCR on DNA extracted from soil were performed using 0.1 µg µl^{-1} bovine serum albumin (BSA), 0.2 mMdNTPs, 3.5 mM of MgCl$_2$, 0.5 µM of each primer (28G1–28G2 and NS31-AMmix) and the supplied reaction buffer, with 2 U of High Fidelity Taq (Roche) to obtain a final volume of 40 µl. Amplifications were carried out in 0.2-ml PCR tubes using a Biometra T Gradient thermocycler in the following steps: 5 min initial denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C; and a final elongation of 7 min at 72°C. A negative control was included in the PCR to check for contamination. The PCR on DNA extracted from root were carried out as described above, except for the annealing temperature that was 58°C. All PCR products were checked using 1.5 % agarose gel with SYBR®safe DNA gel stain (Invitrogen).

Cloning and sequencing

The PCR products were purified using QIAquick (Qiagen, Hilden, Germany), cloned in a pGEM-T Easy vector (Promega, Madison, Wisconsin, USA) and transformed into *Escherichia coli* (XI1 blue). Putative positive transformants were screened in each resulting SSU, LSU, ITS rRNA gene library. The reaction mixture for the PCR amplification of the mycorrhizal root DNA (25 µl) contained: 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 0.1 % gelatin, 0.2 mM of each dNTP, 0.1 µg µl^{-1} BSA, 0.5 µM of each primer and 1 U of Red*Taq* DNA polymerase (Sigma). Amplifications were carried out as described above, except for the cycling parameters which instead were: 35 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C for 35 cycles and a final elongation of 5 min at 72°C after the last cycle. All PCR products were checked using 1.5 % agarose gel with SYBR®safe DNA gel stain (Invitrogen). All clones having inserts of the correct size in each library were sequenced using T7 vector primers by Macrogen sequencing services (Macrogen, Seoul, Korea).

Sequences generated in this study were registered in GenBank under the following accession numbers HM215625-HM216180.

Sequence analyses and phylogenetic inference

Sequence editing was done using Sequencer v4.2.2 (Gene Codes Corporation). Possible chimera sequences were identified using the Chimera Detection programme (Cole et al. *2003*). A search for similar sequences to the ones from this study was conducted with the BLAST tool (Zhang et al. *2000*) provided by GenBank. The sequences were with high similarity and a wide selection of AMF taxa, including representatives of the major clades described by Oehl et al. (*2011*). All the sequences were aligned, separately for the SSU and LSU groups, using the multiple sequence comparison by log-expectation, MUSCLE v3.7, programme (Edgar*2004*).

The alignments obtained using MUSCLE 3.7 were manually edited and the distance matrices constructed using DNAdist from the PHYLIP suite of programmes, version 3.6, with default parameters (Felsenstein*2005*). These pairwise distances were used as input for DOTUR (Schloss and Handelsman *2005*) in order to group the sequences into operational taxonomic units (OTUs) of a defined sequence identity. A threshold of 97 % identity, corresponding to 0.03 dissimilarity (OTU$_{0.03}$), was used to define OTUs, and it was chosen on the basis of previous studies on AMF biodiversity (Pivato et al. *2007*; Santos-González et al. *2007*). Neighbour-joining (NJ) phylogenetic analyses (1,000 bootstrap replicates) were performed with MEGA version 4 programme (Tamura et al. *2007*) using the default parameters. Sequences of *Mortierella verticillata*(AF157145), *Chaetomium globosum* (AB048285), *Trichoderma* sp. (EF672340) and *Fusarium oxysporum*(DQ916150) were used as outgroups in the SSU phylogenetic tree. The

outgroup for the LSU phylogenetic tree was a *Claroideoglomus claroideum* (AF235007) sequence. The same alignments were used to perform a Bayesian inference (BI) approach. BI of phylogeny using Monte Carlo Markov chains (MCMC) was carried out with MrBayes 3.1.2 (Huelsenbeck et al. *2001*). For each alignment, four incrementally heated simultaneous MCMC were run over 1,000,000 generations, under model assumption, using random starting trees and default starting values of the models. Trees were sampled every 100 generations, resulting in an overall sampling of 10,001 trees. The first 2,500 trees were discarded as "burn-in" (25 %). For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian posterior probabilities. Branch lengths were estimated as the mean values over the sampled trees.

GLO_Sx (*Glomerales*), GLO_Lx (*Glomerales*), GIG_Sx (*Gigasporales*) and DIV_Sx (*Diversisporales*) OTUs were named according to their position in the major AMF clades, followed by the letter S (for SSU sequences) or L (for LSU sequences) and a numerical index (x) identifying the OTU number.

Diversity and structure of the AMF communities

In order to analyse the richness and diversity of the AMF communities in the four considered conditions, only sequences belonging to Glomeromycota were selected for the subsequent analyses. The use of DOTUR allowed us to obtain a variety of diversity and richness estimators: the Shannon–Weaver diversity index, a rarefaction curve, bias-corrected Chao1 richness and ACE abundance-based coverage estimators.

A comparison of the AMF spectra was carried out in the collected samples by means of canonical variates analysis (CVA), a multivariate statistical method. CVA was performed on OTU abundances (number of sequences comprising each OTU). The analysis was carried out using the "Canonical Variates" SYN-TAX 2000 package subroutine with the "Spherized scores of objects" (normalization of eigenvectors) option. Correlations with the original variables were also analysed. Only OTUs comprising more than ten sequences were considered for the analysis since the contribution of rare OTUs to community structure is limited. Due to the low number of Glomeromycota sequences obtained from the root samples, it was decided to consider them as subsamples of each soil treatment in the CVA analysis.

Results

AMF assemblage identification in soils and roots

The three primer combinations (NS31/AMmix, 28G1/28G2 and GOCC56/GOCC427) yielded three clone libraries, carrying SSU, LSU and ITS fragments, respectively, from each sampling point. We screened and sequenced 1,209 clones in total from soil and roots: 724 clones from the libraries carrying SSU fragments, 389 clones from the LSU libraries and 96 clones from the ITS libraries.

Apart from sequences showing high similarity (98–99 % identity) with AM fungi (Glomeromycota phylum), sequences corresponding to non-target organisms were also obtained. BLAST searches revealed indeed that out of the 724 SSU sequences obtained using the NS31/AMmix primers, 427 sequences (59 % of the total sequences) featured high similarity to sequences from taxa belonging to the phylum Glomeromycota, whilst the remaining 297 sequences (41 % total sequences) were related to sequences from Ascomycota. The high number of Ascomycota sequences detected analysing the SSU library is due to a not complete specificity of the primers (NS31/AMmix) for the Glomeromycota group, as already shown in other studies (Santos-González et al. *2007*; Toljander

et al. *2008*). Out of the 389 sequences obtained with the 28G1/28G2 primer set, 364 sequences (94 % total sequences) showed high similarity to sequences from Glomeromycota, whilst the remaining 25 sequences (6 % total sequences) showed BLAST similarity with other eukaryotes. All the 96 ITS sequences obtained with the GOCC primers set were found to belong to Glomeromycota. These ITS sequences were obtained from the soils samples and can be referred to only two clades, related to an undescribed *Paraglomus* species and *Paraglomus brasilianum*, respectively (HM215625–HM215633).

The 427 SSU and 364 LSU Glomeromycota sequences were then grouped, using DOTUR software, into OTUs. Considering a similarity threshold of 97 %, the programme identified 27 SSU OTUs (seven of which were singletons) and 55 LSU OTUs (32 of which were singletons). OTUs containing only one sequence (singletons) were discarded, and only OTUs containing two or more sequences retrieved from two different clones were considered for further analyses. This choice allowed us to minimize the possibility to incorporate in the analysis PCR cloning and sequencing artefacts. In conclusion, 20 SSU and 23 LSU OTUs were considered for further analyses.

Phylogenetic analyses of AMF sequences from maize field

Separate phylogenetic analyses were performed for SSU and LSU sequences to assign the different OTUs to the cognate Glomeromycota groups.

Small subunit

The phylogenetic analysis of the SSU rRNA sequences is shown in Fig. 1. By far, the most abundant and diverse AMF taxon in soil samples was Glomeraceae, as defined by Oehl et al. (*2011*), with 276 sequences grouped in 17 OTUs. Three OTUs (GLO_S25, GLO_S22, GLO_S24) were detected in soil under all management conditions. OTU GIG_S26, which the phylogenetic tree showed to be related to *Gigasporales*clade, was also retrieved in the soil under all analysed conditions. OTU DIV_S11, related to *Redeckera fulva*AM418543 (85 % bootstrap support), was only detected in soil subjected to N fertilization.

Phylogenetic tree with associated SOIL and ROOT sample counts.

Tree tip labels (top to bottom):

- AJ301858 Funneliformis verruculosum W3295
- AJ418852 Funneliformis geosporum BEG11
- AJ505619 Funneliformis clarum BEG125
- AJ276085 Funneliformis fragilistratum
- Y17635 Funneliformis caledonium BEG20
- AJ301865 Funneliformis sp WUM3
- AF074360 Funneliformis sp Glo1
- U96139 Funneliformis mosseae BEG12
- AJ315516 Funneliformis sp
- AJ276086 Funneliformis coronatum
- ● GLO_S27
- ● GLO_S17
- ● GLO_S15
- AJ506090 Septoglomus constrictum
- ● GLO_S16
- ● GLO_S12
- ● GLO_S13
- ● GLO_S20
- AF485890 Simiglomus hoi
- AJ301857 Simiglomus sp W3347
- AF485867 Simiglomus sp Glo9
- AF437679 Glomus sp Glo19
- ● GLO_S24
- AY129625 Glomus sp Glo18
- AF437670 Glomus sp Glo17
- AY129620 Glomus sp Glo14
- AF437666 Glomus sp Glo15
- AY512366 Glomus sp Glo20
- AF437669 Glomus sp Glo16
- ● GLO_S22
- AF074353 Glomus sp Glo4
- ● GLO_S10
- ● GLO_S18
- AF437660 Glomus sp Glo13
- ● GLO_S8
- ● GLO_S23
- ● GLO_S9
- ● GLO_S25
- AF074370 Glomus sp Glo7
- EU332715 Glomus sp M27
- AF481623 Glomus sp Glo2
- ● GLO_S21
- AJ133706 Glomus sinuosum MD126
- AY273580 Glomus sp Glo3
- AF213462 1 Glomus proliferum
- AF131053 Glomus sp Glo11
- Y17648 3 Glomus manihotis
- EU332711 Glomus sp G06
- AJ536822 Glomus intraradices BEG121
- AJ309396 Glomus sp Glo10
- L20824 Glomus vesiculiferum
- ● GLO_S19
- X58725 Glomus sp. DAOM197198
- Y17640 Glomus fasciculatum BEG53
- AY129602 Glomus sp Glo8
- ● GLO_S14
- AF074371 Glomus sp Glo5
- Y17652 Viscospora viscosa BEG27
- Y17639 Claroideoglomus etunicatum UT316
- AJ276087 Claroideoglomus lamellosum W3160
- AJ276089 3 Claroideoglomus luteum
- AJ276075 2 Claroideoglomus claroideum
- AF437658 Glomus sp Glo12
- X86687 Diversispora versiformis BEG47
- AM946964 Diversispora versiformis
- AJ276077 Diversispora spurca
- AJ301860 Diversispora sp. W2423
- AF074352 Diversispora sp
- AM418543 Redeckera fulvum
- ● DIV_S11
- AJ306439 Acaulospora longula
- Z14005 Acaulospora rugosa
- Y17633 Acaulospora laevis
- AF131027 Scutellospora dipurpurescens
- AB041344 Dentiscutata cerradensis
- AY635832 Fuscutata heterogama
- AJ306445 Scutellospora calospora
- AJ242729 Orbispora projecturata
- Z14009 Gigaspora albida FL927
- Z14010 Gigaspora gigantea WV932
- U96146 Gigaspora decipiens BEG45
- ● GIG_S26
- AM181144 Gigaspora margarita BEG34
- AF157145 Mortierella verticillata
- AB048285 Chaetomium globosum
- EF672340 Trichoderma sp
- DQ916150 Fusarium oxysporum

Clade labels: Glomerales, Diversisporales, Gigasporales

Taxon	SOIL S0	SOIL S1	SOIL T0	SOIL T1	ROOT S0	ROOT S1	ROOT T0	ROOT T1
GLO_S27			39	23			2	
GLO_S17			2	3			13	
GLO_S15	4	2						
GLO_S16	1				4	1		
GLO_S12		3						
GLO_S13	1	2						
GLO_S20		6	7	7	1			
GLO_S24	1	18	7	6	5	4		1
GLO_S22	2	15	14	4	3	2	1	
GLO_S10						2		
GLO_S18	3	2		4				
GLO_S8	2							
GLO_S23		9	8	4				
GLO_S9	2							
GLO_S25	7	23	4	3	7	1	5	1
GLO_S21	16							
GLO_S19			5	7	1			2
GLO_S14	6							
DIV_S11		1		2				
GIG_S26	21	18	16	24				

Scale bar: 0.01

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Fig. 1

Neighbour-joining phylogenetic analysis of arbuscular mycorrhizal fungal SSU rDNA sequences (~500 bp) obtained from maize root and bulk soil samples. The scale represents substitutions per site. Sequences obtained in the present study are highlighted by a *dot*. Sequences from databases were labelled with the accession number. Bootstrap values from 1,000 replications larger than 50 % are indicated *above branches.Brackets to the right* indicate diverse taxonomic orders as described by Oehl et al. (*2011*). Phylogenetic tree is rooted with outgroup sequences of *M. verticillata* (AF157145), *C. globosum* (AB048285), *Trichoderma* sp. (EF672340) and *F. oxysporum* (DQ916150). The *table beside the phylogenetic tree* shows the distribution of the sequences of each OTU among the different management techniques and between soil and roots. The values represent the sampling point pooled together

Only OTUs related to the Glomeraceae clade were found in the root compartment. GLO_S25 was the only OTU detected in roots under the four different management conditions. GLO_S22 and GLO_S24, which were retrieved in the soil for all the different management conditions, were instead present in the roots of plants grown under only three out of the four management conditions. The other six OTUs retrieved in the roots were associated with one or two management conditions. Phylogenetic analysis, Bayesian inference and neighbour-joining, resulted in the same main tree topology.

Large subunit

The phylogenetic analysis of the LSU rRNA sequences is shown in Fig. 2. The phylogenetic analysis confirmed the exclusive presence of Glomeraceae species, as already suggested by preliminary BLAST analyses. Seven OTU clades can be recognized in the phylogenetic tree receiving bootstrap support ≥96 %.
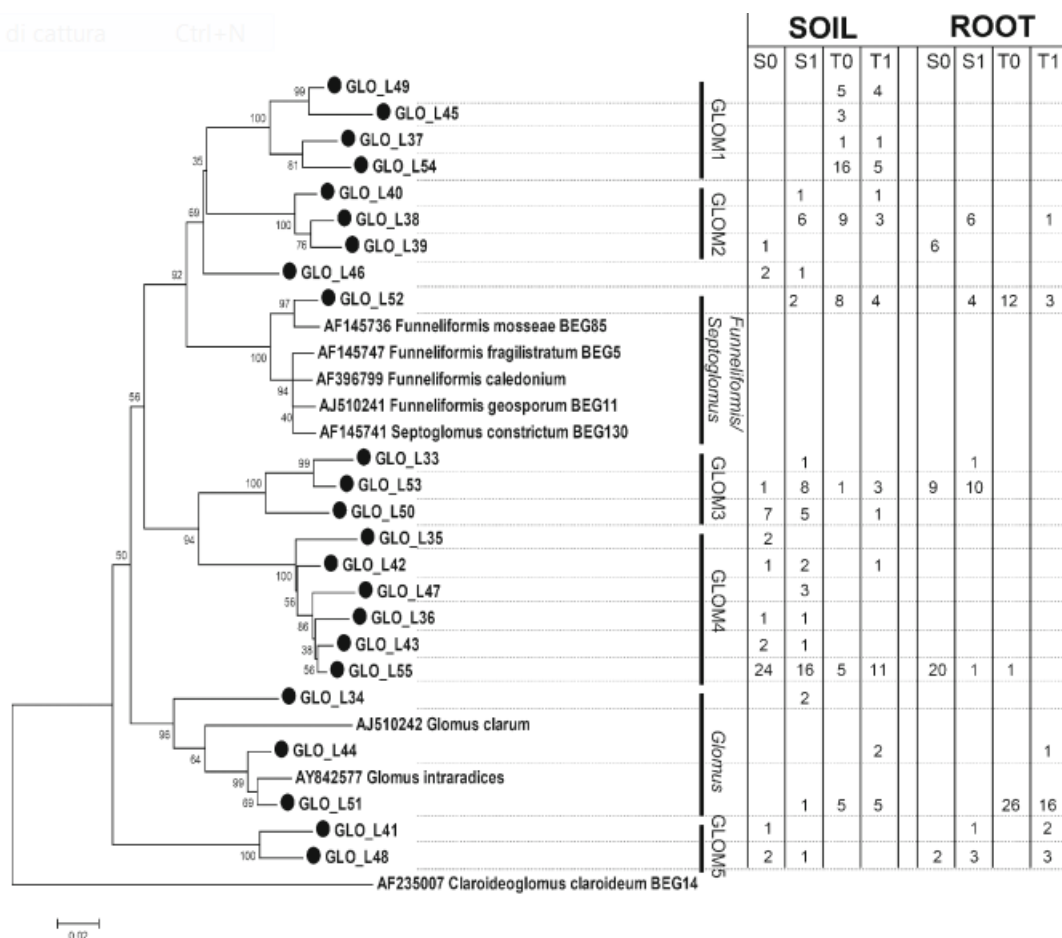
Fig. 2 Neighbour-joining phylogenetic analysis of arbuscular mycorrhizal fungal LSU rDNA sequences (~500 bp) obtained from maize root and bulk soil samples. The scale represents substitutions per site. Sequences obtained in the present study are highlighted by a *dot*. Sequences from databases were labelled with the accession number. Bootstrap values from 1,000 replications larger than 50 % are indicated *above branches*. *Brackets to the right* indicate diverse taxonomic group as described by Oehl et al. (2011) (*italic*) and in the phylotypes identified (*capital letters*). Phylogenetic tree is rooted with outgroup sequences *C. claroideum* (AF235007) which belong to a phylogenetically distinct group inside Glomerales (*Claroideoglomus*; (Oehl et al. 2011). The *table beside the phylogenetic tree* shows the distribution of the sequences of each OTU among the different management techniques and between soil and roots. The values represent the sampling point pooled together

OTU	Clade	SOIL S0	SOIL S1	SOIL T0	SOIL T1	ROOT S0	ROOT S1	ROOT T0	ROOT T1
GLO_L49	GLOM1			5	4				
GLO_L45	GLOM1			3					
GLO_L37	GLOM1			1	1				
GLO_L54	GLOM1			16	5				
GLO_L40	GLOM2		1		1				
GLO_L38	GLOM2		6	9	3		6		1
GLO_L39	GLOM2	1				6			
GLO_L46		2	1						
GLO_L52	Funneliformis/Septoglomus		2	8	4		4	12	3
AF145736 Funneliformis mosseae BEG85	Funneliformis/Septoglomus								
AF145747 Funneliformis fragilistratum BEG5	Funneliformis/Septoglomus								
AF396799 Funneliformis caledonium	Funneliformis/Septoglomus								
AJ510241 Funneliformis geosporum BEG11	Funneliformis/Septoglomus								
AF145741 Septoglomus constrictum BEG130	Funneliformis/Septoglomus								
GLO_L33	GLOM3		1				1		
GLO_L53	GLOM3	1	8	1	3	9	10		
GLO_L50	GLOM3	7	5		1				
GLO_L35	GLOM4	2							
GLO_L42	GLOM4	1	2		1				
GLO_L47	GLOM4		3						
GLO_L36	GLOM4	1	1						
GLO_L43	GLOM4	2	1						
GLO_L55	GLOM4	24	16	5	11	20	1	1	
GLO_L34			2						
AJ510242 Glomus clarum	Glomus								
GLO_L44	Glomus						2		1
AY842577 Glomus intraradices	Glomus								
GLO_L51	Glomus		1	5	5			26	16
GLO_L41	GLOM5	1					1		2
GLO_L48	GLOM5	2	1			2	3		3
AF235007 Claroideoglomus claroideum BEG14									

Among the 23 non-singleton LSU OTUs, only three could be associated to sequences identified with unambiguous species binomials in GenBank. OTU GLO_L52, which was retrieved under the S1, T0 and T1 management treatments in both roots and soils, is related to *Funneliformis mosseae* AF145736 (97 % bootstrap support). In contrast, GLO_L51 and GLO_L44, which are related to *Glomus intraradices* AY842577 (99 % bootstrap support), were detected almost exclusively under tillage conditions. The other OTUs could not be associated to any known Glomeraceae species.

The remaining five OTU clades were progressively numbered from 1 to 5 (GLOM 1–5). Members of the GLOM 1 clade (which comprises GLO_L49, GLO_L45, GLO_L37 and GLO_L54) were only detected in the soil subjected to tillage either with or without N fertilization. In addition, GLOM 1 is the only clade that was not detected in the roots. Both GLOM 3 and GLOM 4 clades contain an OTU (GLO_L53 and GLO_L55, respectively) which was present under all soil managements. The other OTUs comprising these two clades were mainly present in soil under no-till regimens. The GLOM 5 clade, which includes two OTUs (GLO_L41 and GLO_L48), was only retrieved in the soil of non-tilled plots (S1 and S0), whilst it was detected both in maize roots grown in non-tilled plots

(S1 and S0) and in the tilled and N-fertilized plot (T1) roots. Both phylogenetic analysis, Bayesian inference and neighbour-joining, resulted in the same main tree topology.

Analysis of AMF diversity and community structure based on the SSU region

The rarefaction curves related to the 97 % sequence identity SSU OTUs detected in the four soils indicate that our sampling efforts yielded a large proportion of the soil AMF communities diversity, at least for the two tilled plots (T1 and T0), whereas the rarefaction curves relative to the non-tilled plots (S0 and S1) show a decreasing accumulation rate of OTUs, but do not reach the asymptote (Fig. 3). This finding was confirmed by the ACE and Chao1 non-parametric richness estimators, which indeed were in the range to the actually observed OTU numbers in the tilled plots (Table 1).
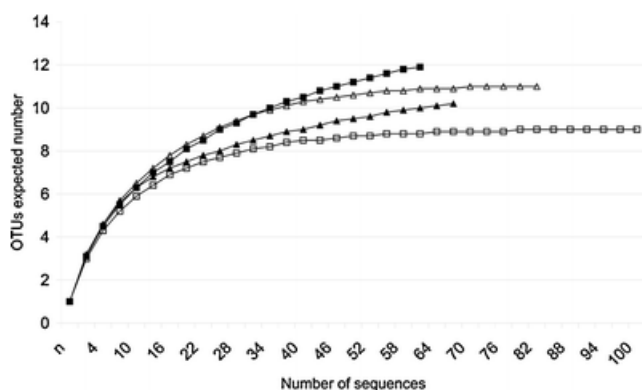
Fig. 3

Rarefaction curves for SSU 97 % sequence identity OTUs detected in S0 (*closed square*), S1 (*closed triangle*), T0 (*open square*) and T1 (*open triangle*) soils, as determined using DOTUR

Table 1

Observed versus estimated (±SE, based on ACE and Chao1 non-parametric richness estimators) 97 % sequence identity OTU numbers for the four soil management conditions

Soil management	Observed OTUs	Estimated OTUs	
		ACE	Chao1
S0	12	14.2 (±2.6)	13.5 (±2.3)
S1	11	11.5 (±1)	11.2 (±0.5)
T0	9	9 (±0)	9 (±0)
T1	11	11 (±0)	11 (±0)

The Shannon–Weaver index, calculated on the SSU data, was not significantly different ($df = 3$, $F = 3.205$, $P = 0.361$) among the four soils under analysis. The values detected were: 1.59 ± 0.45 for S0, 1.68 ± 0.34 for S1, 1.10 ± 0.39 for T0 and 1.54 ± 0.24 for T1.

The CVA analysis is shown in a biplot (Fig. 4), where Axis1 and Axis2 account for 94.72 and 3.47 % of the total variance, respectively. The CVA biplot (Fig. 4) clearly shows that replicates of the same cultural conditions are well grouped together. The analysis discriminates the different

groups mainly on the basis of N fertilization (mainly due to OTUs GLO_S27, GLO_S17, GLO_S24 and GLO_S23). In detail, OTUs GLO_S23 and GLO_S24, belonging to unidentified *Glomus*, seem to be enhanced by N fertilization, whilst GLO_S27 and GLO_S17, belonging to the *Funneliformis* clade, by the lack of fertilization. Both Axis1 and Axis2 discriminate between the AMF communities present in the tilled and the no-tilled plots for a given fertilization treatment.
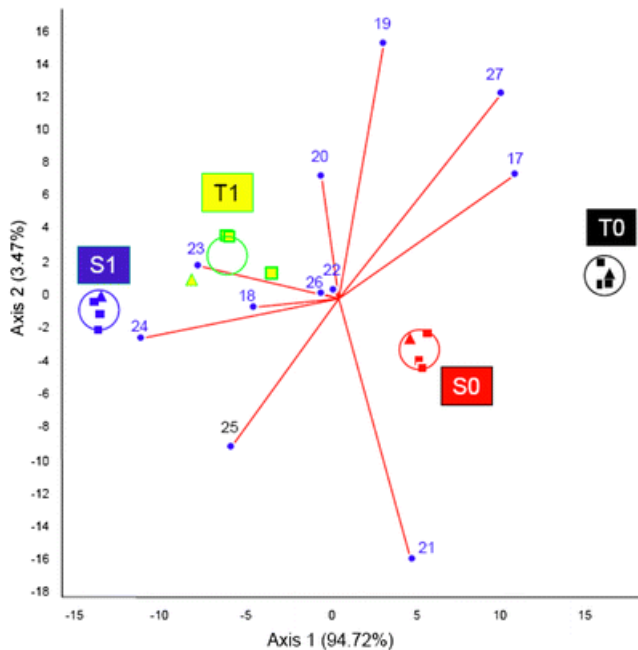

Fig. 4

CVA biplot of the AMF communities found in the soil (*square*) and maize roots (*triangle*) for the four different plots. The ellipses represent 90 % confidence intervals. The percentage of the total variance accounting for each canonical variate is reported. Treatments: tilled and fertilized (*T1*); tilled and non-fertilized (*T0*); no-tilled and fertilized (*S1*); no-tilled and non-fertilized (*S0*). The vectors represent the correlations of SSU OTUs with the canonical axes: GLO_S17 (*17*), GLO_S18 (*18*), GLO_S19 (*19*), GLO_S20 (*20*), GLO_S21 (*21*), GLO_S22 (*22*), GLO_S23 (*23*), GLO_S24 (*24*), GLO_S25 (*25*), GIG_S26 (*26*), GLO_S27 (*27*)

Discussion

In this work, the diversity of AMF communities occurring in a maize field subjected to different agricultural management practices was investigated using molecular markers based on rRNA gene regions. Since no primer combination designed until now is able to detect all Glomeromycota fungi in environmental conditions, we decided to use three primer combinations (NS31/AMmix, 28G1/28G2 and GOCC56/GOCC427) in order to detect a wide spectrum of AMF species difficult to cover with a single primer set. The molecular analysis of DNA directly extracted from soil allowed investigating which AMF were present as mycelia and spores in soil avoiding the selection effects due to trap plants or the tedious and time-expensive morphological observation of the spores. To our knowledge, only a few other papers (Pivato et al. *2007*; Hempel et al. *2007*; Cesaro et al. *2008*; Balestrini et al. *2010*) have dealt with the analysis of AMF assemblages detected using DNA extracted directly from soil.

Each primer combination contributes to AMF diversity description

Even if the comparison of the different primer set performances was not the main aim of this study, we provide some general considerations to better understand the results. The small

subunit rRNA gene was examined because it is the most widely used marker in field studies analysing AMF communities, also due to the availability of primer sets which are able to detect a wide range of glomeromycotan genera as well as the increasing number of available AMF sequences in GenBank following large-scale 454 pyrosequencing (Öpik et al. *2009*; Lumini et al. *2010*). In particular, the NS31-AMmix primer set should allow the detection of Glomeraceae, Entrophosporaceae, Diversisporaceae, Acaulosporaceae, Gigasporaceae, Scutellosporaceae, Dentiscutaceae, Racocetraceae and Archaeosporaceae (Santos-González et al. *2007*; Schechter and Bruns*2008*). As expected, the phylogenetic analyses of 427 SSU AMF sequences obtained from soil and maize roots using this primer set allowed us to identify sequences belonging to Glomeraceae (79 %), Entrophosporaceae (1.4 %), Diversisporaceae (0.8 %) and Gigasporaceae (18.8 %). Lack of detection of Archaeosporaceae in both samples (maize roots and soils) did not probably depend on the primers used since NS31/AM1 successfully amplified many *Archaeospora* sequences from *Collinsia sparsiflora* roots in Californian soils (Schechter and Bruns *2008*). On the other hand, whilst *Archaeospora* species have frequently been found in trap cultures, they have never been detected in arable soils (Hijri et al. *2006*), which is in agreement with the results of other molecular studies on AMF communities in Central Europe (Daniell et al. *2001*; Vandenkoornhuyse et al. *2002*; Gollotte et al. *2004*; Scheublin et al. *2004*; Renker et al. *2005*; Börstler et al. *2006*).

The second primer set used (28G1/28G2), amplifying a region of the LSU rDNA, was chosen because this region is claimed to exhibit a higher phylogenetic resolution than the SSU, and it has recently been used to design new primer sets for AMF species-level identification (Krüger et al. *2009*). Even though this primer set also amplifies *Diversisporales* (da Silva et al. *2006*), we only amplified sequences from fungi belonging to*Glomerales*, probably due to a major affinity of the primers towards this group of AMF. Despite the relatively restricted range of sequences detected, the phylogenetic analysis highlighted six well-supported clades inside the Glomeraceae showing different distributions across the different agricultural practices. However, the lack of LSU sequences of reliably identified species in public databases does not allow affiliating these clades to other subgroups already described, with the exception for two of them (*Funneliformis/Septoglomus*subgroup and *Glomus* subgroup).

The third primer set targeting the ITS rDNA region of Paraglomeraceae was used since this family was not detected by the other two primer sets.

AMF biodiversity in maize field

AMF diversity, as expressed by Shannon–Weaver diversity indices, was almost consistent for the different soil management conditions (1.10–1.68) and definitely higher than those obtained from other fields studies based on plant root molecular analysis (Daniell et al. *2001*; Hijri et al. *2006*; Alguacil et al. *2009*). Moreover, rarefaction curves, together with ACE and Chao1 indices, suggested that the number of observed OTUs is close to the estimated number, indicating good sampling efficiency for AMF diversity in soil. However, other field studies based on spore isolation and characterization from agricultural soils showed slightly higher Shannon–Weaver diversity indices (Oehl et al. *2010*). Even though the results obtained with these two different approaches cannot be easily compared, it is possible that AMF biodiversity based on spore morphological characterization allows catching some morphospecies probably biased by molecular analysis. On the other hand, the molecular approaches allow detecting putative cryptic species not yet characterized as morphospecies because these are present in soil as mycelia and not as spores.

Our results show a predominance of OTUs belonging to the family Glomeraceae, demonstrating that the members of this genus were the main colonizers of the maize field. Of the 18 SSU OTUs belonging to Glomeraceae, only OTU GLO_A$_S$19 could be ascribed to a known isolate, *Glomus* sp. DAOM197198. The other 17 Glomeraceae OTUs found in both soil and maize roots were often closely related to other root-derived sequences from various ecosystems around the world, but none was closely related to any described AMF species in GenBank. The higher phylogenetic resolution power of the LSU region allowed us to recognize seven well-supported subgroups inside the Glomeraceae clade. Two subgroups out of seven were clearly affiliated with *Funneliformis/Septoglomus* and *Glomus*, respectively. Within such subgroups, only two phylotypes, GLO_L52 and GLO_L51, could be identified at the species level, as *F. mosseae* and *G. intraradices*, respectively. These OTUs were mainly retrieved from the soil and roots subjected to tillage. The absence, in public databases, of fully identified sequences clustering in the remaining five subgroups (GLOM1, GLOM2, GLOM3, GLOM4, GLOM5) did not allow us to ascribe such subgroups to known species. OTU GLO_L55, which was comprised within such subgroups, was the most frequent OTU retrieved from the soil (24 % of the total sequences) as well as the main colonizer of maize roots analysed in the S0 treatment along with GLO_L53 (9.7 % of the total sequence).

The SSU OTU comprising the Entrophosporaceae group (GLO_S14) was only detected in S0, suggesting a low tolerance to perturbed conditions. Moreover, OTU DIV_S11, related to *R. fulva*, only seems to be associated with the N-fertilized samples (S1 and T1), even though the low number of sequences comprising this OTU does not allow firm conclusions to be drawn. In addition, OTU GIG_S26, grouping many sequences related to Gigasporales, was found in soil under all treatments. This finding was rather unexpected since members of this family are not usually detected (either as spores or sequences obtained from crop plants) in agricultural fields upon soil disturbance imposed either through agricultural use or by heavy landscaping machinery (Jansa et al. *2003*; Mathimaran et al. *2007*), but are in line with the results of Oehl et al. (*2005,2010*) based on spore identification tools from field samples. The results obtained with the GOCC56/GOCC427 primer pair highlights the difficulty of detecting Paraglomeraceae in root samples, as already reported by (Hijri et al. *2006*). In addition, Hempel et al. (*2007*) observed that these fungi can be dominant in certain soils. In the current study, some sequences related to this taxon were found under all soil management conditions, in particular sequences with high similarity to an undescribed *Paraglomus* species, and to *P. brasilianum*, which has already been reported in maize roots (Millner et al. *2001*; Morton and Redecker *2001*).

The explorative analysis of the AMF community present in the roots highlighted a strong predominance of sequences belonging to Glomeraceae, ascribable to the most frequent OTUs retrieved in soil. The predominance of species belonging to Glomeraceae detected in soil and roots confirm the well-known characteristics of the member of this family, adaptability and stress tolerance, confirming that they can be retrieved across a wide range of habitats, either natural or agricultural (Öpik et al. *2006*).

Agricultural practice impacts AMF distribution

The observed dominance of Glomeraceae sequences is in agreement with previous reports concerning agricultural lands (Öpik et al. *2006*). For example, Helgason et al. (*1998*) found a dominance of *F. mosseae* or closely related species, whereas Daniell et al. (*2001*) mostly found *Funneliformis caledonium–Funneliformis geosporum* sequences. The dominance of Glomeraceae species has also been reported in studies based on spore morphology, *F. mosseae* being the most frequently observed species (Oehl et al. *2003*; Cheng and

Baumgartner *2004*; Sjöberg et al. *2004*; Wang et al. *2008*). In other studies, instead, *G. intraradices* was reported as the dominant species especially in heavy textured agricultural soils (Mathimaran et al. *2005*; Hijri et al. *2006*). The presence and abundance of sequences related to *G. intraradices* and *F. mosseae* in perturbed environments is not surprising in light of the many studies reporting a "ruderal" lifestyle for these species (Oehl et al. *2003*, *2004*; Hijri et al. *2006*). The dominance of a few Glomeraceae species is probably a consequence of the strong selection pressure imposed by agricultural practices which leads to the predominance of fast root-colonizing species (Oehl et al. *2004*) and species that are able to tolerate, among others, the repeated disruption of external hyphal networks, periods without mycorrhizal host plants, and the application of fertilizers and fungicides (Gosling et al. *2006*). The analysis conducted using the 28G1–28G2 primer set highlighted several different Glomeraceae OTUs that show strong differences in their response to tillage and N fertilization. Although the effects of mineral N on AMF community composition at present are not well understood, a number of studies have indicated that N fertilization can have a negative impact on the functional structures of AMF and on the richness of AMF colonizing plant roots (Santos et al. *2006*). As to the relative importance of the different agricultural practices (N fertilization and tillage), CVA indicated that the AMF assemblage structure and composition in the maize field plots examined are primarily influenced by N fertilization and, to a lesser extent, by tillage, as indicated by the shift in the dominant groups of AMF that occur in the different treatments.

In conclusion, the results obtained in the maize fields under study highlighted the stronger effect of N fertilization to shape the AMF communities with respect to tillage. High-input agricultural practices force the selection of AMF species showing generally non-beneficial traits, for example high investment in reproduction at the expense of nutrient scavenging and transfer process that are beneficial for hosts. Instead, a lower selection pressure imposed by a modulation of management practices could allow the presence of AMF species showing beneficial–complementary functional traits necessary to guarantee an improvement of the agroecosystem in which they are present (Verbruggen and Kiers *2010*; Barret et al. *2011*). This information is a prerequisite to achieve efficient use and manipulation of AMF communities for long-term agricultural stability and productivity in light of a desirable shift from conventional agricultural systems to more sustainable agricultural models.

Note added in proof

The authors would like to indicate that since the submission of this paper, Krüger M, et al (2012) New Phytologist 193:970–984, in March 2012, reported an extensive molecular phylogeny of arbuscular mycorrhizal fungi (AMF) commented on the same issue by P. Young (2012) New Phytologist 193:823–826.

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