Vertical distribution of arbuscular mycorrhizal fungi in agricultural soil

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Foreword

This dissertation is a cumulative work of manuscripts from my publication list, either published or submitted. The thesis is based on the following papers:

I. **Sosa-Hernández, M. A.**, Roy, J., Hempel, S., Kautz, T., Köpke, U., Uksa, M., et al. (2018). Subsoil arbuscular mycorrhizal fungal communities in arable soil differ from those in topsoil. Soil Biol. Biochem. 117, 83–86. doi:10.1016/j.soilbio.2017.11.009.

II. **Sosa-Hernández, M. A.**, Roy, J., Hempel, S., and Rillig, M. C. (2018). Evidence for Subsoil Specialization in Arbuscular Mycorrhizal Fungi. Front. Ecol. Evol. 6. doi:10.3389/fevo.2018.00067.

III. **Sosa-Hernández, M. A.**, E. F. Leifheit, R. Ingraffia, M. C. Rillig. (201X) Subsoil arbuscular mycorrhizal fungi for sustainability and climate smart agriculture: a solution right under our feet. *To be submitted*.

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I dedicate this work to Sir David Attenborough, with my thanks for a life of dedicated work that has led me, as many others, to the path of biology.

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Chapter 1

General introduction

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are a widespread group of filamentous fungi that form symbiotic relationships with the vast majority of land plants (Brundrett and Tedersoo, 2018). They form a monophyletic clade currently placed in the Glomeromycotina subphylum within the Mucoromycota phylum (Spatafora et al., 2016), but in the past they have been classified as a phylum basal to Dikarya (Hibbett et al., 2007) or within the now extinct Zygomycetes (Morton and Benny, 1990) among others. These migrations in the phylogenetic position of AM fungi correspond to recent profound changes in the fungal systematic with the arrival of molecular tools, but also illustrate considerable gaps in our knowledge about some basic aspects of this group's biology. For instance, characteristics as wether these fungi exhibit heterokaryosis or even ploidy number are still under debate (Bruns et al., 2017; Pandey and Garg, 2017). This notwithstanding, the arbuscular mycorrhizal symbiosis has been extensively researched and is fairly well understood. The fungus colonizes the root developing an intraradical

mycelium and eventually penetrates the cortical cells, where it forms highly specialized exchange structures: the arbuscules (Luginbuehl and Oldroyd, 2017). At the same time an extraradical mycelium is formed to explore the soil, and taking up nutrients such as phosphorous (P), zinc (Zn) or nitrogen (N), to deliver them to the plant in exchange of carbon (C) compounds (Smith and Read, 2008; Smith and Smith, 2011). AM fungi are obligated biotrophs and depend entirely on their plant host for their C supply, receiving up to 20% of the plant's photoassimilates (Bago et al., 2000) in form of monosaccharides and perhaps also lipids (Rich et al., 2017). Plant's benefits from the AM symbiosis in terms of nutrient uptake, particularly P, are well documented (Smith and Smith, 2011), but AM fungi have also proven influence on a variety of ecosystem processes (Powell and Rillig, 2018; Rillig, 2004). Most notably they increase plant productivity (Lekberg and Koide, 2005), protect the plant against pathogenic fungi and nematodes (Veresoglou and Rillig, 2012), promote soil aggregation (Leifheit et al., 2014), improve water uptake (Lazcano et al., 2014) and decrease soil nutrient loss (Cavagnaro et al., 2015). All these features makes them highly relevant in agroecosystems, particularly in the pursuit of sustainable agriculture (Rillig et al., 2016; Thirkell et al., 2017).

The subsoil environment

Subsoil in agriculture is defined as the portion of soil beneath the tilled or formerly tilled horizon, typically starting at a depth from 20 – 30 cm. The differences between top- and subsoil vary greatly with soil type; generally speaking however, subsoil is characterized by lower amounts of organic

carbon (Chen et al., 2018; Schmidt et al., 2011), higher bulk densities (Kautz et al., 2013), smaller fluctuations in temperature and moisture (Fierer et al., 2003; Weil and Brady, 2016), lower pH (Lynch and Wojciechowski, 2015) and comparatively low microbial biomass (von Haden and Dornbush, 2017; Hsiao et al., 2018; Jones et al., 2018); but see (Parvin et al., 2018), where the highest microbial biomass was measured at a depth of 50 cm in a peatland. Microarthropod (Ellers et al., 2018) and microbial communities are typically different across the soil profile (e.g. Bahram et al., 2015; Fierer et al., 2003; Moll et al., 2016; Schlatter et al., 2018; Uksa et al., 2015, **Chapter 2**), and have been shown to be limited by C (Jones et al., 2018) or by P and N (Hsiao et al., 2018). Important processes such as ammonia oxidation, a key element of the N cycle, are mediated by fundamentally different groups (i.e. Archaea and Bacteria) in subsoil as in topsoil (Mushinski et al., 2017; Tao et al., 2018), exemplifying again the uniqueness of deep soil as a microbial environment.

Plants can face a varied amount of challenges in subsoil depending on soil type, including hypoxia, aluminum toxicity and physical resistance to root growth among others (Lynch and Wojciechowski, 2015). However, subsoil can hold up to two thirds of the nutrient pool, and it can contribute to 10-80% of the plant's nutrition (Kautz et al., 2013). Additionally, deep soil layers often act as a moisture reservoir, allowing deep rooting plants to cope better with surface drought (Bardgett et al., 2014). The implications of plants' adequate subsoil access for a sustainable agriculture are discussed in **Chapter 4**.

Vertical distribution of AM fungi

Early research on AM fungi in agriculture already observed abrupt declines in spore numbers and infection levels with depth (Jakobsen and Nielsen, 1983; Smith, 1978; Sutton, 1973; Sutton and Barron, 1972), and similar declines in colonized root length and spore numbers were observed in grasslands (Koide and Mooney, 1987; Zajicek et al., 1986). Notably Zajicek *et al.* (1986) observed that some species disappeared from the spore pool with depth and hypothesized a special adaptation in the ones that were present. Kabir *et al.* (1998) compared two tillage systems at a 5 cm depth resolution down to 25 cm, and found only significant changes between the two systems in the first layer, and the usual decline in spore numbers with depth. However, deep rooting plants in an arid ecosystem had AM roots down to 4.8 m deep in the soil (Virginia et al., 1986). Notably, in a study assessing fumigation effects in a soybean plantation, spores from species that were more abundant at 35 cm were dominant in the upper layer when the communities recovered, suggesting that the deeper layer acted as a reservoir and that those species where able to colonize the superficial soil in the absence of competition (An et al., 1990).

Later on, Oehl *et al.*(2005) analyzed AM communities based on spore morphology in agricultural land and a close by grassland down to 70 cm. They were able to show that spore pools changed with depth, and despite decreasing spore numbers, diversity was still considerable in the deepest layers. Moreover under maize in a plowed system, the greatest spore

diversity was found under the plough layer, and overall some species sporulated predominantly or even exclusively at depths different than topsoil. Similar results were found in a coffee plantation, with a peak in spore numbers in the 20-30 cm layer, and again depth specific sporulation (Muleta et al., 2008), but in a greenhouse study only the general pattern of spore abundance and diversity decrease could be found (Liu et al., 2013). Comparing reduced and conventional tillage through an extensive spore sampling and identification effort, Säle *et al.* (2015) found effects on the topsoil AM community and shifts but no diversity or abundance changes in deeper layers. In an arid ecosystem, AM colonization declined with depth, but was maintained as high as 50% at depth of 1 meter (Taniguchi et al., 2012). In contrast, a T-RFLP (terminal restriction fragment length polymorphism) analysis in a grassland showed no significant differences between the communities at 0-40 and 40-80 cm depth, and the authors attribute this lack of changes to a fairly constant pH through the profile (Montero Sommerfeld et al., 2013). In contrast, in a heavy metal contaminated site, spore numbers and root colonization increased with depth down to 60 cm (Gucwa-Przepióra et al., 2007, 2013).

A group of studies assessed AM abundance trough the profile using fatty acids as biomarkers. Studying a chronosequence after various tillage events, it was found that AM biomass decreased after tillage down to a depth of 30 cm, but the effects were less pronounced below 5 cm (Wortmann et al., 2008). In the same study, the ratio AM to other fungi increased with soil depth, and AM was the group suffering the greatest impact after tillage and the slowest to recover to previous abundances. With a combination of

biomarkers, spore morphology and hyphal length assessment, Tian *et al.* (2011) found the usual declines in abundance and richness with depth but also stated that these parameters were dynamic with time and mirrored the responses in the topsoil, evidencing an active community down to 90 cm. Moreover, in a wheat-soybean rotation it was found that despite a marked decrease in AM biomass with depth, as measured with biomarkers, down to 1 m approximately 50% of the AM stocked biomass could be found below 35 cm (Higo et al., 2013), pointing at an important contribution of deep soil communities in plant's nutrition.

Interestingly, Rillig and Field (2003) observed in a pot experiment with artificially elevated CO_2 levels, no significant changes in AM root colonization in the first 15 cm, but a marked increase in colonization in the 15-45 cm layer. This result suggest that top- and subsoil AM communities may respond differently to aboveground environmental changes.

By the time of the beginning of my work this was the state of the art in vertical distribution of AM fungi. Later published work, as my own research, will be commented in **Chapter 5**.

In **Chapter 2** we present high-throughput sequencing evidence on top- and subsoil differences in AM fungal communities, with the presence of subsoil unique phylotypes. **Appendix 2.2**, **Appendix 2.3** and **Appendix 2.4** expand on these observations. **Chapter 3** studies the inability of some subsoil phylotypes to persist in topsoil, evidencing some level of specialization.

Molecular fungal ecology

Traditionally, AM fungal ecology has been studied through the assessment of relative abundances of different spores characterized by their morphology (e.g. Muleta et al., 2008; Säle et al., 2015). Molecular tools such as Sanger sequencing, which requires either DNA from a single organism or a cloning step, were of limited use as AM fungi typically occur as diverse assemblages even inside an individual root (Valyi, 2016). With the development of high-throughput sequencing techniques in the last decades, multispecies pools from environmental samples could be sequenced in parallel, but often only relatively small DNA fragments could be obtained. Illumina sequencing allows for affordable parallel sequencing, with unprecedented sequencing depths, producing several millions of reads per run. In fact, the sequencing depth of Illumina might be more than enough to capture the diversity of AM fungal communities (Vasar et al., 2017). However, the length of the fragment that can be sequenced is limited, in for instance Illumina MiSeq pair ended technology, to approximately 500 base pairs (bp), depending on the overlap of the two fragments. This poses a challenge for selecting an appropriate genetic marker with sufficient taxonomic resolution and the right length. The ribosomal DNA (rDNA, a DNA sequence that codes for the ribosomal RNA) is the most widespread target for taxonomic identification in fungi, especially the internal transcribed spacer (ITS) contained in it. Illumina technology does not allow for the sequencing of such a long fragment, so smaller regions as fragments of the SSU (small subunit), LSU (large subunit) or ITS1 are commonly used (Hart et al., 2015). Multiple primers have been developed to target these different regions in AM fungi, and direct comparison of them

shows consistent ecological patterns, but differences in representation for particular groups (Lekberg et al., 2018).

It is worth noting that this methodology is not free of some inconveniences that hinder the interpretation of the results. For instance, targeting rDNA carries an inherent bias, as multiple copies of this gene occur in every single nucleus, and the number of copies per nucleus can vary between different species (Hart et al., 2015). Moreover, even intraspecific variability in this gene has been reported, with distinct species exhibiting different degrees of variability (Thiéry et al., 2012, 2016). All of these notwithstanding, Illumina analysis of mock communities have proven to produce consistent and adequate ecological patterns (Egan et al., 2018), validating the use of this technology in molecular fungal ecology.

All the work presented here was performed with Illumina MiSeq pair ended sequencing. **Chapter 2** and **Chapter 3** present data based on partial LSU sequences clustered in OTUs at 97% similarity. **Appendix 2.2** re-analyzes the data presented in **Chapter 3** using an exact sequence variant (ESV) approach. **Appendix 2.3** is based on ITS1 sequences processed with an ESV approach, **Appendix 2.4** is based on partial LSU sequences processed also with a ESV approach.

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Chapter 2

Subsoil arbuscular mycorrhizal fungal communities in arable soil differ from those in topsoil

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Abstract

Arbuscular mycorrhizal fungi are recognized as important drivers of plant health and productivity in agriculture but very often existing knowledge is limited to the topsoil. With growing interest in the role of subsoil in sustainable agriculture, we used high-throughput Illumina sequencing on a set of samples encompassing drilosphere, rhizosphere and bulk soil, in both top- and subsoil. Our results show subsoil AMF communities harbor unique Operational Taxonomic Units (OTUs) and that both soil depths differ in community structure both at the OTU and family level. Our results emphasize the distinctness of subsoil AMF communities and the potential role of subsoil as a biodiversity reservoir.

Chapter 3

Evidence for subsoil specialization in arbuscular mycorrhizal fungi

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Abstract

Arbuscular mycorrhizal (AM) fungal communities are now known to vary with depth in arable land. Here we use two previously published high-throughput Illumina sequencing data sets, and compare a 52 year long chronosequence of recultivated agriculture fields after a topsoil and subsoil mixing event, with a set of undisturbed topsoil and subsoil samples from a similar field. We show that AM taxa identified as subsoil indicators are exclusively present in early stages of the chronosequence, whereas topsoil indicator taxa can be found across the chronosequence, and that similarities from the chronosequence fields to the subsoil communities decrease with time. Our results provide evidence on the ecological specialization of certain AM fungal taxa to deep soil layers.

1. Introduction

Arbuscular mycorrhizal (AM) fungi belong to the monophyletic subphylum Glomeromycotina (Spatafora et al., 2016) and form a symbiotic relationship with most land plants (Brundrett and Tedersoo, 2018). These fungi can increase plant productivity (Lekberg and Koide, 2005), enhance nutrient uptake (Smith and Smith, 2011), promote soil aggregation (Leifheit et al., 2014), boost pathogen protection (Veresoglou and Rillig, 2012), and are therefore considered important factors in agriculture. AM fungal communities in arable land have been characterized both with spore identification techniques (Antunes et al., 2012; Köhl et al., 2014) and molecular methods (e.g. Alguacil et al., 2008; Van Geel et al., 2017) but with few exceptions, existing information is limited to the first 30 cm of the soil profile. Subsoil (i.e. beneath the plough layer) AM fungal communities, however, differ from those in topsoil in diversity, species composition and community structure (Muleta et al., 2008; Oehl et al., 2005; Yang et al., 2010) and even exhibit contrasting patterns of distribution at higher taxonomic levels (Sosa-Hernández et al., 2018). We hypothesize that these differences are caused by Grinellian ecological specialization (Devictor et al., 2010), i.e. top- and subsoil represent two different environments to which particular AM taxa have adapted.

A recent study by Roy et al. (2017) used high-throughput Illumina sequencing to analyze AM fungal communities in a series of agricultural fields in western Germany forming a re-cultivation chronosequence (hereafter referred to as “chronosequence fields”). In short, following mining operations, pits were closed and restored with local soil and after a 3-year period of alfalfa (*Medicago sativa*) cultivation, reconverted to conventional agriculture. The restoration was carried out with a mixture of former agricultural soil and loess parent material from various depths. Therefore, we assume that directly after conversion AM fungal communities from different depths experience a community coalescence event (Rillig et al., 2015), i.e. taxa from different depths are mixed in the newly deposited top layers. This event provides excellent opportunity to trace the fate of subsoil-specific AM fungal

taxa along the re-cultivation chronosequence, which allows testing our hypothesis of ecological specialization of certain AM fungal taxa to deep soil layers. In a recent study, we characterized AM fungal communities in an agricultural field both in top- (10-30 cm deep) and subsoil (60-75 cm deep) (Sosa-Hernández et al., 2018), hereafter referred to as "unmixed field". We identified subsoil and topsoil indicator AM fungal taxa. Here, we traced those taxa along the chronosequence fields. According to our hypothesis, AM fungal taxa identified as subsoil indicators would decrease in abundance in the topsoil along the chronosequence as a function of time since the mixing event occurred, while taxa identified as topsoil indicators will maintain their abundance, and ii) the early mixed community would resemble subsoil communities and this similarity would decrease through time.

2. Material and methods

2.1 Study sites

Both study sites are located in the southwest of the state of North Rhine-Westphalia, Germany, and in both, soil has been characterized as Haplic Luvisol (FAO, 1998). The distance from the unmixed field to the area where the chronosequence fields are located is roughly 55 km. The chronosequence fields (Roy et al., 2017) consist of a re-cultivation chronosequence after open mining, comprising 10 fields each approximately 6 ha in size. The newly deposited soil profile is about 2 m deep and consists of a mixture of the previous soil (1 m deep) and loess substrate in a 1:5 ratio. For the first three years after the mixing event fields are covered permanently with alfalfa (hereafter referred to as phase 1), for the two following years barley (*Hordeum vulgare*) was cultivated (hereafter referred to as phase 2) and after the fifth year conventional agriculture was resumed, with a sugar beet (*Beta vulgaris vulgaris* var. *altissima*) - winter wheat (*Triticum aestivum*) crop rotation (hereafter referred to as phase 3). From 'these chronosequence fields five samples per field were taken at a 0-10 cm depth, adding up to a total of 50 samples. In the unmixed field (approximately 1 ha in size), nine samples each were taken at depths from 10-30 cm and 60-75 cm as

described in Uksa et al. (2014), adding up to a total of 18 samples. Chicory (*Cichorium intybus*) was grown on this field for the third year.

2.2 Sequencing

DNA was extracted from the chronosequence fields' samples using the PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol, as for the unmixed field samples, DNA was extracted using the FastDNA Spin Kit for Soil (MPBiomedicals, Eschwege, Germany) following the manufacturer's protocol. In both studies AM fungal communities were characterized with primers targeting the large ribosomal subunit LSU including the variable D1-D2 region, using similar protocols (see Roy et al. (2017) and Sosa-Hernández et al. (2018) for details). In short, after DNA extraction, PCR was carried out using AM fungal specific primer sets described in Krüger et al. (2009). The product of this amplification was used as a template in a follow up PCR using the general fungal primers LR3 and LR2rev (Hofstetter et al., 2002). Amplicons from the two different studies were sequenced independently but with identical protocols on an Illumina MiSeq platform at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv, Berlin, Germany).

2.3 Bioinformatics processing of amplicon sequences

A total of 2,377,171 raw sequences from the chronosequence experiment and 1,876,440 raw sequences from the unmixed experiment were processed separately as follows: Paired-end sequences were merged and quality filtered (maximum error rate of 1) using USEARCH v8.1.1861 (Edgar, 2010). Sequences were dereplicated and singletons were removed. Further quality filtering was performed by aligning those sequences to an AM fungal ribosomal DNA reference database (Krüger et al., 2012) using mothur v.1.38.1 (Schloss et al., 2009), this process also eliminated the primer sequence. Sequences not overlapping the region were discarded.

Quality filtered and dereplicated sequences from the chronosequence experiment (58,686 sequences) and from the unmixed experiment (53,595

sequences) were pooled together and clustered into operational taxonomic units (OTUs) at a 97% similarity level using UPARSE (Edgar, 2013), which includes internal chimera removal. OTU centroids were identified and non-dereplicated filtered sequences from both experiments including previously discarded singletons, were mapped to those OTUs centroids at a 97% similarity level. Various format editing steps such as sequence counting were performed with OBITools 1.2.9 (Boyer et al., 2016). Representative sequences of these OTUs have been deposited at ENA under accession numbers LT993068-LT993221.

Taxonomic assignment of the OTUs was carried out using BLAST+ (Camacho et al., 2009) against Glomeromycotina reference sequences published in Krüger et al. (2012) and against the EMBL nucleotide database (Kanz et al., 2005). Alignments below 70% similarity and/or shorter than 300 bp were discarded. Results from both databases were checked for consistency and matches contained in Krüger et al. (2012) were used to assign the OTUs. We decided to favor matches in Krüger et al. (2012) over EMBL, due to the often imprecise description of the match in the latter (e.g. “soil fungus”, ”uncultured Glomeromycota”). When the taxonomic resolution of the match was sufficient, we followed a similar approach to that used in Martínez-García et al. (2015) for SSU sequences, and assigned OTUs with ≥ 97% similarity match to a species, ≥90% to a genus, ≥80% to a family and ≥70% to the subphylum. In cases with insufficient resolution in the match description, the OTUs were assigned to the closest available taxonomic level. A species level match refers to how confidently we assign a name to our OTU based on known sequences, and does not imply that these OTUs are to be considered equivalent to those species.

2.4 Statistical analysis

All subsequent analyses were performed with R version 3.3.1 (R Core Team, 2016). Community analyses were performed with the package “vegan” (Oksanen et al., 2016). Before conducting the statistics, five samples belonging to the 45-year old samples in the chronosequence experiment,

were excluded from any subsequent analysis due to very low numbers of AM fungal sequence reads. After this removal, the lowest amount of reads in a sample was determined as 559 and all samples were normalized to this number by random subsampling without replacement with the function “rrarefy”. Rarefaction curves and OTU accumulation curves were generated with the functions “rarecurve” and “specaccum”, respectively.

Using the sequences retrieved from the unmixed field samples we identified sub- and topsoil indicator OTUs using the function multipatt() in the package “indicspecies” (Cáceres and Legendre, 2009) and traced their fate in the chronosequence since the coalescent event.

Compositional changes between samples were measured with Bray-Curtis (Bray and Curtis, 1957) and Jaccard (Jaccard, 1912) dissimilarities with the function “vegdist” and visualized with a non-metric multidimensional scaling (NMDS) using the function “metaMDS”. Additionally, we compared these Bray-Curtis and Jaccard distances between unmixed topsoil or unmixed subsoil samples to the samples from the chronosequence to test for changes in multivariate distances over time. Comparisons between dissimilarities in different phases were performed with pairwise Mann–Whitney tests with correction for multiple testing, as implemented with the function “pairwise.wilcox.test”.

3. Results

After taxonomic assignment and normalization, we identified a total of 136 AM fungal OTUs. Details on the taxonomic assignment of each OTU can be found in **Table S1**. The chronosequence fields yielded a richness of 123 OTUs and the unmixed fields a richness of 73 OTUs. Between the two experiments 60 OTUs were shared, representing 44.12% of the total richness but 93.49% of the reads in “unmixed” fields and 76.53% of the reads in “chronosequence” fields. Both rarefaction curves (**Fig. S1**) and OTU accumulation curves (**Fig. S2**) were past the linearity point, indicating that the sequencing depth and number of samples were appropriated to capture the majority of the diversity.

We identified three subsoil indicator OTUs (**Table 1**), and we detected two of these subsoil indicator OTUs in topsoil from chronosequence fields with time since the mixing event up to five years (**Fig. 1a**). However, we did not detect these OTUs in chronosequence fields older than five years, neither in the rarefied nor in the non-normalized raw OTU tables. Similarly we identified nine topsoil indicator OTUs (**Table 1**). Those topsoil indicators could be detected in all chronosequence fields and they showed a tendency to increase in relative abundance after the first two years since the mixing event (**Fig. 1b**). A complete table with relative abundances of each OTU can be found in **Table S1**.

AM fungal communities in recently restored chronosequence fields (i.e. shortly after the mixing event) are more similar to unmixed subsoil communities, and with increasing time since mixing, chronosequence communities show increasing dissimilarity to unmixed subsoil communities (**Fig. 2).**

Bray-Curtis distances from chronosequence fields to the unmixed subsoil samples increase with time, forming two significantly different groups (phase 1 + phase 2, and phase 3; **Fig. 3A**, for statistics see **Table S2**). Analogous results are obtained when considering Jaccard distances (**Fig. S3A,** for statistics see **Table S2**). Bray-Curtis distances to the unmixed topsoil communities follow a unimodal trend with intermediate values in phase 1, minimum values in phase 2, and maximum dissimilarity values in phase 3 (**Fig. 3B**, for statistics see **Table S2**). Similarly, Jaccard distances to unmixed topsoil follow a unimodal trend with minimum values in phase 2, but phases 1 and 3 are not significantly different **(Fig. S3B,** for statistics see **Table S2**).

Table 1. Identified sub- and topsoil indicators.

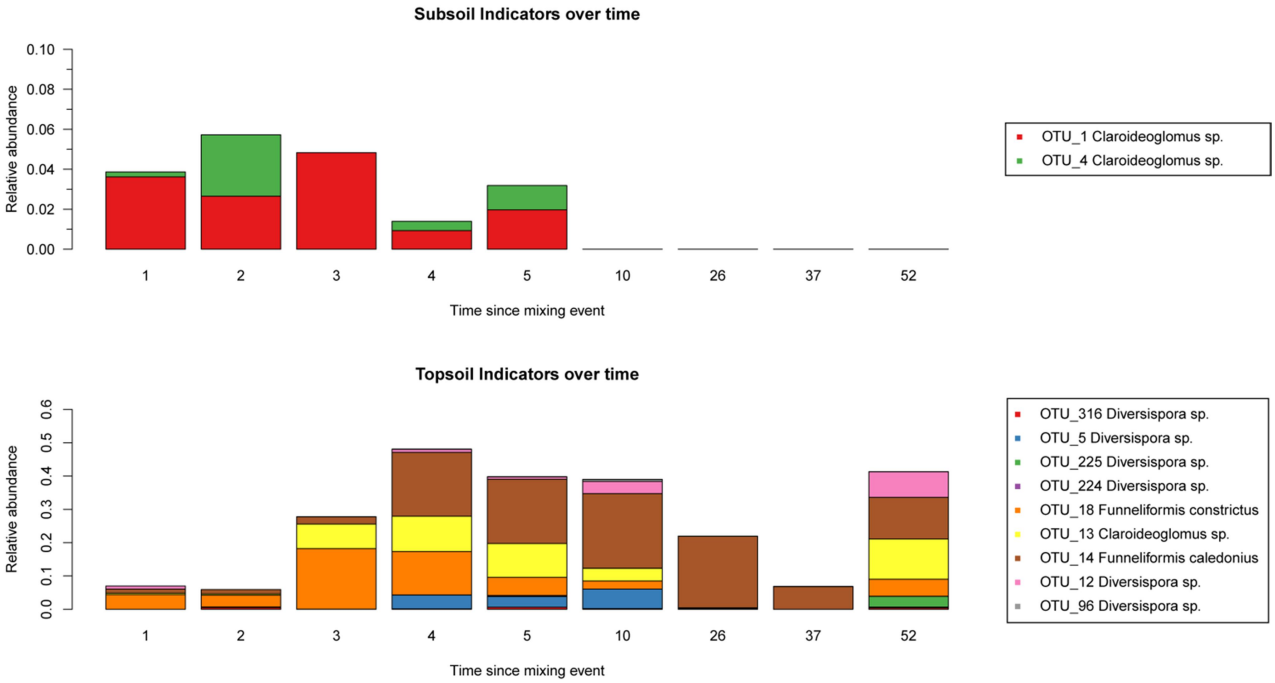
		Subsoil indicators	
OTU	**Taxonomic assignment**	**Stat**	**p value**
OTU_1	Claroideoglomus sp.	0.937	0.005
OTU_7	Claroideoglomeraceae	0.878	0.035
OTU_4	Claroideoglomus sp.	0.877	0.01

		Topsoil indicators	
OTU	**Taxonomic assignment**	**Stat**	**p value**
OTU_316	Diversispora sp.	0.955	0.005
OTU_5	Diversispora sp.	0.953	0.005
OTU_225	Diversispora sp.	0.947	0.005
OTU_224	Diversispora sp.	0.943	0.005
OTU_18	Funneliformis constrictus	0.933	0.005
OTU_13	Claroideoglomus sp.	0.841	0.02
OTU_14	Funneliformis caledonius	0.836	0.02
OTU_12	Diversispora sp.	0.824	0.05
OTU_96	Diversispora sp.	0.816	0.015

4. Discussion

We show that i) AM fungal taxa identified as subsoil indicators are present only in young fields (1-3 year since the mixing event), while taxa identified as topsoil indicators are present across the entire chronosequence and ii) early mixed communities from the chronosequence resembled to some extent unmixed subsoil communities and this similarity decreased with time after the mixing event. These results strongly suggest the inability of subsoil-specific AM fungal OTUs to persist in topsoil after a subsoil-topsoil mixing event. AM fungal richness in the chronosequence fields follows a unimodal trend with highest values during phase 2 (Roy et al., 2017); however, the detection of topsoil indicators through the entire chronosequence suggests that the observed loss of subsoil indicators was specific to subsoil phylotypes rather than a generalized diversity loss due to soil treatment during initial deposition or subsequent management. The fact that the three identified

Figure 1. Sub- and topsoil indicators over time. Number of reads detected in the chronosequence fields, for each of the subsoil **(A)** and topsoil **(B)** indicators identified in the unmixed field. Horizontal axis represents the time since the recultivation started, in years. Different indicator OTUs are coded by color.



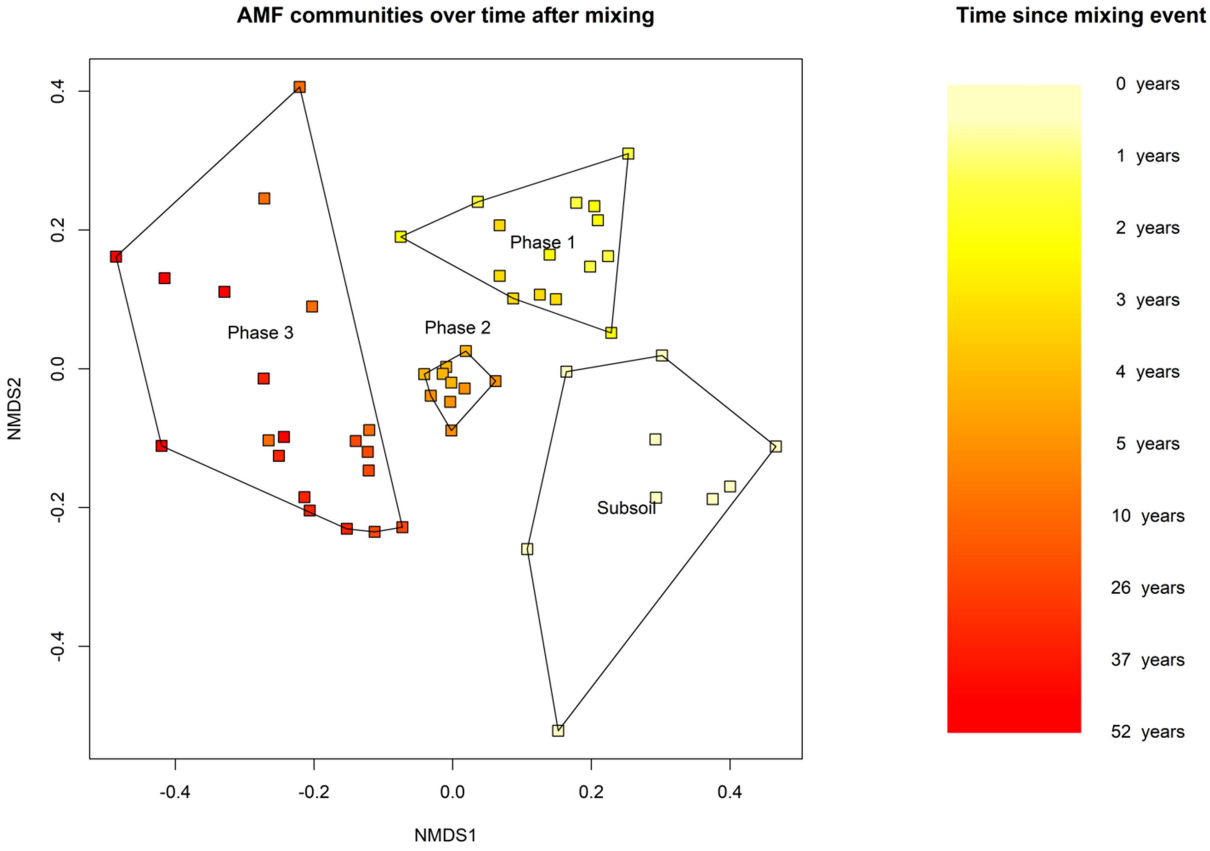
subsoil OTUs were all assigned to the family Claroideoglomeraceae is in line with previous results where this family showed a significant increase in relative abundance (Sosa-Hernández et al., 2018) or sporulated predominantly (Oehl et al., 2005; Yang et al., 2010) in deeper soil layers.

There are essentially two, not mutually exclusive hypotheses to explain this inability to coexist in the topsoil: abiotic filtering and biotic interactions (Vályi et al., 2016). Possible abiotic filters to subsoil AM fungal taxa in topsoil layers include disturbance in the form of tillage (Kabir, 2005) and greater diurnal and seasonal variations in temperature and moisture (Fierer et al., 2003). Alternatively, possible biotic filters are competitive exclusion by topsoil AM fungal taxa, increased grazing pressure or differential partner selection by the plant due to different nutrient availability. Particularly interesting is the notion that plants might demand different services from AM fungal communities at different depths. By allocating carbon selectively to the

desired phylotypes (Werner and Kiers, 2015) plants may shape the observed vertical distribution in AM fungal taxa. It is not clear what the relative importance of abiotic filtering and biotic interactions in driving this species loss is, that is to say, whether subsoil is for this AM taxa a fundamental or a realized niche (Devictor et al., 2010). Equally unknown is whether the subsoil phylotypes established in topsoil of the chronosequence fields and disappeared after a period of time or if they never did establish and the sequences we detect represent dormant inoculum or relic DNA (Carini et al., 2016). We believe that patterns in dissimilarity from "chronosequence" fields to the unmixed topsoil and subsoil communities with time can be interpreted as indirect evidence of the fate of these respective communities across the chronosequence. The slow increase in dissimilarities to unmixed subsoil with time may point at an inactivity and/or slow decline of these OTUs in topsoil, regardless of the host plants or the management. Nonetheless, the observed pattern could as well be explained by the presence and slow decay of relic DNA, as mentioned above. In contrast, the dissimilarities to unmixed topsoil are more responsive to the changes in management in the different phases, suggesting that the members of these communities were active and their populations were part of dynamic turnovers.

Overall, our results support our hypothesis of an ecological specialization of certain AM fungal taxa to deep soil layers. Identifying the specific mechanisms driving the observed patterns will require experimental approaches such as greenhouse reverse transplant experiments or in-vitro competition trials. Nonetheless, our results provide a first snapshot of the outcome of top- and subsoil community coalescence events. They show that AM fungal taxa found in subsoils are not able to persist in topsoil layers for longer periods of time. Some deep tillage practices, including deep ploughing or deep mixing, can have positive effects on yield under particular scenarios (Schneider et al., 2017); however, our results suggest that any practice inverting the soil profile has the potential for deleterious effects on AM

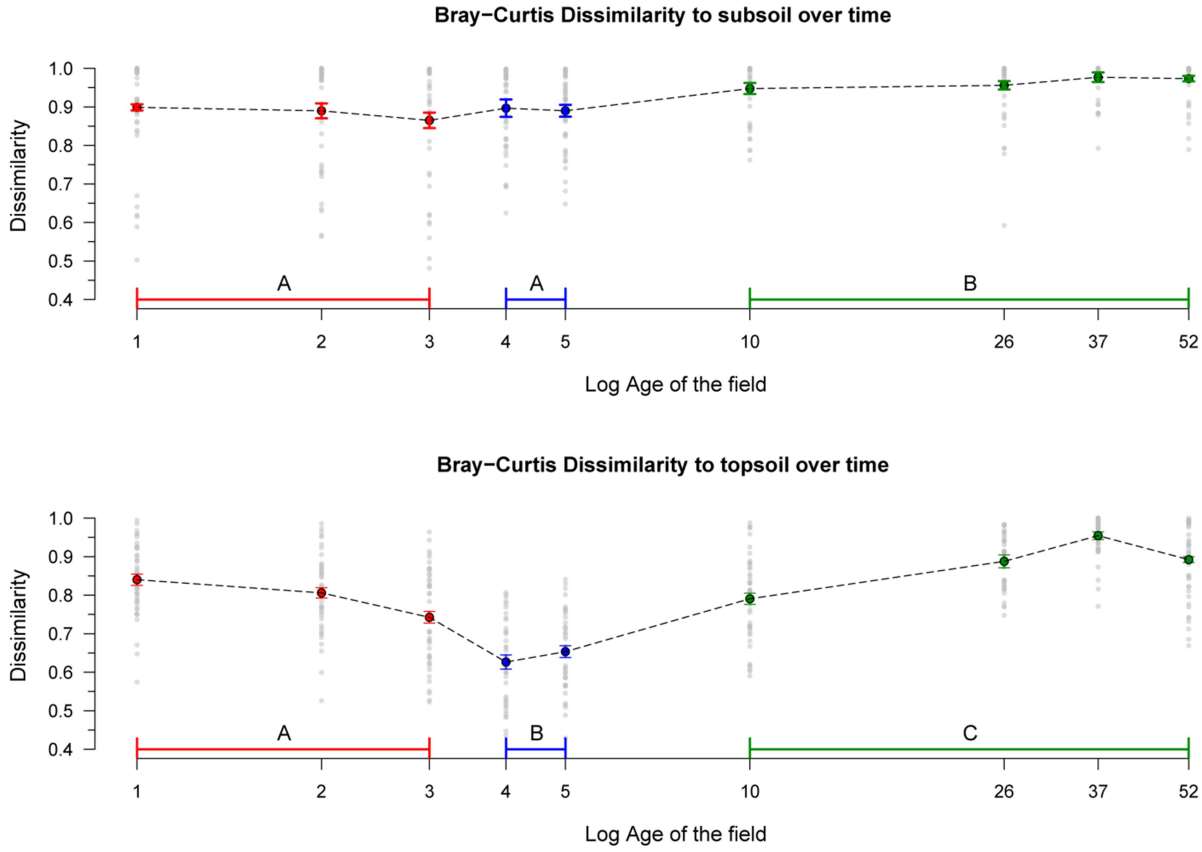
Figure 2. Community ordination of AMF over time. Non-metric multidimensional scaling (NMDS) of a Bray-Curtis pairwise dissimilarity of the AMF communities. The OTU table was rarefied to 559 reads, the minimum amount of reads per sample and includes all chronosequence samples and subsoil samples from the unmixed field. Time since start of the recultivation is coded by color. The polygons encompass all samples from that group. Subsoil = 60–75 cm, n = 9. Phase 1: 1–3 years, n = 15. Phase 2: 4–5 years, n = 10. Phase 3: 10–52 years, n = 20.



fungal diversity. Therefore, we suggest that such practices should only be considered as extraordinary measures in soils with root-restricting layers that meet the criteria for potential benefits of deep tillage (Schneider et al., 2017). Whenever possible, subsoiling (i.e. deep ripping) should be preferred over any practice that inverts or mixes the soil profile. With growing awareness of the potential role of AM fungi in sustainable agriculture (Thirkell et al., 2017) acquiring fine-tuned knowledge about the response of particular AM fungal phylotypes to tillage and soil mixing events is crucial if we are to exploit the potential of mycorrhizal technology (Rillig et al., 2016).

Caution is needed while handling subsoil AM fungal communities if we are to not irrevocably alter them even before unearthing their ecology and functional potential.

Figure 3. Dissimilarities to sub- and topsoil over time. Bray-Curtis distances (i.e., dissimilarities in both community composition and relative abundances) between chronosequence fields and (A) subsoil communities and (B) topsoil communities. Dotted lines link the means, bars represent the standard error. Different phases are coded by color, significant differences between phases are represented by different letters. Details on the statistics are presented in Table S2. Phase 1: 1–3 years, n = 135. Phase 2: 4–5 year, n = 90. Phase 3: 10–52 years, n = 180.



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Supplementary data to this chapter can be found in Appendix 3.

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Chapter 4

Subsoil arbuscular mycorrhizal fungi for sustainability and climate smart agriculture: a solution right under our feet?

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Abstract

With growing populations and climate change, assuring food and nutrition security is an increasingly challenging task. Climate smart and sustainable agriculture, that is, conceiving agriculture to be resistant and resilient to an adverse or changing climate while keeping it viable in the long term, is probably the best solution. The role of soil biota and particularly arbuscular mycorrhizal (AM) fungi in this new agriculture is believed to be of paramount importance. However, the large nutrient pools and the microbiota of subsoils are rarely considered in the equation. Here we explore the potential contributions of subsoil AM fungi to this agriculture and suggest future research goals that would allow us to maximize their benefits.

1. Introduction

Assuring food and nutrition security has long been one of the greatest challenges for humanity and given current population growth and climate change scenarios, this is an increasingly challenging task. Some of the latest estimates predict the need to increase agricultural productivity by at least 70% by 2050, and the focus shifts increasingly to the role of soil biodiversity in general (Bender et al., 2016) and particularly arbuscular mycorrhizal (AM) fungi (Thirkell et al., 2017), in achieving this in a sustainable way. Moreover, agricultural productivity needs to become more **resistant** and **resilient** to the increasingly common and severe extreme climate events, that is, agriculture needs to get **climate smart** (Lipper et al., 2014).

Arbuscular mycorrhizal fungi are a monophyletic, widespread group of fungi that form a mutualistic relationship with most land plants, including many agricultural crops (Brundrett and Tedersoo, 2018; Smith and Read, 2008). While predominantly known for their ability to increase plant nutrient uptake and productivity (Smith and Smith, 2011), they influence a wide range of ecosystem processes (Powell and Rillig, 2018; Rillig, 2004). AM fungal biomass abundance (Higo et al., 2013), spore numbers (Jakobsen and Nielsen, 1983; Muleta et al., 2008; Oehl et al., 2005; Säle et al., 2015), and root colonization levels (Jakobsen and Nielsen, 1983; Sutton, 1973), typically decline with increasing soil depth, but over 50% of AM fungal total biomass can be found below 30 cm (Higo et al., 2013), and outside of agriculture, AM

roots have been reported as deep as 8 meters (de Araujo Pereira et al., 2018). AM fungal communities below 30 cm have also been shown to differ from those in topsoil both in spore morphology based studies (e. g. Muleta et al., 2008; Oehl et al., 2005; Säle et al., 2015) and sequencing studies, with some phylotypes being exclusively detected in subsoil (Moll et al., 2016; Sosa-Hernández et al., 2018a). There is also growing evidence for subsoil ecological specialization in some AM fungal taxa (Sosa-Hernández et al., 2018b). Moreover, in an elevated CO_2 experiment by Rillig & Field (2003) AM root colonization increased in subsoil (here 15-45 cm) but not in topsoil, suggesting that topsoil and deeper soil AM communities might respond differently to environmental changes. Altogether, AM fungal communities below the plow layer are an often overlooked but probably highly relevant component of agroecosystems that holds opportunities for management. In this paper, we review the different potential benefits of subsoil AM for agriculture, summarize the knowledge about them, and provide suggestions for future research on this topic.

2. Subsoil and climate smart agriculture

In agriculture, the term subsoil refers to the soil beneath the Ap horizon, i. e. beneath the tilled or formerly tilled horizon. Considering that tillage depth is usually 20-30 cm, the vast majority of the volume of agricultural soil can be defined as subsoil, which makes even more remarkable the comparatively scarce knowledge we have and attention we pay to it as compared to topsoil. Subsoil contributions to plant nutrition ranges between 10-80%, and is

expected to increase when topsoil is dry or nutrient depleted (Kautz et al., 2013). Unsurprisingly, several studies have shown no yield increase after fertilization even in nutrient poor soils, as nutrient availability is typically characterized in topsoil and potential nutrient delivery from subsoil was not considered (Kautz et al., 2013). Guaranteeing plant access to the subsoil nutrient and water reservoir greatly increases the resistance of the system, making a greater pool of resources available and allowing the plant to avoid detrimental conditions in the topsoil e.g. during a drought event.

Biodiversity is assumed to stabilize ecosystem functioning under fluctuating environmental conditions, known as the insurance hypothesis (Yachi and Loreau, 1999), and Isbell et al. (2015) showed that biodiversity adds to the resistance of ecosystem productivity under climate extremes. We now also start realizing the potential impacts of soil biodiversity loss or alteration on human health (Wall et al., 2015) and food properties and quality (Rillig et al., 2018). While microbial abundances commonly decrease with increasing soil depth, subsoils can also be a microbial biodiversity reservoir and harbor unique taxa (Fierer et al., 2003), and subsoil communities have been hypothesized to contribute to the re-colonization of topsoil after perturbation (An et al., 1990; Verbruggen et al., 2012), adding resilience to the system.

3. Subsoil AM fungi for sustainable agriculture

3.1 General aspects

Subsoil AM fungi communities can be abundant (Higo et al., 2013; Wortmann et al., 2008), unique (Moll et al., 2016; Sosa-Hernández et al., 2018a) and they likely contribute to plant performance and ecosystem functioning in an underappreciated manner. In contrast with topsoil, subsoils are typically characterized by higher bulk densities and compaction, reduced pore spaces and lower oxygen concentrations (Lynch and Wojciechowski, 2015; Weil and Brady, 2016), altogether representing a suboptimal environment for roots. Although we still lack empirical evidence of subsoil AM fungal specific traits, it is a fair assumption that they are adapted to these environmental conditions. Among the hypothesized traits of these subsoil-specialized AM fungi would be an increased ability to colonize even the smallest soil pores, enhanced tolerance to anaerobic conditions and, due to the general scarcity and uneven distribution of roots, greater persistence in time in the form of resting structures or long-lived mycelium. All these traits could be well-matched to the intrinsic problems a plant faces in subsoil, and could become particularly important under certain circumstances, such as present in clay soils, soils with high compaction or soils with aeration problems. Moreover, applying a CSR (competitors, stress tolerators, ruderals) framework to AM fungi (Chagnon et al., 2015), subsoil AM fungi are expected to follow a stress tolerator life strategy. As such, deeper soil AM fungal phylotypes are expected to exhibit greater resource use efficiency and production of long-lived biomass, representing an advantageous carbon cost/benefit investment

for the plant. These slow growing communities would initially represent a carbon sink for the plant with little immediate benefits, but once the fungal network has been established, a long-lasting mycelium would provide its services to the plant at perhaps relatively little additional cost. Following the same rationale, the observed decrease in AM fungal spores with depth (e.g. Muleta et al., 2008; Oehl et al., 2005; Säle et al., 2015) might be less related to a decrease in abundance than to a change in both environment and life history strategy. AM fungal spores can be dispersed by wind (Egan et al., 2014), small mammals (Janos et al., 1995), earthworms (Reddell and Spain, 1991) or arthropods (McIlveen and Cole Jr., 1976), but all these vectors seem unlikely to be relevant in subsoils, with perhaps the exception of earthworms. With less disturbance and decreased microbial activity, probably a long-lived mycelium is in itself the best option for dispersal in time, and at larger time scales, also in space. This again represents a potential advantage for the plant symbiont, since AM fungal spores are particularly large and filled with lipids and carbohydrates with a high metabolic cost (Giovannetti, 2000), and ultimately it is the plant that provides this carbon and energy. While the same holds true for the production of mycelium, plants obtain a direct profit from this carbon investment, because it is the mycelium that explores the soil and captures and transports nutrients to the plant. Summing up, plants may receive greater returns for every unit of carbon they provide to AM fungi in subsoil, as compared to in topsoil.

Last but not least, subsoil arbuscular mycorrhizae may have a significant role in the very formation of soil. The importance of the biological component in pedogenesis has long been identified (Jenny, 1994) and while bacteria tend to have greater geochemical capabilities, fungi can weather rocks too, especially mycorrhizal fungi (Hoffland et al., 2004). In fact, it is difficult to understand pedogenesis throughout earth's history without considering the coevolution of plant roots and mycorrhizal fungi (Leake and Read, 2017). The ability of ectomycorrhizal (EC) fungi to release low molecular weight organic chelators in soil, which enhances mineral weathering, remains to be shown in AM fungi. However, AM fungi affect mineral weathering through various indirect pathways, including increased respiration, soil stabilization, enhanced evapotranspiration and exudation (Taylor et al., 2009), and differences in the mineral weathering abilities of AM and EM roots might be less pronounced than previously assumed (Koele et al., 2014). When it comes to deeper soil layers, biological activity is generally lower and despite potential accumulation of clay minerals from upper horizons, usually it comprises larger amounts of primary minerals, posing great potential for mineral weathering and nutrient release. AM fungi greatly expand the volume of soil under the influence of the symbiosis, often referred to as the mycorrhizosphere (Linderman, 1988), and in subsoil this likely means fostering microbial activity in a greater volume of soil. This combined action of roots, AM fungi and the associated microbial community has the potential to favor soil development, and in shallow soils where the parent material or the bedrock are close to the surface, this process could increase soil formation and deepening.

3.2 Efficient fertilization

Probably the most widely appreciated contribution of AM fungi to plant performance is their ability to increase plant nutrient uptake, particularly of P (Smith and Smith, 2011). Harnessing the nutrient supply by AM fungi the amount of applied fertilizer and the energy linked to its production can be reduced. A major issue in optimizing efficient fertilization is reducing the amount of nutrients lost to the system via leaching. AM fungi decrease nutrient leaching not only expanding the nutrient interception zone due to the development of a mycorrhizosphere, but also thanks to increased nutrient uptake, enhanced soil structure and fostering of the microbial community with associated nutrient immobilization (Cavagnaro et al., 2015). Köhl and van der Heijden (2016) demonstrated that different AM fungal species differ in their ability to decrease nutrient leaching, highliting the potential importance of AM fungal diversity. In fact, the observed increase in nutrient leaching in highly fertilized agroecosystems may be explained not only due to greater soil nutrient content, but also due to a typically reduced abundance and diversity of AM fungi (van der Heijden, 2010).

AM fungi have been shown to stabilize community productivity across gradients of nutrient availability, and to reduce plant tissue nutrient content variability along such gradients in a grassland (Yang et al., 2016). If transferable to agricultural systems, these effects would be crucial in achieving food and nutrition security particularly in regions where access to fertilizers might be limited or irregular. Moreover, expanding the available

soil nutrient pool to deep soil further increases resistance, allowing for instance the maintenance of plant growth under drought conditions, where nutrients in topsoil might be present but not accessible (positionally unavailable) for the roots. Altogether, with the continuously increasing prices of fertilizers and their predicted scarcity in a near future, making the most out of these resources is the only way forward and subsoil and subsoil AM fungi may prove important in this task.

Nitrogen

Nitrogen (N) applied in agricultural fields can be lost via leaching or in form of gaseous emissions. The influence of AM fungi on gaseous loss of N will be discussed later in this article, in the context of greenhouse gas emissions. As for leachate N, it occurs mostly in form of dissolved nitrate (NO_3^-), a particularly mobile form of N in soil. AM fungi promote soil aggregation (Leifheit et al., 2014) by improving soil structure and therefore increasing soil water holding capacity. Additionally, AM fungi take up N preferentially in form of ammonium (NH_4^+), reducing the pool of N available for **nitrification** and consequently reducing the mobility of N. In subsoil, AM fungi could intercept N that migrated down the profile and immobilize it or deliver it to the plant, thus avoiding N losses. Moreover, the proportion of NH_4^+ to other N sources increases in subsoil (Kautz et al., 2013), increasing the potential role of subsoil AM fungi in mobilizing and delivering this N to the plant, assuring access to a previously unavailable pool and reducing the need for N fertilization.

A particularly relevant role of subsoil AM fungi might be the capture and delivery to the plant of N weathered from rocks. Recently, Houlton et al. (2018) demonstrated that bedrock weathering might be a significant source of active N in various terrestrial environments. When this weathering occurs in deep soil layers, a big proportion of this N may be released to groundwater and ultimately to the sea (Houlton et al., 2018). In such scenarios the presence of an active microbial community, together with deep soil root proliferation, are crucial to capture this N before it is lost from the system. Due to their unique ability to capture and transport nutrients from the soil directly to plant roots, including N (Smith and Smith, 2011), AM fungi are promising candidates for maximizing the benefits obtained from this previously ignored resource, both reducing the need of N input and avoiding the contamination of groundwater.

Phosphorus

When it comes to P it is generally assumed that due to its low mobility in soils, leaching is of no importance and most effort has been spent on avoiding P loss and P mediated eutrophication via topsoil erosion. However, we now know that excessive manuring, the existence of preferential pathways or a sandy soil texture can lead to significant P leaching (Djodjic et al., 2004; Schoumans, 2015), with its associated economic and environmental consequences. The role of AM fungi in P uptake has been extensively researched (Smith and Smith, 2011), and they can reduce the need of heavy manuring due to increased and efficient P uptake. As for subsoils, here AM

fungi can again increase water holding capacity, reducing the risk of leaching, but these fungi also can intercept P that has migrated down the profile and deliver it to the plant. Inputs of organic P in subsoil, mostly via roots but also with direct injection of organic matter, can remain inaccessible to the plant due to decreased decomposition and mineralization rates. The role of subsoil AM fungi may be particularly important in acquiring this otherwise unavailable P. Moreover, Wang *et al.* (2017) found some evidence that AM fungi in subsoil might contribute more to plant P nutrition than topsoil AM fungi, under heavy P fertilization. Consequently, subsoil AM fungi have potential to be of great relevance in the avoidance of P loss, particularly in sandy soils or when the topsoil is P saturated.

Re-allocation of nutrients

More generally, fostering the proliferation of roots and AM fungi in deeper soil layers expands the volume of biologically active soil, increasing nutrient mineralization and immobilization rates. Thanks to their unparalleled ability to penetrate even the smallest soil pores such as in high density environments like subsoil, these fungi reach nutrients beyond the rhizosphere and transport them to the plant and topsoil again. This notwithstanding, no microorganism can increase the net content of nutrients in soil, other than with their own biomass if they were applied as inoculum, with the exception of N-fixing bacteria. Therefore, even the most sustainable and efficient agricultural practices will eventually need to resupply nutrients to the soil in order to maintain productivity and avoid soil exhaustion. The

same applies to subsoils: gaining access to this nutrient pool does not exempt farmers from the need to eventually replenish it. Natural migration of nutrients from topsoil to subsoil typically occurs via root exudates, dead roots, the action of anecic earthworms and the deposition of nutrients dissolved in water that reach subsoil through preferential flow pathways (Kautz et al., 2013). Therefore, enhancing the formation and maintenance of biopores is crucial for a proper replenishment of the subsoil. Additionally, the presence of an extensive mycorrhizosphere with its associated exudates can foster the return of some nutrients to the subsoil. Apart from these natural processes, direct inclusion of nutrients in deeper layers, such as injection of organic matter into subsoil, should be considered. Recent studies have shown positive effects of the admixing of organic matter in subsoil on the performance of barley (Jakobs et al., 2017), but understanding the long term effects of these on subsoil diversity and sustainability requires further research.

4. Greenhouse gases emission in agriculture

Modern agriculture is responsible for an estimated 12% of the global anthropogenic greenhouse gases emissions (Linquist et al., 2012). Some of these emissions are associated with fertilizer production and the use of heavy machinery, but most of them occur in the form of direct emissions from the field. The potential benefits of subsoil AM fungi in alleviating emissions related to fertilizer application were discussed in the previous section. Next, we will address the role of subsoil AM fungi in reducing the

release of two important greenhouse gasses associated with agriculture: carbon dioxide (CO_2) and nitrous oxide (N_2O).

4.1 Carbon dioxide: Subsoil AMF and carbon farming

The traditional view of very stable carbon in subsoil is questioned in recent findings. Stable subsoil carbon may be readily decomposed when fresh carbon is added. We propose that AM fungi have the potential to counteract this phenomenon due to their function in soil structure and in the capture of nutrients.

The traditional view of Carbon in subsoil

In depths of up to 3 m, the pedosphere stores more carbon (C) than the biosphere and the atmosphere combined (Jobbágy and Jackson, 2000). With increasing depth throughout the soil profile the mean residence time of C increases, reaching up to 10,000 years (refs 2-4 in Fontaine 2007). In the past, it was generally assumed that the age of C is connected to its stability, i.e. older C is also more stable. Indeed, we do find more stable compounds in the subsoil compared to topsoil. This could be attributed to several reasons:

i. Subsoils usually have reduced amounts of nutrients, especially N and P, which limit microbial activity and thus the turnover of OM. ii. Subsoils have a higher soil density with smaller pore volumes that decrease overall habitat space for soil organisms, thus reducing their abundance. iii. Subsoils often

show a change in texture, i.e. increased amounts of clay that can bind organic matter (OM) in organo-mineral complexes with stable bonds resulting from, e.g. ligand exchange or polyvalent cation bridges. As environmental conditions such as temperature and moisture are usually more stable in subsoil (Weil and Brady, 2016), the importance of soil mineral chemistry for OM stabilization becomes more pronounced. Iv. In subsoil a greater proportion of OM is located in microaggregates as compared to topsoil, allowing for slower turnover times (Torres-Sallan et al., 2017).

Recent findings question the stability of C in subsoil

However, in more recent studies the stability of old C in the subsoil has been questioned and a number of studies have shown that subsoil C is susceptible to decomposition when fresh C is added to the soil (e.g. Fontaine et al., 2007; Hobley et al., 2017). The majority of these studies extracted the soil for use in pot studies, where single and sometimes easily degradable substances were added to the soil. The soil extraction represents a massive disturbance, changes temperature, soil density and moisture conditions, which can possibly boost microbial activity and thus degradation of OM. Therefore, the instability of subsoil OM might have been overestimated due to methodological flaws and could be much less in the field under realistic conditions.

The role of AMF in subsoil carbon cycling – soil structure

One factor usually not included in previous experiments considering subsoil C cycling is AM fungi. In numerous studies they have been shown to improve

soil aggregate stability through hyphal enmeshment of soil aggregates and the production of extracellular polymers (Rillig and Mummey, 2006). Compared to topsoil, subsoil is subject to less disturbance that can disrupt hyphal networks, leading to a longer residence time of aggregate-protected OM (Lehmann et al., 2017). Therefore, stabilization of soil aggregates by mycorrhizal hyphae in the subsoil can contribute substantially to the protection and thus sequestration of soil organic matter (SOC). A better soil structure also improves soil pore connectivity, leading to increased interactions between soil microbes, and, consequently, likely increased competition for nutrients. If AM fungi could outcompete decomposers for nutrients, they would be able to indirectly reduce decomposition activity and thus potential loss of added or stabilized carbon.

The role of AM fungi in subsoil carbon cycling – nutrient additions

More specifically, competition for nutrients can also be induced directly by AM fungi, as they acquire nutrients and water for themselves, thereby reducing the nutrient and water availability for other microbes, which could reduce the activity of decomposers due to nutrient or water deficiency (Jansa and Treseder, 2017; Verbruggen et al., 2016). However, nutrient additions, as single or combined additions of N and P increase SOC decomposition, an effect called priming (Kuzyakov, 2010). Meyer et al. (2018) concluded that both the current soil nutrient conditions and microbial nutrient demand must be considered when predicting the effect of N addition on SOC turnover. According to the authors, the importance of the subsoil as a long-term C sink

is unclear when there is also increased input of additional N and P. The nutrient capture by AM fungi could be important for minimizing the stimulating effect that additional nutrients have on decomposition of SOM, particularly after the admixing of organic matter in subsoil, as performed by Jakobs *et al.* (2017).

In addition to capturing nutrients, AM fungi can reduce the availability of carbon compounds in the rhizosphere, because plants provide carbon to AM fungi in exchange for nutrients delivered. In the absence of AM, higher rhizodeposition would stimulate microorganisms in the rhizosphere, and thereby possibly stimulate SOM decomposition as microorganisms mine for nutrients in stabilized SOM. AM fungi receive up to 20 % of a plants' assimilates (Bago et al., 2000), which they first use for their own metabolism, before mycelial exudates are released. In this way, the mycorrhizal extraradical mycelium can be an important pathway of C to the SOM pool, when they exude mycelial organic compounds to soil parts more distant from the root system, but also via mycelium turnover. In topsoil, the C input by mycorrhiza can sometimes exceed the input of leaf litter and fine root turnover (Godbold et al., 2006). In a boreal forest, Clemmensen *et al.* (2013) found that in subsoil up to 70% of soil C can be root-derived, especially when root densities were high in deep horizons. In this study, and several others, mycorrhizal and other endophytic fungi dominated the subsoil, but decomposer fungi were only abundant in upper soil horizons (Bödeker et al., 2016; Lindahl et al., 2007). This suggests that decomposition processes

controlled by microbial community composition *in situ*, might be dominant in topsoil but subordinate in subsoil.

The role of AMF in subsoil carbon cycling – Litter decomposition

Although AM fungi may increase litter decomposition in short-term laboratory experiments, they probably have positive long-term effects on soil C (Verbruggen et al., 2013; Wilson et al., 2009). In the short-term, AM fungi are able to enhance OM degradation through the stimulation of decomposers, but we do not know whether this stimulation is permanent. Moreover, microbial metabolites are not necessarily lost, they can be integrated into very stable compounds such as mineral-associated SOM fractions, which have the longest mean residence times in soil (Cotrufo et al., 2013; Ehleringer et al., 2000). This stabilization mechanism could be especially important in subsoil, because here, the amount of clay minerals increases, representing a great potential for long-term stabilization of (fresh) C.

The potential contribution of AM fungi in subsoil carbon storage

Some efforts are made to find ways to increase SOC storage, e.g. in subsoil by increasing the presence of plants throughout the year with catch crops, by the use of undersown crops or deep rooting plants (Jakobs et al., 2017; Kell, 2011). Without further management, however, this could stimulate soil microbial activity and thus also decomposition of freshly added OM as well as

stabilized OM (Kong 2017). To counteract this effect, AM fungi could be fostered in order to reduce rhizodeposits, by including mycorrhizal crops together with a low management intensity (e.g. no tillage) and adapting a low fertilization level, as mycorrhizal fungi are more abundant in no-tillage systems and their effects are more pronounced in nutrient limited systems (Jansa et al., 2002, 2006). However, the triangular interaction of plants, AM fungi and other microbes in relation to SOC storage in soil particles or microbial biomass is still not very well understood. For instance, although AM fungi have been observed to induce smaller priming effects on SOM than roots, they might still promote soil respiration and thus increase SOC losses (Verbruggen et al., 2017). Therefore, future research should adopt a comprehensive approach for studying plant—fungal mediated processes in C cycling, considering the influxes (e.g. photosynthetic assimilation, root exudation, mycelial exudation, litter fall, soil organism detritus and fecal residues), effluxes (e.g. all parts of soil respiration, decomposition, leaching), as well as immobilization and storage of C in SOM and microbial biomass. These processes are especially interesting to study with respect to long-term C gains, e.g. through plant growth promotion effects, soil aggregation or the production of microbial products.

Data on the sensitivity of stored deep C are limited; we need further on-site research (with a low level of disturbance and alteration of environmental conditions) to evaluate the impact and importance of management strategies such as deep rooting plants, and effects of microbial community properties.

4.2 Nitrous oxide emissions

Agriculture is a major source of anthropogenic N_2O emissions (Linquist et al., 2012), a potent greenhouse gas with tremendous global warming potential 280-310 higher than CO_2 and a lifetime in the atmosphere that ranges from 118 to 131 years (Fleming et al., 2011; IPCC, 2001). Multiple pathways of N_2O production co-occur in soil and their relative contribution to its emission is poorly understood. Ammonia oxidation, dissimilatory nitrate reduction to ammonium (DNRA) and various denitrification pathways have been identified as microbially mediated processes with significant contributions to N_2O emission in agricultural soils (Baggs, 2011; Zhu et al., 2013). Under low oxygen concentrations, such as those expected in subsoil, typically anaerobic processes, such as DNRA or denitrification, are expected to prevail (Baggs, 2011), with significant denitrification rates having being reported in subsoil (Cleemput, 1998; Clough et al., 2005). Since NO_3^- is the primary substrate for both processes, we can expect that the reduction in NO_3^- leachate arriving at the subsoil due to the effect of AM fungi would also have a negative impact on DNRA and denitrification rates in subsoil. Furthermore, in grassland subsoil the addition of easily available C increased N_2O production, suggesting again that the reduced secretion of simple carbohydrate exudates in an AM root would further reduce this process. Ammonia oxidation is an aerobic process mediated by autotrophic organisms, in which the concentrations of oxygen and the substrate ammonia (NH_3) influence process rates. AM fungi were shown to have a direct negative effect on N_2O emission following N fertilization in a pot trial using agricultural soil, and the

competition with nitrifiers for NH_4^- was identified as the main driver (Storer et al., 2017). While the presence of high NH_4^- concentrations in subsoil is unlikely due to its limited mobility, this might not be the case following the mineralization of admixed organic matter in subsoil. Under such scenarios, where additionally considerably less anaerobic conditions prevail due to the deep tillage, the presence of subsoil AM fungi to readily take NH_4^- up and outcompete nitrifiers would be potentially important.

5. The way forward

Achieving food security at a global scale is a complex task with several fronts to work on. As for increasing and securing agricultural productivity, climate smart agriculture offers the best perspectives for success (Lipper et al., 2014). Much more research is needed to fully understand the role of subsoil and subsoil AM fungi in plant performance and to what extent we can manage them for sustainable intensification. This notwithstanding, evidence begins to accumulate pointing at particular agricultural practices that may help make our yields more sustainable and climate smart. First and foremost, we need to approach these challenges in a well informed and integrated way, as optimizing only some aspects of productivity ignoring others will certainly be counterproductive (Rillig et al., 2016). In fact, there is no one-size-fits-all solution and required management components are highly context dependent. This is why sustainable intensification has been defined as an increase of knowledge per hectare (Buckwell et al., 2014), stressing the importance of fine-tuned information.

Plant breeding and choice

Clearly, a fundamental prerequisite for the exploitation of subsoil is the presence of deep roots. Thus, crop rotations or catch cropping with deep rooting plants is essential to access deep soil resources and to create biopores that subsequent crops can use to grow into subsoil (Kautz et al., 2013). For instance, deep rooting and mycorrhizal plants, such as wheat, have been shown to increase AM fungal abundance through the soil profile (Higo et al., 2013). Additionally, crop breeding and crop selection can be done considering a set of traits that favor the plant's abilities to access subsoil, as reviewed by Lynch & Wojciechowski (2015) and Bishopp & Lynch (2015). However, it is very unlikely that any one given cultivar will possess all the traits required to fully optimize the use of subsoil. Therefore, while developing crop rotations or intercropping systems, it is desirable to look closely at the roots and select for a varied and balanced set of traits that better suits our goals (Rillig et al., 2015), aiming not only for a diversity of aboveground characteristics but a diversity of root architectures and abilities that can sustain the desired ecosystem services (Bardgett et al., 2014; Bardgett and van der Putten, 2014). Plant domestication has produced high yielding and resistant phenotypes that perform better than their wild relatives in the context of high input agriculture. This selective breeding has often come at a cost of neglected impacts on the soil microbiome (Pérez-Jaramillo et al., 2016). In the particular case of AM fungi, an extensive analysis comparing domesticated plants with their wild relatives found that

under limited P availability both phenotypes profit from AM colonization, but under high P fertilization regimes (such as in conventional agriculture) the symbiosis was less efficient in domesticated plants (Martín-Robles et al., 2018). In addition to deep rooting traits, we recommend accounting for mycorrhizal responsiveness in future plant breeding efforts to assure that crops can benefit the most from the local AM fungal communities (Rillig et al., 2016).

Subsoil management

Access to subsoil can be limited by physical properties, such as the existence of a hard plow pan that prevents root growth. The benefits of deep tillage and other subsoil tillage management options can be controversial and highly context dependent; but on average, given the existence of a plow pan, yields can be substantially increased after deep plowing (Schneider et al., 2017). The existence of subsoil specific AM fungal phylotypes and their inability to survive soil mixing events, however, calls for precaution and the general avoidance of any method that inverts the soil profile (Sosa-Hernández et al., 2018b). Intensive tillage has been identified as a major factor reducing AM fungal abundance and diversity in agriculture (Kabir, 2005). Recently, Säle *et al.* (2015) compared the effects of reduced and conventional tillage, down to 40 cm in the soil profile using spore-based community analysis. Their results confirm the expected shifts in spore abundance and diversity in topsoil but those effects were not significant in deeper layers, despite a shift in community composition. The absence of spore abundance shifts does not

necessarily imply a lack of effect on hyphal abundance or colonization rates, but changes in subsoil community composition highlight that tillage can affect AM fungi in deeper layers, with unknown consequences for their functionality. No-till or reduced till systems however typically face another set of problems that may include increases in bulk soil density, limited nutrient mobility through the profile or the use of agrochemicals for weed control, plus a set of economic and technical constraints that are more pronounced on small farms (Giller et al., 2015).

AM fungal inoculum

Assembling the right consortia of plant phenotype and rhizosphere microbiome has also been postulated as one of the means for a new underground revolution that aims at an ecological intensification in agriculture (Bender et al., 2016). This approach is very promising but holds intrinsic associated risks (Machado et al., 2017). The benefits of mycorrhizal inoculum can be highly context dependent (Hoeksema et al., 2010) and the use of non-native genotypes carries always the possibility of associated environmental impacts (Schwartz et al., 2006). This variability (but often not uncertainty, Lehmann and Rillig, 2014)) in response to AM inoculation often leads to a lack of trust in its general efficiency by the agricultural community. We think AM fungal inoculum should not be used indiscriminately in general, or substitute for other AM-promoting management options. When it comes to subsoils, the evident existence of a specific AM fungal community calls for

even greater caution, and at present our knowledge is too limited to encourage the use of inoculum for the subsoil.

Future research challenges

Early research on AM fungi already observed abrupt decreases of spore abundance and colonization levels with increasing depth in agriculture (Sutton, 1973; Sutton and Barron, 1972). This could have led to a subsequent lack of interest in studying the arbuscular mycorrhizal symbiosis in deeper layers. However, outside the realm of agriculture, evidence of AM colonization was found down to 4.8 m in honey mesquite (Virginia et al., 1986) and this depth record has been recently updated to 8 m in an eucalyptus and acacia plantation (de Araujo Pereira et al., 2018). Very little research has been conducted on the community composition of AM fungi across different depths in agriculture, with few notable exceptions (e.g. Muleta et al., 2008; Oehl et al., 2005; Säle et al., 2015), and these spore-based studies have only recently been supported by molecular based research (Moll et al., 2016; Sosa-Hernández et al., 2018a; Wang et al., 2017). Moreover, the only assessment on subsoil AM functionality was performed by Hafner *et al.* (2014), who compared root-derived C in the rhizosphere as influenced by AM fungi from two different depths in a greenhouse experiment.

Consequently, we believe that more basic, descriptive research, both spore- and molecular based, needs to be performed to better understand the

vertical distribution of AM fungi in agriculture and to confirm some of the already obtained knowledge across different regions and crops. We think it is particularly important to start linking agricultural management with responses in AM fungi across the entire soil profile, as exemplified by Säle *et al.* (2015), ideally covering aspects such as tillage, fertilization and crop rotations. Furthermore, we also need to learn about the functioning of AM fungal communities in the subsoil, since AM fungi and roots face a very different environment than in topsoil. Rooting depth and architecture is one of the niche axes that allows plant coexistence in natural habitats (Silvertown, 2004), and roots at varying depths may forage for different resources (e. g. shallow roots acquiring P and deeper roots acquiring water). We can assume that, similarly, what the plant demands from its mycorrhizal partner might vary with soil depth, opening the possibility for specialized or even new functionality of subsoil AM phylotypes. Experiments assessing these potential differences in mycorrhizal functionality across depths are crucial and the isolation of deep soil AM fungi would go a long way towards the understanding of these communities.

Discerning the assemblage mechanisms, ecosystem role and phylogenetic structure of AM fungi in deeper soil layers will help us answer important questions about AM fungal biogeography and diversity maintenance. Despite the three-dimensional nature of soil, to date we have centered most our efforts on a shallow soil layer with virtually no understanding of the ecosystem contributions of deeper AM fungi (Powell and Rillig, 2018), even if most evidence points to greater vertical than horizontal variation in fungal

community composition (Bahram et al., 2015). Routinely including the vertical axis in AM studies across different biomes and in our theoretical frameworks will deepen our overall understanding of the biology of this relevant group of plant symbionts. Increasing our knowledge and expanding our perspective to include subsoil and subsoil AM fungal communities will not solve our problems on its own; however, an integrated subsoil management that takes AM fungi into account can bring us one step further in achieving sustainable and stable yields.

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Chapter 5

General discussion

Vertical distribution of arbuscular mycorrhizal fungi in agriculture

The main aims of this thesis were i) to describe the distribution of arbuscular mycorrhizal (AM) fungi across the soil profile in arable land using molecular methods ii) to gain insights into AM fungal community assemblage processes in subsoil. In this section I discuss my results and put them in the broader context of previously existing and recently published literature on this topic.

Community composition

Early research on AM fungi already revealed that spore numbers and mycorrhization percentages declined with increasing soil depth (Jakobsen and Nielsen, 1983; Smith, 1978; Sutton, 1973; Sutton and Barron, 1972). Similar observations were also made in grasslands (Koide and Mooney, 1987), but some species' spores could only be found at depths other than topsoil (Zajicek et al., 1986). It was only later that a number of studies started assessing the changes in community composition with increasing depth in agriculture, based on spore morphology (Muleta et al., 2008; Oehl et al., 2005; Säle et al., 2015; Tian et al., 2011; Yang et al., 2010). In these studies

AM fungal spore pools changed with increasing depth and often, certain spore morphotypes were restricted to a particular depth. Additionally, while spore abundance and diversity tended to decrease in deeper soil, considerable spore diversity could be found below 30 cm (i.e. in subsoil). The drawback of these studies is the impossibility to determine whether the absence of a species' spores in a certain depth corresponds with the absence of that species in that layer, or if that species is present but not sporulating. The high-throughput sequencing data presented in this thesis provides strong evidence for the shift in community composition with depth. In **chapter 2** we could observe how in the studied site, subsoil communities were significantly different from those in topsoil both in species composition and in relative abundances of the present phylotypes. We were also able to corroborate with molecular methods that certain phylotypes could only be found in subsoil, indicating that deeper soil communities were not just a diluted analog of the ones found in topsoil. In **appendix 2.3** and **2.4** we explored the vertical distribution of AM fungi with greater sample numbers and increased vertical resolution, and obtained similar results. Remarkably, in appendix 2.3 we can observe how the ordination of the communities points at an abrupt change between topsoil and subsoil, with a more gradual change within the different depths studied in subsoil. A similar change can also be observed in **appendix 2.4**. Probably, the observed pattern can be explained by the particularities of agricultural topsoil, where AM fungi are exposed to different management techniques, such as tillage or fertilization. Tillage in the first 30 cm of the profile, often with the subsequent formation of a plow pan, creates dramatically different environments in top and subsoil. Research

on fine scale community composition at this top- subsoil interface in no-till systems or natural grasslands would expand our understanding of tillage effects on AM fungal community composition.

A recent study by Schlatter *et al.* (2018) used Illumina sequencing to characterized fungal communities at different soil depths in agriculture, down to 1 m deep. In this study the use of general fungal primers led to the detection of very few AM fungal phylotypes (7 OTUs), and unsurprisingly their relative abundance was highest in topsoil. In contrast, Wang *et al.* (2017) studied AM fungal communities with Illumina sequencing at 2 different depths (0-20 and 20-40 cm) in a field planted with maize, and found no significant shifts in the communities with depth. These contrasting results could be explained by the depth intervals selected. The field was ploughed before sowing at a depth of 15 cm, but previous tillage management is not mentioned, and it is probable that the 20-40 cm interval represented the interface between top- and subsoil. With AM fungal abundances being typically higher in the first 30 cm of the profile, if the interface between top- and subsoil is sampled in a combined sample, it is to be expected that the signal of topsoil communities will override the less abundant subsoil phylotypes and result in no measurable differences between the two profiles. Despite the lack of significant community shift with depth, this study found differences in colonization percentage and response to P fertilization with soil depth. Similarly, in a grassland, Montero Sommerfeld *et al.* (2013) found no differences in AM fungal communities at 0-40 and 40-80 cm depth. The authors attribute this lack of community shift to a fairly constant pH through

the profile. Additionally, the community characterization technique used (LSU T-RLFP) might not have had the adequate resolution to detect those changes.

Subsoil exclusive phylotypes

Several studies reported that certain species' spores could only be found at depths other than topsoil, both in agriculture (Muleta et al., 2008; Oehl et al., 2005; Säle et al., 2015; Tian et al., 2011) and in other ecosystems (Becerra et al., 2014; Zajicek et al., 1986). Moll *et al.* (2016) used SSU cloning to characterize AM fungal communities at 4 different depths in a maize plantation, and despite being able to identify only 5 different OTUs, they found that one of them was only detected below the plow layer. This thesis provides strong high-throughput evidence for the existence of subsoil exclusive phylotypes. In **chapter 2,** out of a total of 64 OTUs detected, 2 were exclusive for subsoil, and 17 exclusive for topsoil. In **appendix 2.4** the use of an ESV approach and greater sample numbers allowed for the detection of 197 phylotypes exclusive for subsoil, and 313 exclusive for topsoil. Here, 2 depth intervals for topsoil and 2 for subsoil were studied and therefore, sampling effort for both layers is comparable. In **appendix 2.3** however, 1 depth interval corresponds to topsoil and 4 to subsoil. Here general fungal primers were used and consequently the sequencing depth for AM fungi is lower as compared to appendix 2.4. This notwithstanding, 35 phylotypes were subsoil exclusive as compared to 29 in topsoil. These observations suggest that increasing sampling effort in subsoil leads to the detection of greater numbers of subsoil exclusive phylotypes, and that this number might

be greater than topsoil exclusive phylotypes. This idea is supported by previous observations of subsoil fungal communities being less species rich but more beta-diverse (i.e. exhibiting greater beta diversity) as compared to topsoil communities (Powell et al., 2015; Schlatter et al., 2018). If this proves true, future research with increased subsoil sampling efforts might prove the existence of a hitherto unknown rich and diverse group of AM fungi.

Family level changes

In **chapter 1** a remarkable effect of depth at the family level was observed, with Claroideoglomeraceae OTUs being dominant in subsoil and Diversisporaceae OTUs being dominant in topsoil. Using an ESV approach and assessing the effect of depth at different taxonomic levels confirmed the magnitude of the shift at the family level (Roy et al. unpublished). This is the first time that such a pattern is reported. A similar pattern was observed in **appendix 2.4**, with Claroideoglomeraceae again increasing in relative abundance in subsoil, but in this case Diversisporaceae, being overall less dominant than in the previous study, had greater relative abundances in deeper layers. Little is known about AM fungal traits at the family level (Powell et al., 2009) and therefore, at this point it is difficult to make conclusions about the implications and mechanisms behind these shifts.

Different soils

When studying vertical distribution in soil it is paramount to acknowledge that different soil types have contrasting soil profiles, and that the accompanying changes in environmental conditions in each horizon might have an impact on AM fungal community assemblage. Data presented in this thesis cover a Luvisol (**chapter 1**), a Cambisol (**appendix 2.3**) and a Vertisol (**appendix 2.4**). Major conclusions regarding community shifts with depth and presence of exclusive subsoil phylotypes are supported in the three different soil types. However, the relative abundance of different families and the nature of indicator species for each layer differ, highlighting the need to put these results in the context of the studied soil type. Future research on the distribution of fungal communities along the soil profile needs to assess the presence of different horizons, and whenever possible, sample them separately. Sampling for instance at defined depth increments is suboptimal, if one of those intervals mixes two horizons with contrasting soil parameters.

Subsoil specialization

In **chapter 3** we present evidence for subsoil specialization in certain AM fungal phylotypes. Since the identification of AM fungal spores that occurred only in subsoil, it has been suggested that certain AM fungal species might be adapted to this environment (Zajicek et al., 1986). The observed inability of some subsoil AM fungal phylotypes to persist in topsoil after a top- subsoil mixing event could be explained by environmental filtering or by out-competition by topsoil phylotypes. In a study assessing AM fungal community dynamics across depth after topsoil fumigation, some species that were

dominant in subsoil before fumigation became more abundant in topsoil afterwards, suggesting that in the absence of competition they were able to colonize shallow soil (An et al., 1990). The family level changes in community composition discussed above, point at a deep phylogenetic signal in the set of traits needed to be adapted to subsoil. However, disentangling the relative contribution of both biotic and abiotic factors driving the observed specialization requires experiments, such as *in vitro* competition trials or reverse transplant assays.

Subsoil community assemblage

Outside of agriculture it has been observed that neutral processes (such as dispersal limitation and stochasticity) are more prevalent in fungal community assemblage in deeper soil, whereas deterministic processes (such as abiotic and biotic filtering) are of greater relevance in shallow soil (Powell et al., 2015). Data presented in **chapter 2** show how subsoil AM fungal communities in subsoil are less even and exhibit greater beta diversity than in topsoil, an observation expected under the prevalence of neutral processes. Similar increases in beta diversity and decreases in evenness with depth where observed in fungal communities in agriculture (Schlatter et al., 2018). Subsoil is a heterogeneous environment with typically very low root abundance, which creates a suboptimal environment for AM fungi due to their obligate dependency of roots for carbon uptake. Moreover, subsoil exclusive phylotypes are most probably limited in their dispersal, as typical vectors of AM fungal dispersion like wind, small mammals or arthropods are not expected to frequently occur in subsoil. Altogether, stochasticity and

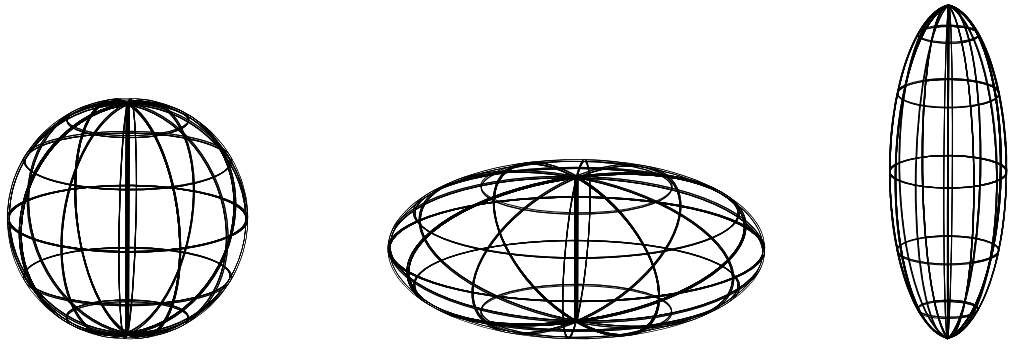
priority effects might play a great role in the assemblage of subsoil AM fungal communities.

Growth strategies

In **chapter 2**, we observe how some AM fungal phylotypes can be detected at relatively constant relative abundances at different depths, whereas others occur at different depths but with contrasting relative abundances. This raises a question about different growth strategies in AM fungi. In the 2-D space of a petri dish fungi grow typically forming a more or less circular colony, whereas in the 3-D space of liquid cultures they typically form a hemisphere cut by the medium surface. It is therefore assumed that in soil fungi grow in every dimension forming a rather spherical mycelium. The presence of obstacles, varying nutrient densities, foraging by fungal consumers and competition with other fungi results almost certainly in an irregular and amorphous mycelium but conceptually, the spherical growth strategy can be used. Much like different plants express contrasting root architectures according to different foraging strategies, one might expect that AM fungal species differ in their growth strategies. I propose the existence of AM fungi that explore the soil in every direction (forming a sphere-like mycelium), others that explore horizontally in a restricted depth (forming an oblate) and a third group that explores the soil vertically (forming a prolate) **Fig. 1**. These different growth strategies could explain AM fungi being exclusive and locally dominant at one depth (oblate growth) and others being found at different depths at similar relative abundances (oblate). Testing this hypothesis is challenging, due to the technical limitations to

observe fungal growth in soil, but in the future, high resolution mapping of fungal communities using molecular tools might allow us to test it.

Figure 1. Suggested growth strategies



Sphere Oblate Prolate

Subsoil and AM fungal biogeography

Recently it has been shown that despite putatively low dispersal abilities, AM fungi exhibit very low endemism at a global scale (Davison et al., 2015). In this study, phylotypes present on multiple continents had an evolutionary origin more recent than the last separation of continents, pointing at unexpectedly efficient dispersal in recent time. With the expected very low dispersal ability of subsoil AM fungi, I hypothesize that this endemism level might be higher in subsoil. Sampling subsoil AM fungi in previously merged but now separated land masses (e.g. eastern South America and western Africa, Madagascar and India) could show that subsoil AM fungal communities reflect to a certain extent paleogeography. If those communities are similar to each other, with phylotypes originated previous to the separation of the continents, it would mean that those communities

have had very little dispersal and evolutionary change. On the other hand, if they are dissimilar but with recently evolved phylotypes, it would probably mean that they have evolved in isolation with little dispersal. Similar communities with recent evolutionary origins would point at unexpectedly high dispersal efficiency, whereas dissimilar communities with origin previous to the continental split would contradict all previous hypotheses.

Subsoil AM fungi and agriculture

In **chapter 4** we discuss the potential benefits of subsoil AM fungi for sustainable and climate smart agriculture. AM fungal standing biomass in subsoil can be comparable to that in topsoil (Higo et al., 2013), and their abundance dynamics can mimic those in topsoil (Tian et al., 2011). As obligate biotrophs, AM fungi obtain all their carbon from the plant, which can amount up to a 20% of all the plant's photoassimilates (Bago et al., 2000), and the plant has the ability to allocate carbon selectively to different strains (Werner and Kiers, 2015). Altogether, the observed abundance and abundance dynamics in subsoil AM fungi point to an active community providing a service to the plant.

Subsoil AM fungi potentially grant the plant access to a large nutrient and water pool, which increases the resistance of the system to unfavorable conditions in topsoil. Due to increased nutrient uptake efficiency and their positive effects on soil structure, they can prevent nutrient leaching, and capture nutrients that migrated down the soil profile, avoiding detrimental environmental impacts and reducing the need for fertilization.

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Summary

The major aims of this thesis were to describe how arbuscular mycorrhizal (AM) fungal communities vary with depth in agricultural land, and to gain insights into AM fungal community assemblage processes in subsoil. In **chapter 1** I introduce basic aspects of AM fungal biology, review existing literature on the topic of this thesis and introduce the molecular methodology used in my studies. **Chapter 2** and its appendices provide high-throughput sequencing evidence for the differences in AM fungal community composition in top- and subsoil, such as for the existence of phylotypes exclusively found in subsoil. As compared to topsoil, studied subsoil communities are less species rich, less even and have higher community turnover. However, they are not just a subset of topsoil communities and harbor unique taxa. In **chapter 3** a top- subsoil mixing event is studied to trace the fate of subsoil phylotypes in topsoil. Here we observe the inability of these subsoil phylotypes to persist in topsoil, and interpret this as evidence for subsoil specialization in certain AM fungal taxa. **Chapter 4** discusses the potential roles of subsoil AM fungi in agriculture, with a focus on sustainability and climate smart approaches. The major potential roles identified concern the access to greater nutrient and water pools, the resistance of the system to unfavorable conditions in topsoil, and the avoidance of nutrient leaching and greenhouse gas emissions. We also point out future research needs in this field. **Chapter 5** puts the results of this thesis in the broader context of the literature, summarizing the conclusions

of previous chapters. In this chapter I also introduce ideas on AM fungal growth strategies and implications of subsoil AM fungi for the understanding of AM fungal biogeography.

Zusammenfassung

Die Hauptziele dieser Arbeit waren die Beschreibung, wie arbuskuläre Mykorrhiza (AM) Pilzgemeinschaften mit der Tiefe in landwirtschaftlichen Flächen variieren, und das Verstehen der Prozesse, die die Zusammensetzung von AM Pilzgemeinschaften im regulieren. In **Kapitel 1** stelle ich grundlegende Aspekte der AM-Pilzbiologie vor, bespreche bestehende Literatur zum Thema dieser Arbeit und führe in die molekulare Methodik meiner Studien ein. **Kapitel 2** und seine Anhänge liefern einen Hochdurchsatzsequenzierung-Nachweis für die Unterschiede in der AM-Pilzgemeinschaften im Ober- und Unterboden sowie für die Existenz von Phylotypen, die ausschließlich im Unterboden vorkommen. Im Vergleich zu Oberboden sind die untersuchten Unterbodengemeinschaften weniger artenreich, weniger gleichmäßig und zeigen größere beta-Diversität. Sie sind jedoch nicht nur eine Untermenge von Oberbodengemeinschaften, sondern beherbergen einzigartige Taxa. In **Kapitel 3** wird ein Ober- Unterboden Mischungsevent untersucht, um das Schicksal von Unterbodenphylotypen im Oberboden nachzuvollziehen. Hier beobachten wir das Scheitern dieser Unterboden-Phylotypen im Oberboden zu bestehen, und interpretieren dies als starkes Indiz für die Unterbodenspezialisierung bestimmter AM-Pilz-Taxa. **Kapitel 4** diskutiert die möglichen Rollen von AM-Pilzen in der Landwirtschaft mit Fokus auf Nachhaltigkeit und klimaintelligente Ansätzen. Die wichtigsten identifizierten potenziellen Rollen betreffen den Zugang zu größeren Nährstoff- und Wasser-Pools, die Widerstandsfähigkeit des Systems gegenüber ungünstigen Bedingungen im Oberboden und die Vermeidung von Nährstoffauswaschung und Treibhausgasemissionen. Wir weisen auch auf künftige Forschungsbedürfnisse in diesem Bereich hin. **Kapitel 5** stellt die Ergebnisse dieser Arbeit in den breiteren Kontext der Literatur und fasst die Schlussfolgerungen der vorangegangenen Kapitel zusammen. In diesem Kapitel stelle ich auch Ideen zu AM-Pilzwachstumsstrategien und

Implikationen von AM-Unterbodenpilzen für das Verständnis der AM-Pilzbiogeographie vor.

List of publications and contributions

I. Rillig MC**, Sosa-Hernández** MA, Roy J, Aguilar-Trigueros CA, Valyi K, Lehmann A. 2016. Towards an integrated mycorrhizal technology: harnessing mycorrhizae for sustainable intensification in agriculture. Frontiers in Plant Science 7: 1625. doi: 10.3389/fpls.2016.01625.
Own contributions: I contributed ideas and text.

II. **Sosa-Hernández MA,** Roy J, Hempel S, Kautz T, Köpke U, Uksa M, Schloter M, Caruso T, Rillig MC**.** 2018. Subsoil arbuscular mycorrhizal fungal communities in arable soil differ from those in topsoil. Soil Biology & Biochemistry 117: 83-86.
Own contributions: I performed molecular work and data analysis, and wrote the manuscript..

III. **Sosa-Hernández MA,** Roy J, Hempel S, and Rillig MC (2018a). Evidence for subsoil specialization in arbuscular mycorrhizal fungi. Front. Ecol. Evol. 6:67. doi:10.3389/fevo.2018.00067.
Own contributions: I performed data analysis and wrote the manuscript.

IV. **Sosa-Hernández MA** Leifheit EF, Ingraffia R, Rillig MC. Subsoil arbuscular mycorrhizal fungi for sustainability and climate smart agriculture: a solution right under our feet? (to be submitted).
Own contributions: I conceived the ideas of the paper, performed literature research and wrote the manuscript.

V. Roy J, Mazel F, **Sosa-Hernández MA**, Duenas J, Zinger L, Hempel S, Rillig MC. A hierarchy of ecological filters acts at a hierarchy of phylogenetic resolutions to shape the spatial distribution of arbuscular mycorrhizal fungi. New Phytologist (submitted).
Own contributions: I contributed ideas, text, and a previously published dataset.

Appendix 2.1

Amplicon generation and Illumina paired-end sequencing

We performed amplicon-based AMF-specific metabarcoding by implementing a nested-PCR approach, using the primer sets SSUmAf-LSUmAr and SSUmCf-LSUmBr (Krüger *et al.*, 2009). These primer sets target the nuclear rDNA amplifying the partial SSU, the entire ITS region and the partial LSU region in Glomeromycotina. DNA extracts were normalized to a concentration of 20 ng μl^{-1}. All PCRs were performed with the Kapa HiFi PCR Kit (Kapa Biosystems, Woburn, MA, USA) following manufacturer's recommended procedures. For the first PCR (25 cycles 98ºC - 20 s., 60ºC - 30 s., 72ºC - 50 s., primer set SSUmAf-LSUmAr), 1 µl of normalized DNA extract was used as DNA template. For the second PCR (25 cycles 98º C-20 s.,60º C-30 s., 72º C- 50 s., primer set SSUmCf-LSUmBr), 1 µl of a 1:10 dilution of the previous PCR result was used as DNA template. 1 µl of a 1:10 dilution of the ~1.5 Kbp product was used as template in a third PCR (20 cycles 98ºC -20 s.,47ºC - 30 s., 72ºC - 50 s.) using the primers LR3 and LR2rev (Hofstetter *et al.*, 2002) containing the adaptors for the follow up indexing (see below). These relatively universal fungal primers amplify a 350-420 bp region in the LSU including the variable D1-D2 region. LR2rev is a modification of LR2R, with the sequence 5'- GAAAAGAACTTTGAAAA -3'. Amplicons were afterwards gel-separated, band-excised and purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). The resulting purified DNA was tagged with indexing sequences and adaptors suited for Illumina MiSeq sequencing during a fourth PCR using the sequencing primers. The product was purified using magnetic beads (GC Biotech, Alphen aan den Rijn, The Netherlands), DNA concentration was determined and a library was created pooling equimolar amounts of each sample. The amplicons pool was sequenced on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv, Berlin, Germany) to generate 2x300 bp paired-end reads. A

total of 1,876,440 sequences were obtained. After two rounds of sequencing we could not obtain acceptable read numbers for one sample (rhizosphere, topsoil), so this sample was excluded from any subsequent analysis.

Bioinformatics

Raw paired-ended reads were merged, quality-filtered (maximum error rate of 1), resulting in 1,195,469 reads. Quality-filtered reads were dereplicated and singletons were removed using USEARCH v8.1.1861 (Edgar, 2010), resulting in 265,833 unique sequences. Further quality filtering was performed by aligning those sequences to a AMF ribosomal DNA reference database (Krüger *et al.*, 2012) using mothur v.1.38.1 (Schloss *et al.*, 2009), this process also eliminated the primer sequence. Sequences not overlapping with any sequences in the database were discarded, resulting in 53,595 sequences. These quality-filtered sequences were clustered at a 97% similarity level using USEARCH v8.1.1861 (Edgar, 2010) which includes internal chimera removal, resulting in 71 OTUs (5,131 chimeras removed). 94.4% (1,128,187) of sequences filtered in the first step including singletons, were now successfully assigned to those 71 OTUs at a 97% similarity level. Various format editing steps such as sequence counting were performed with OBITools 1.2.9 (Boyer *et al.*, 2016).

Taxonomic assignment of the OTUs was carried out using BLAST+ (Camacho *et al.*, 2009) against Glomeromycotina reference sequences published in Krüger *et al.* (2012) and against the EMBL nucleotide database (Kanz *et al.*, 2005). Matches below 70% similarity and/or shorter than 220 bp were discarded. Results from both databases were checked for consistency and matches contained in Krüger *et al.* (2012) were used to assign the OTUs. We decided to favor matches in Krüger *et al.* (2012) over EMBL, due to the often imprecise description of the match in the latter (e.g. “soil fungus”, ”uncultured Glomeromycota”). Matches for OTU_12 and OTU_43 however, were dissimilar for both data bases (Krüger: Gigaspora margarita, EMBL: uncultured Archaeosporaceae) and both similarity and alignment length were higher in EMBL database. Consequently, OTU_12 and OTU_43 were assigned to the family

Archaeosporaceae. We believe this issue is explained by an underrepresentation of the Archaeosporaceae family in Krüger *et al.* (2012). When the taxonomic resolution of the match was sufficient, we followed a similar approach to that used in Martínez-García *et al.* (2015) for SSU sequences, and assigned OTUs with ≥ 97% similarity match to a species, ≥90% to a genus, ≥80% to a family and ≥70% to the subphylum. In cases with insufficient resolution in the match description, the OTUs were assigned to the closest available taxonomic level (see **Table S1** below). Five OTUs could not be satisfactorily assigned due to poor matches against both reference databases, additionally two OTUs were assigned to non Glomeromycotina groups (Ascomycota and Basidiomycota respectively). All these OTUs were removed from any subsequent analysis. At this point 1,125,415 reads conform the OTU table, representing ~60% of the unprocessed sequences and 99,7% of the quality filtered sequences, pointing to a high specificity of the amplification process.

Community analysis

All subsequent analyses were performed with R version 3.3.1 (R Core Team, 2016). Community analyses were performed with the package "vegan" (Oksanen *et al.*, 2016). The lowest number of reads in a sample was determined as 34,281 and all samples were normalized to this number by random subsampling without replacement with the function "rrarefy". OTUs accumulation curves were generated with the function "specaccum". A Bray-Curtis (Bray & Curtis, 1957) dissimilarity matrix was generated with the function "vegdist", nonmetric multidimensional scaling (NMDS) was generated with the function "metaMDS", multivariate permutational analysis of variance (PERMANOVA) was carried out with the function "adonis", using the three sampled sites as a random factor and difference in dispersion of the communities was determined with the function "betadisper". Graphical representations were generated using packages "ade4" (Dray & Dufour, 2007) and "vegan". Univariate analysis on the effect of depth and compartment were calculated using a generalized linear model (GLM) with a quasi-Poisson distribution. We renounced testing for interactions between both depth and compartment, due to the limited replication at that level.

Extended Results

OTU accumulation curves for both top- and subsoil were past the linearity point, indicating that we captured the majority of the diversity (Fig. S3). Overall, the families Claroideoglomeraceae, Diversisporaceae and Glomeraceae accounted together for 93.4% of all the reads.

In our subsoil samples (60 – 75 cm) we detected a richness of 49 OTUs. Claroideoglomeraceae is here the most abundant family with 60% of the reads. The family Glomeraceae was the second most abundant in the subsoil with 28% of the reads, followed by Diversisporaceae with 7.3% of the reads (Fig. 1). Gigasporaceae accounted for 1.1% and Paraglomeraceae for another 1.2% of the reads, 1.9% of the reads belonged to OTUs assigned at the subphylum level. Ambisporaceae and Archaeosporaceae represented each less than 1% of the subsoil reads. Two OTUs (OTU_40 assigned to the subphylum Glomeromycotina and OTU_68 assigned to the genus *Glomus*) were exclusively detected in the subsoil, also before rarefaction dowsampling (data not shown). The overall most abundant OTU (OTU_1, *Claroideoglomus sp.*) was virtually solely present in the subsoil (99.6% of its reads), where it dominated across the different compartments representing 35.0% of the reads (Fig. 2).

In the topsoil (10 – 30 cm) 62 OTUs were detected with 15 OTUs exclusively found in this environment. The family Diversisporaceae is here the most abundant and accounts for 42% of the reads. Again the second most abundant family was Glomeraceae represented with 34.1% of the reads followed by Claroideoglomeraceae with 15.1% (Fig. 1). Archaeosporaceae represented 6.6% of the reads, Paraglomeraceae 1.5%; Gigasporaceae, Ambisporaceae and OTUs assigned at the subphylum level accounted each for less than 1% of the topsoil reads. Here the two most abundant OTUs were OTU_4 (13.7%) and OTU_2 (13.0%), both assigned to the genus *Diversispora* (Table S4).

Beta diversity (i.e. spatial variation in species composition as measured by Jaccard index) showed a tendency towards increase in the subsoil, but this trend was not significant ($F_{1,15}$= 2.54, P=0.13, Fig. 3). Topsoil communities were, however, more even (Fig. S6), with a mean Shannon-index of 2.42 ±

0.24 compared to 1.53 ± 0.24 in subsoil (GLM, $F_{1,15}$=16.02 P=0.001). In subsoil, OTU richness (27.4 ± 5.9) was significantly lower than in topsoil (41.6 ± 6.0; GLM, $F_{1,15}$=23.83 P<0.001).

No significant differences in Shannon-index were found across bulk soil (2.14 ± 0.50), drilosphere (1.78 ± 0.86) and rhizosphere (1.92 ± 0.51) as well (GLM, $F_{2,14}$=0.45 P=0.65). Again, mean richness was not significantly related to any compartment (rhizosphere 28.4±6.9, bulk soil 39.3±7.6, drilosphere 33.7±10.8; GLM, $F_{2,14}$=2.17 P=0.15).

Table S1.

Table with taxonomic assignment for each OTU and the proportion of the total reads for that OTU detected in each soil compartment. Topsoil = 10-30 cm, subsoil = 60-75 cm. Red cells highlight OTUs exclusively found at one depth, yellow OTUs with over 0.9 of their reads found at one depth. Evenly distributed OTUs (at least 0.3 of their reads at each depth) are highlighted in green.

OTU	Proportion of reads		Taxon	Taxonomic Resolution	Family
	SUBSOIL	TOPSOIL			
OTU_1	0.996	0.004	*Claroideoglomus* sp.	Genus	Claroideoglomeraceae
OTU_2	0.212	0.788	*Diversispora celata*	Species	Diversisporaceae
OTU_3	0.996	0.004	*Claroideoglomus* sp.	Genus	Claroideoglomeraceae
OTU_4	0.094	0.906	*Diversispora* sp.	Genus	Diversisporaceae
OTU_5	0.513	0.487	Claroideoglomeraceae	Family	Claroideoglomeraceae
OTU_6	0.990	0.010	Claroideoglomeraceae	Family	Claroideoglomeraceae
OTU_7	0.446	0.554	*Glomus macrocarpum*	Species	Glomeraceae
OTU_8	0.468	0.532	*Funneliformis mosseae*	Species	Glomeraceae
OTU_9	0.373	0.627	*Diversispora* sp.	Genus	Diversisporaceae
OTU_10	0.139	0.861	*Claroideoglomus* sp.	Genus	Claroideoglomeraceae
OTU_11	0.263	0.737	Funneliformis caledonius	Species	Glomeraceae
OTU_12	0.015	0.985	Archaeosporaceae	Family	Archaeosporaceae
OTU_13	0.584	0.416	*Funneliformis* sp.	Genus	Glomeraceae
OTU_14	0.910	0.090	Glomeromycotina	Subphylum	Undetermined
OTU_15	0.000	1.000	Claroideoglomeraceae	Family	Claroideoglomeraceae
OTU_16	0.898	0.102	*Funneliformis constrictus*	Species	Glomeraceae
OTU_17	0.954	0.046	*Glomus macrocarpum*	Species	Glomeraceae
OTU_18	0.642	0.358	*Scutellospora* sp.	Genus	Gigasporaceae
OTU_19	0.003	0.997	*Funneliformis* sp.	Genus	Glomeraceae
OTU_20	0.001	0.999	*Funneliformis* sp.	Genus	Glomeraceae
OTU_21	0.677	0.323	*Rhizophagus* sp.	Genus	Glomeraceae
OTU_22	0.254	0.746	*Rhizophagus* sp.	Genus	Glomeraceae
OTU_23	0.015	0.985	*Ambispora gerdemannii*	Species	Ambisporaceae
OTU_24	0.004	0.996	Diversisporaceae	Family	Diversisporaceae
OTU_25	0.481	0.519	*Paraglomus* sp.	Genus	Paraglomeraceae
OTU_26	0.009	0.991	*Funneliformis constrictus*	Species	Glomeraceae
OTU_27	0.001	0.999	*Diversispora* sp.	Genus	Diversisporaceae
OTU_28	0.118	0.882	*Diversispora* sp.	Genus	Diversisporaceae
OTU_29	0.197	0.803	*Funneliformis* sp.	Genus	Glomeraceae
OTU_30	0.000	1.000	Glomeraceae	Family	Glomeraceae
OTU_31	0.000	1.000	*Funneliformis constrictus*	Species	Glomeraceae
OTU_32	0.019	0.981	*Diversispora* sp.	Genus	Diversisporaceae
OTU_33	0.000	1.000	*Archaeospora* sp.	Genus	Archaeosporaceae
OTU_34	0.999	0.001	Claroideoglomus sp.	Genus	Claroideoglomeraceae
OTU_35	0.036	0.964	*Funneliformis* sp.	Genus	Glomeraceae
OTU_37	0.025	0.975	*Diversispora* sp.	Genus	Diversisporaceae
OTU_38	0.008	0.992	Ambisporaceae	Family	Ambisporaceae
OTU_39	0.000	1.000	Diversisporaceae	Family	Diversisporaceae
OTU_40	1.000	0.000	Glomeromycotina	Subphylum	Undetermined
OTU_41	0.000	1.000	*Diversispora* sp.	Genus	Diversisporaceae
OTU_42	0.090	0.910	*Diversispora* sp.	Genus	Diversisporaceae
OTU_43	0.091	0.909	Archaeosporaceae	Family	Archaeosporaceae
OTU_44	0.000	1.000	*Claroideoglomus* sp.	Genus	Claroideoglomeraceae
OTU_47	0.998	0.002	Claroideoglomeraceae	Family	Claroideoglomeraceae
OTU_48	0.009	0.991	*Funneliformis caledonius*	Species	Glomeraceae
OTU_49	0.000	1.000	*Diversispora* sp.	Genus	Diversisporaceae
OTU_50	0.108	0.892	Claroideoglomeraceae	Family	Claroideoglomeraceae
OTU_53	0.000	1.000	*Funneliformis* sp.	Genus	Glomeraceae
OTU_54	0.000	1.000	*Rhizophagus* sp.	Genus	Glomeraceae
OTU_55	0.429	0.571	Glomeraceae	Family	Glomeraceae
OTU_56	0.996	0.004	*Glomus macrocarpum*	Species	Glomeraceae
OTU_57	0.719	0.281	*Scutellospora* sp.	Genus	Gigasporaceae
OTU_59	0.794	0.206	*Glomus* sp.	Genus	Glomeraceae
OTU_60	0.000	1.000	Archaeosporaceae	Family	Archaeosporaceae
OTU_61	0.000	1.000	Claroideoglomeraceae	Family	Claroideoglomeraceae
OTU_62	0.121	0.879	*Diversispora* sp.	Genus	Diversisporaceae
OTU_63	0.000	1.000	*Diversispora* sp.	Genus	Diversisporaceae
OTU_64	0.080	0.920	*Diversispora* sp.	Genus	Diversisporaceae
OTU_65	0.038	0.962	*Diversispora* sp.	Genus	Diversisporaceae
OTU_66	0.000	1.000	Claroideoglomeraceae	Family	Claroideoglomeraceae
OTU_68	1.000	0.000	*Glomus* sp.	Genus	Glomeraceae
OTU_69	0.000	1.000	Claroideoglomeraceae	Family	Claroideoglomeraceae
OTU_70	0.001	0.999	*Funneliformis* sp.	Genus	Glomeraceae
OTU_71	0.998	0.002	Glomeromycotina	Subphylum	Undetermined

Figure S2.

OTU accumulation curves for top- and subsoil based on random sampling order with 100 permutations. Curve represents the mean OTUs number and error bars represent standard deviation.

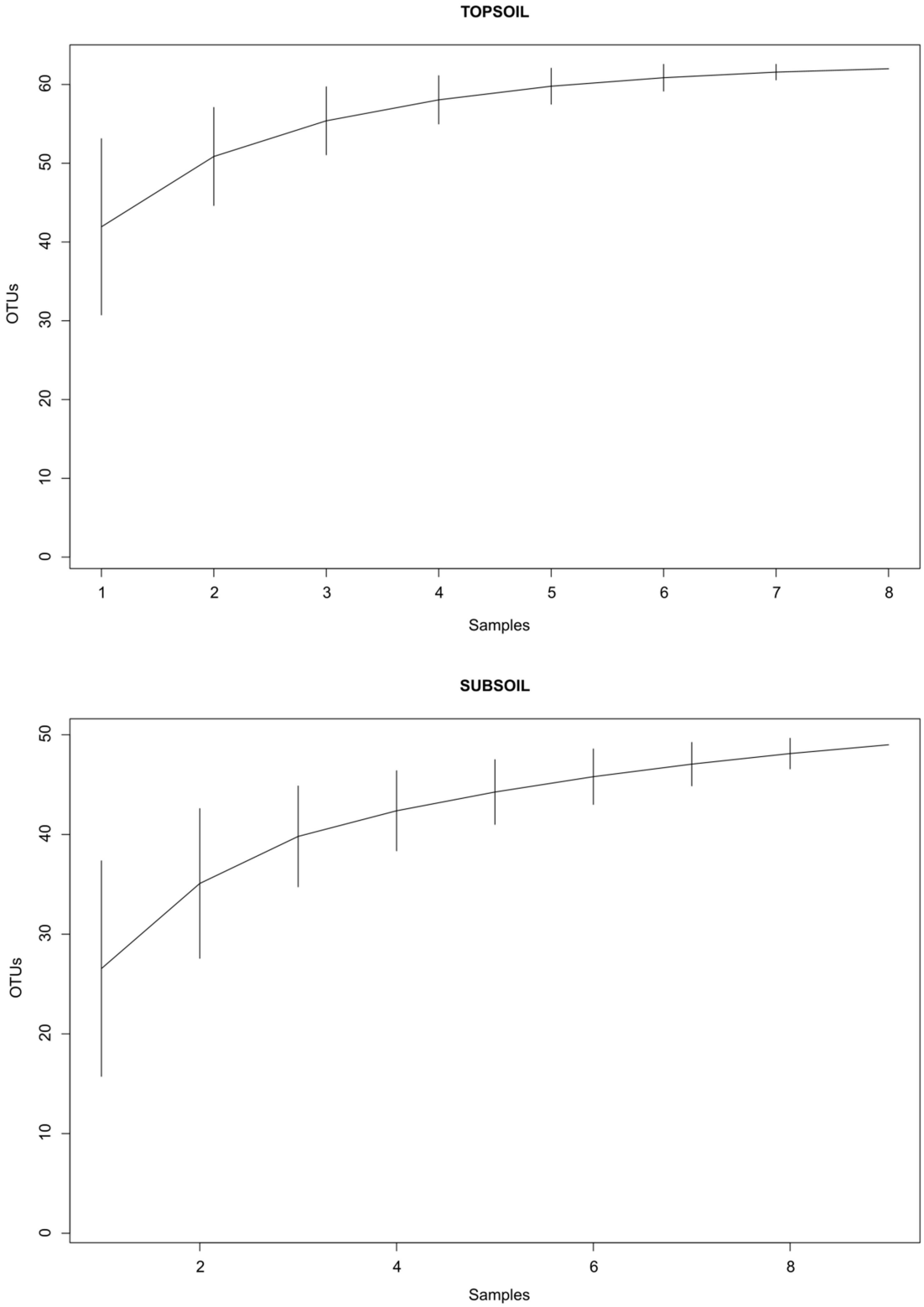
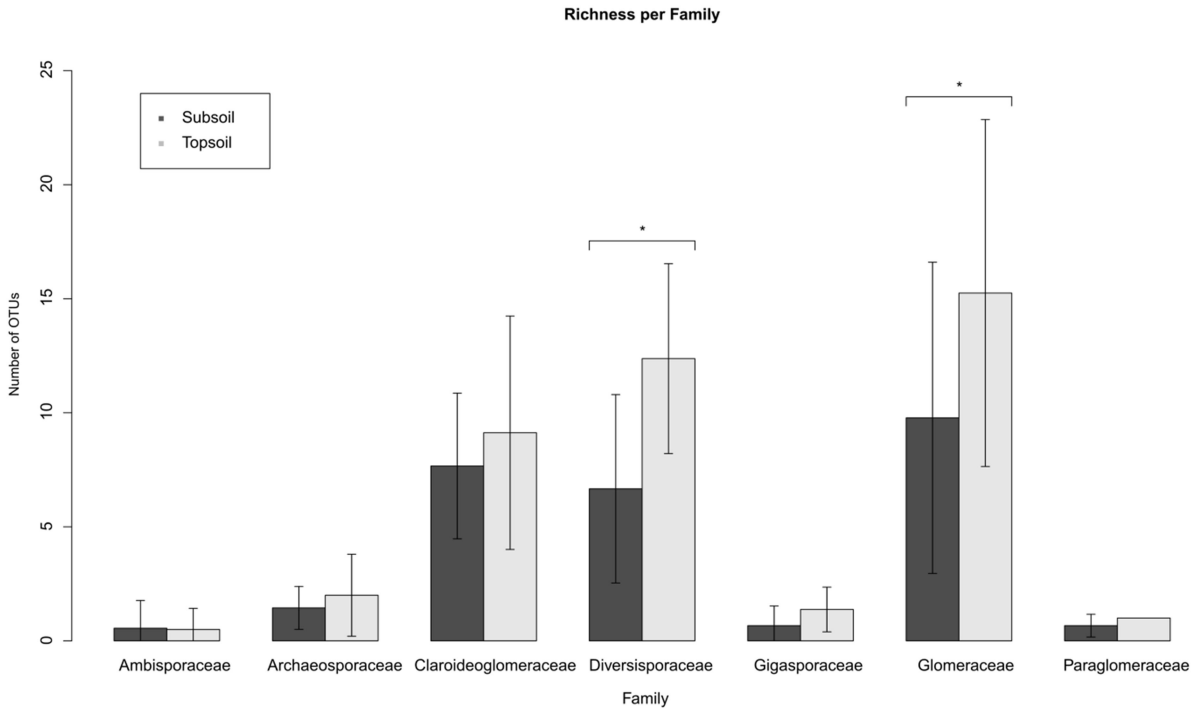


Figure S3.

a) Mean absolute richness and **b)** mean relative richness, for each family and depth. Error bars represent standard deviation, asterisk denote statistically significant differences.

a)



b)

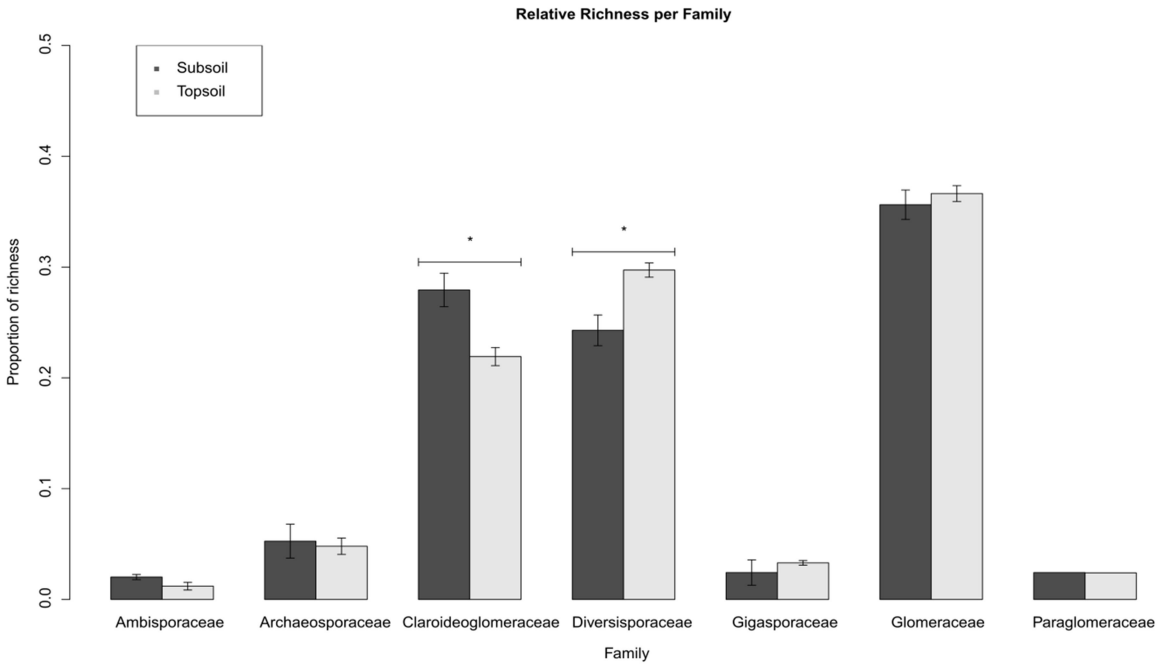


Table S4.

Relative abundance of each OTU for the given soil compartments. Numbers are rounded and refer to the proportion of the community represented by the given OTU. Red cells highlight the absence of the OTU, yellow relative abundances below 0.001 and green relative abundances over 0.01.

OTU	TOPSOIL	SUBSOIL	BULKSOIL	DRILOSPHERE	RHIZOSPHERE
OTU_1	0.002	0.350	0.188	0.255	0.101
OTU_2	0.130	0.031	0.097	0.070	0.064
OTU_3	<0.001	0.110	0.068	0.069	0.035
OTU_4	0.137	0.013	0.081	0.077	0.052
OTU_5	0.033	0.031	0.018	0.019	0.066
OTU_6	0.001	0.072	0.046	0.044	0.023
OTU_7	0.070	0.050	0.089	0.028	0.063
OTU_8	0.098	0.077	0.025	0.120	0.122
OTU_9	0.033	0.017	0.039	0.024	0.008
OTU_10	0.072	0.010	0.017	0.058	0.042
OTU_11	0.075	0.024	0.047	0.053	0.043
OTU_12	0.044	<0.001	0.052	0.008	0.001
OTU_13	0.022	0.027	0.018	0.009	0.050
OTU_14	0.001	0.011	0.015	<0.001	0.003
OTU_15	0.013	0.000	0.008	0.010	0.000
OTU_16	0.002	0.012	0.002	<0.001	0.022
OTU_17	0.004	0.073	<0.001	0.014	0.120
OTU_18	0.007	0.010	0.005	0.003	0.019
OTU_19	0.004	<0.001	0.006	<0.001	0.000
OTU_20	0.004	<0.001	0.004	0.001	0.000
OTU_21	0.001	0.002	0.003	<0.001	0.001
OTU_22	0.004	0.001	0.002	0.001	0.005
OTU_23	0.002	<0.001	0.001	<0.001	0.002
OTU_24	0.004	<0.001	<0.001	0.002	0.003
OTU_25	0.015	0.012	0.013	0.021	0.004
OTU_26	0.012	<0.001	0.008	0.003	0.007
OTU_27	0.003	<0.001	0.003	<0.001	0.000
OTU_28	0.004	0.001	0.004	0.002	0.001
OTU_29	0.030	0.007	0.003	0.007	0.047
OTU_30	0.003	0.000	<0.001	0.004	0.000
OTU_31	0.002	0.000	0.001	0.001	0.000
OTU_32	0.003	<0.001	<0.001	0.004	<0.001
OTU_33	0.001	0.000	0.001	0.000	0.000
OTU_34	<0.001	0.010	<0.001	0.015	<0.001
OTU_35	0.002	<0.001	<0.001	<0.001	0.003
OTU_37	0.003	<0.001	0.002	0.001	0.001
OTU_38	<0.001	<0.001	<0.001	<0.001	0.001
OTU_39	<0.001	0.000	<0.001	<0.001	<0.001
OTU_40	0.000	0.001	0.000	0.001	<0.001
OTU_41	0.001	0.000	0.001	<0.001	0.000
OTU_42	0.001	<0.001	0.001	<0.001	<0.001
OTU_43	0.021	0.002	0.023	0.005	0.003
OTU_44	<0.001	0.000	0.000	<0.001	0.000
OTU_47	<0.001	0.011	0.016	<0.001	0.002
OTU_48	0.001	<0.001	<0.001	0.001	<0.001
OTU_49	<0.001	0.000	<0.001	<0.001	0.000
OTU_50	0.030	0.003	0.009	0.014	0.026
OTU_53	0.001	0.000	0.001	0.001	0.000
OTU_54	<0.001	0.000	0.000	<0.001	0.000
OTU_55	<0.001	<0.001	<0.001	<0.001	<0.001
OTU_56	<0.001	0.002	0.000	<0.001	0.003
OTU_57	<0.001	<0.001	<0.001	<0.001	0.001
OTU_59	0.003	0.009	0.015	<0.001	0.002
OTU_60	<0.001	0.000	<0.001	0.000	0.000
OTU_61	<0.001	0.000	<0.001	<0.001	0.000
OTU_62	0.089	0.011	0.052	0.045	0.046
OTU_63	<0.001	0.000	<0.001	<0.001	0.000
OTU_64	0.005	<0.001	0.002	0.003	0.003
OTU_65	0.004	<0.001	0.003	0.001	0.001
OTU_66	<0.001	0.000	<0.001	<0.001	0.000
OTU_68	0.000	<0.001	0.000	0.000	<0.001
OTU_69	<0.001	0.000	<0.001	<0.001	0.000
OTU_70	0.003	<0.001	<0.001	0.003	0.001
OTU_71	<0.001	0.008	0.009	0.000	0.002

Figure S5.

Rarefaction curves for each sample before normalization. Green lines represent topsoil samples, brown lines represent subsoil samples.

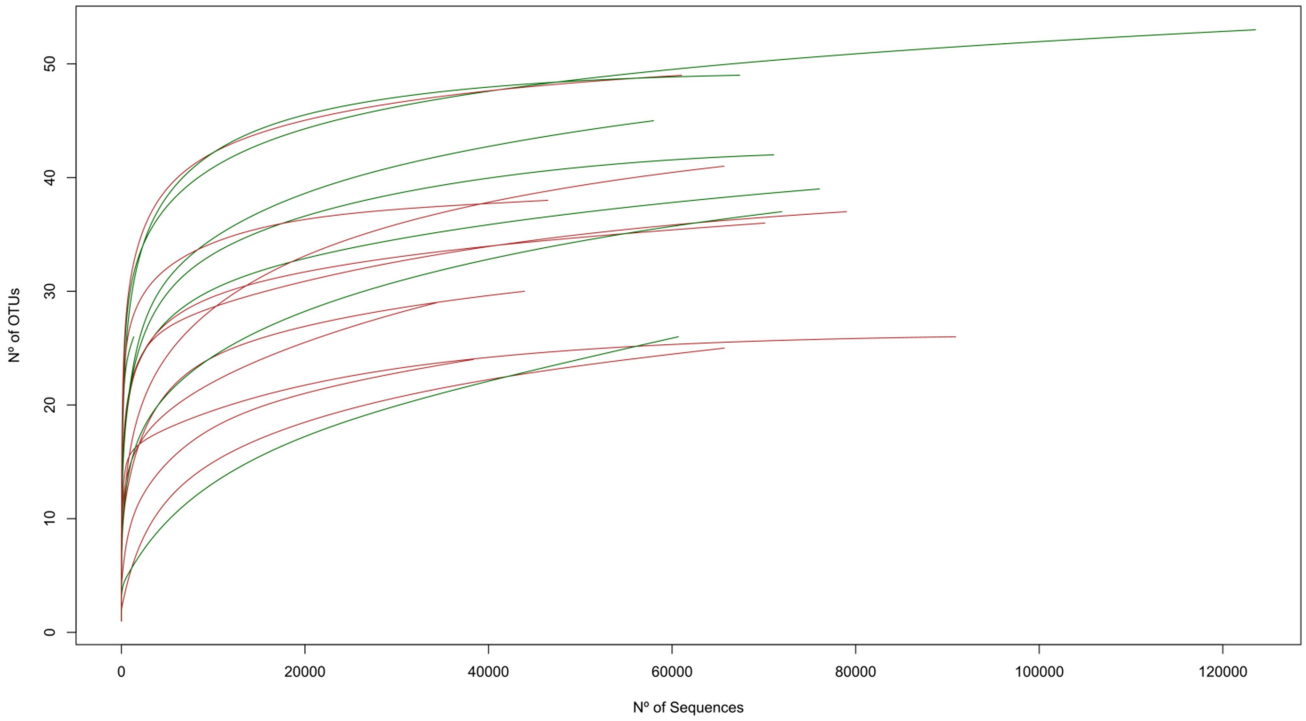
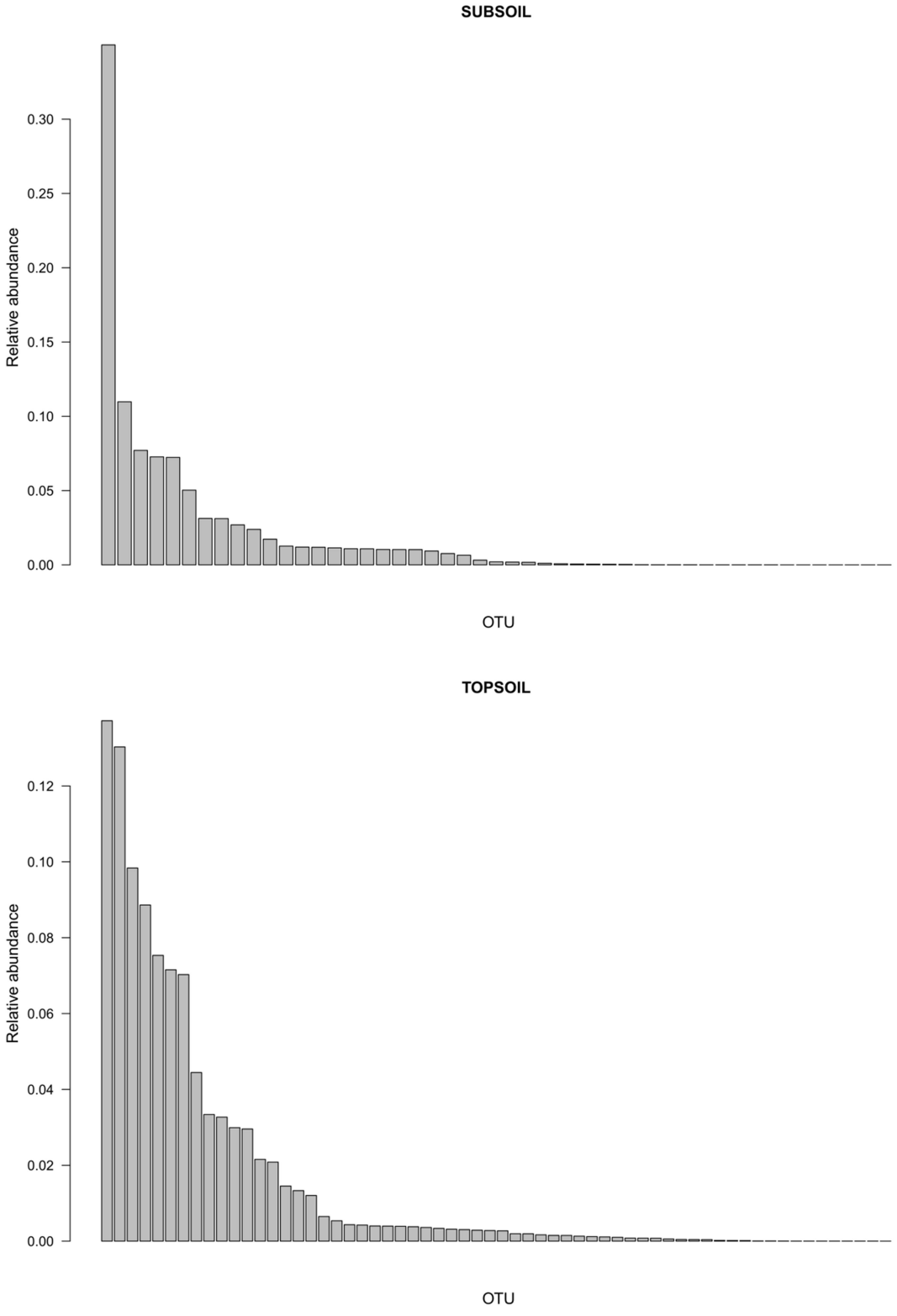


Figure S6.

OTU relative abundance distribution graphs for a) subsoil and b) topsoil.



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Appendix 2.2

The sequences presented in **chapter 2** are here re-analyzed using a single sequence variant (SSV) approach, as implemented by Callahan *et al.* (2016). In short, here sequences are not clustered at a given similarity threshold, but the quality of each sequence is carefully addressed to identify "true" sequences (where we can confidently assume that there are no PCR or sequencing artifacts in the sequence), and these are used as the operational taxonomic unit. Therefore, SSV provides the greatest taxonomic resolution possible for a given marker.

Non metric multidimensional scaling (NMDS) of the AM fungal communities was performed following the same criteria presented in chapter 2 with the newly created SSV abundance table (**Fig S1**). To compare the two results I carried out a procrustes test with the newly created and the 97% similarity based NMDS (**Fig S2**) using the formulas "procrust" and "protest" from the "Vegan" package (Oksanen et al., 2016), using the software R (R Core Team, 2016). The community ordination obtained with SSV is comparable to the previously obtained results (PROCRUSTES, sum of squares=0.296, r=0.840, P=0.001). Permutational multivariate analysis of variance was repeated with the new table, using the formula "adonis". Again results are fully consistent with the previously obtained, with a significant effect of depth (PERMANOVA, $F_{1,16}$= 4.471, P<0.001), and no significant effect of compartment (PERMANOVA, $F_{2,16}$= 0.971, P<0.001). Overall these results confirm that in this set of samples, even at greater taxonomic resolutions no differences can be found in the community composition of the different soil compartments.

Figure S1.

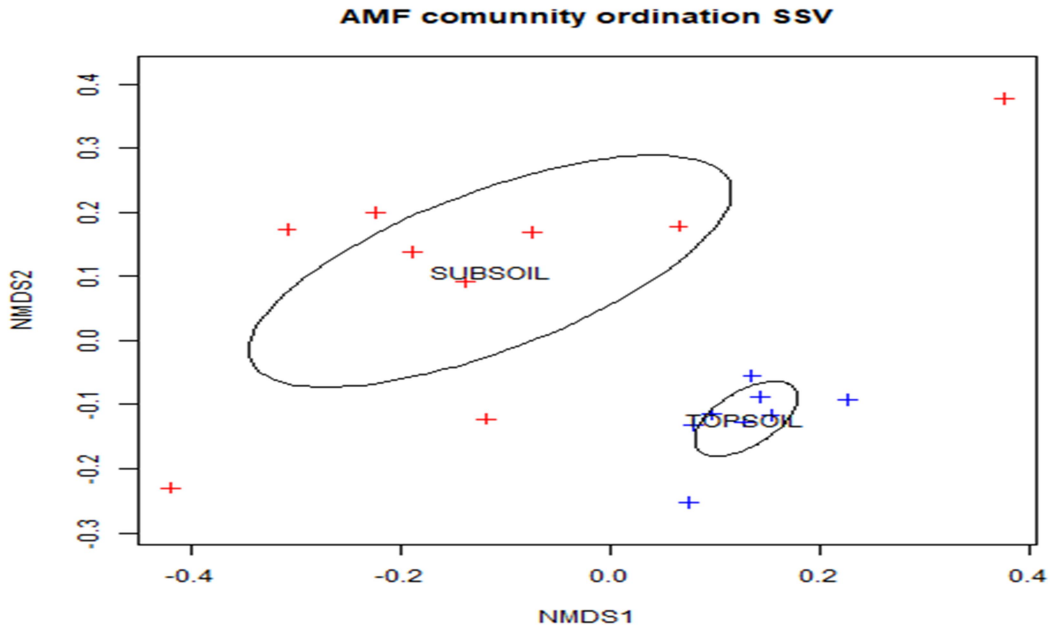
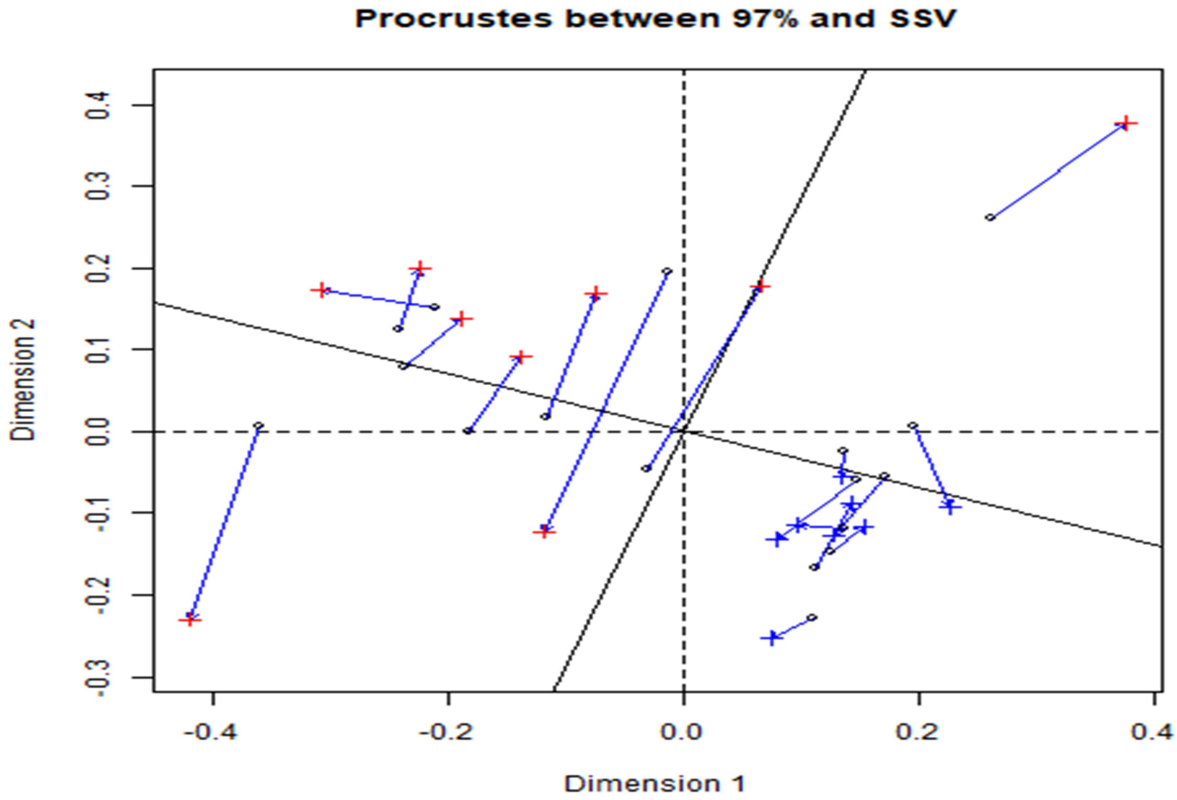


Figure S2.



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Appendix 2.3

Here I present preliminary data on a high-throughput study of arbuscular mycorrhizal (AM) fungi across different depths in an agriculture field. First results confirm a significant shift of AM fungal communities with depth, and the existence of subsoil specific AM fungal phylotypes. Furthermore, previous results on the greater proportion of reads assigned to the family Claroideoglomeraceae in subsoil are here again supported.

Material and methods

Samples were taken from a long-term fertilizer trial in Gießen (Germany) at 0–30, 30–40, 40–50, 50–70 and 70–100 cm depth, in a Fluvic Gleyic Cambisol. Details on the sampling can be consulted in Bauke *et al.*(2018). In short, 20 soil cores were sampled, DNA was extracted and PCR was performed using general fungal primers that target the ITS1 region (fITS7 (Bertini et al., 1999), ITS4 (White et al., 1990)). Amplicons were sequenced on a Illumina MiSeq platform, and sequences were filtered using a single sequence variant (SSV) approach as implemented with the R package "dada2" (Callahan et al., 2016). Community analysis was performed using the R software (R Core Team, 2016) and the package "vegan" (Oksanen et al., 2016). The identification of SSVs specific for each depth was performed on a non-normalized SSV table. Subsequent analyses were performed on a SSV table normalized by the minimum amount of reads per sample (250).

Results

A total of 323 AM fungal SSVs were identified. From those, 29 were exclusively found at a depth of 30cm, and 13, 9, 9 and 4 at depths of 40, 50 70 and 100 cm respectively (**Figure S1**). In total, 35 SSVs were exclusively found in subsoil (below 30 cm) vs 29 in topsoil. Richness and Shannon diversity values for each depth after normalization are presented in **table S2**. Community ordination shows a shift in community composition from top- to subsoil, and a minor shift between the different depths in subsoil (**Figure S3**). The effect of depth on community composition is significant (PERMANOVA, $F_{1,99}$= 3.73, P<0.001). The proportion of reads assigned to the family Claroideoglomeraceae was highest at 70 and 100 cm, with 32% and 48% of the reads respectively (**Figure S4**).

Figure S1. Number of SSVs found exclusively at each depth using a non-normalized table (i.e. without accounting for different sampling efforts).

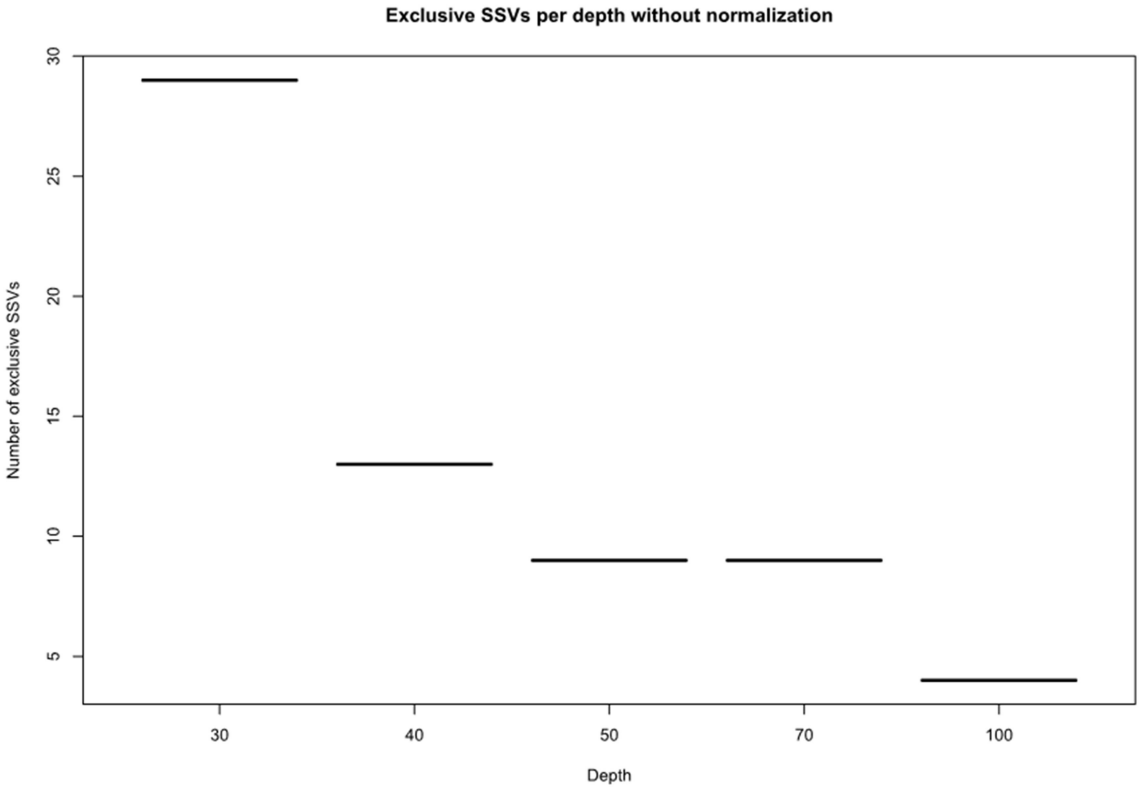


Table S2. Diversity indexes for various depths. Depth is given in cm, richness in number of SSVs, sd stands for standard deviation.

Depth	Mean richness	Richness sd	Mean Shannon	Shannon sd
30	28.25	13.39628778	2.690850041	0.566812413
40	19.55	7.19996345	2.174997488	0.591861156
50	14.85	4.682273402	1.988907901	0.455840867
70	14.7	5.151392249	2.028717595	0.378480051
100	10.25	4.919082071	1.767691496	0.487191188

Figure S3. Non metric multidimensional scaling (NMDS) of the AM fungal communities at different depths, based on Bray-Curtis dissimilarities.

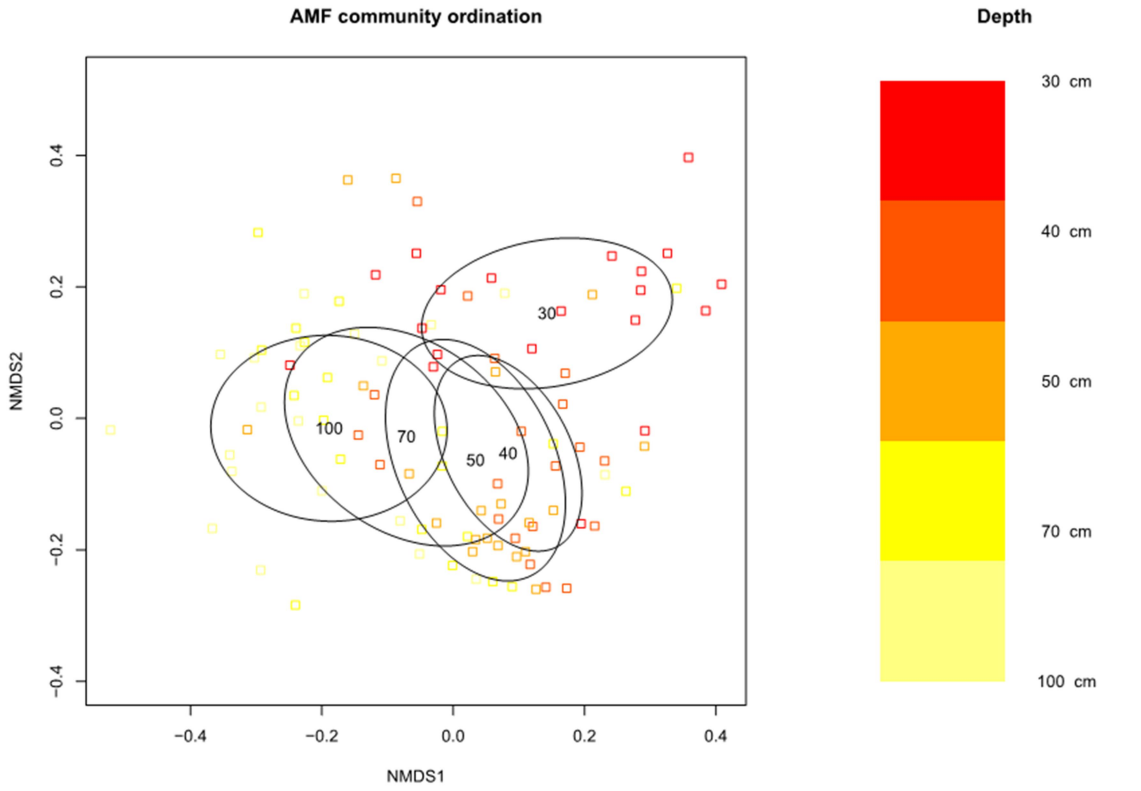
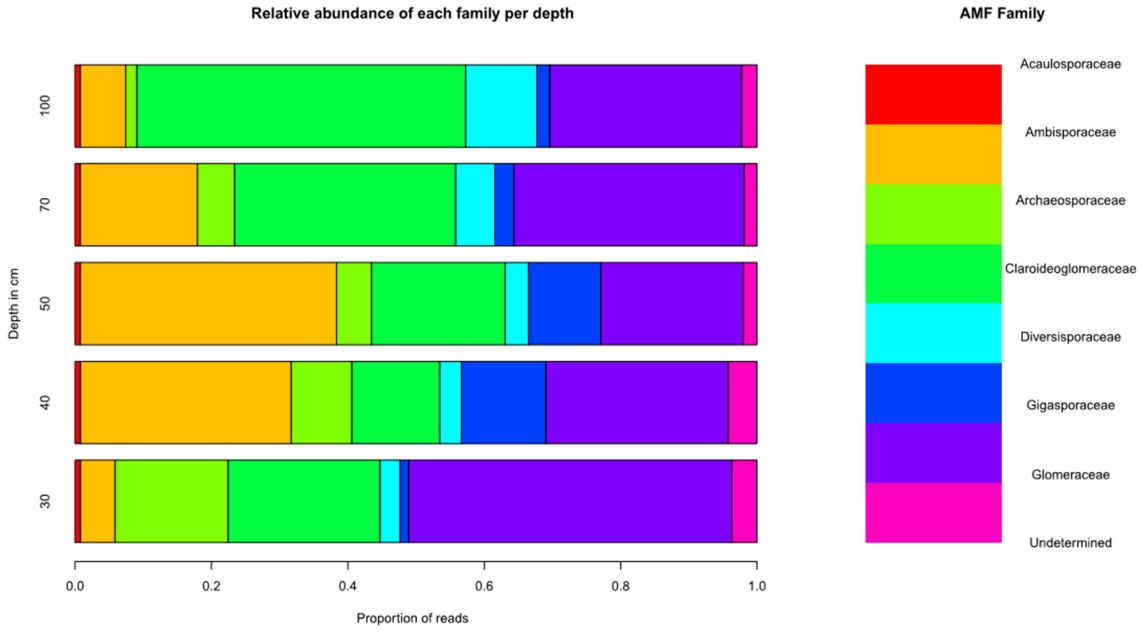


Figure S4. Relative abundance of each family at different depths.



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Appendix 2.4

Here I present preliminary data on a high-throughput study of arbuscular mycorrhizal (AM) fungi across different depths in an agriculture field. First results confirm a significant shift of AM fungal communities with depth, and the existence of subsoil specific AM fungal phylotypes.

Material and methods

Samples were taken in a long-term tillage trial in Sicily (Italy) at 0-15, 15-30, 30-60 and 60-90 cm depth (from now on depths A, B, C and D respectively), in a Chromic Haploxeret Vertisol. In short, 30 plots were sampled at the given depths, DNA was extracted and PCR was performed using AM fungal specific primers following the protocol described in (Sosa-Hernández et al., 2018). Amplicons were sequenced on a Illumina MiSeq platform, and sequences were filtered using a single sequence variant (SSV) approach as implemented with the R package “dada2” (Callahan et al., 2016). Community analysis was performed using the R software (R Core Team, 2016) and the package “vegan” (Oksanen et al., 2016). The identification of SSVs specific for each depth was performed on a non-normalized SSV table. Subsequent analyses were performed on a SSV table normalized by the minimum amount of reads per sample (1471).

Results

A total of 2162 AM fungal SSVs were identified. From those, 282 were exclusively found at a depth A, and 203, 110, and 87 at depths B, C and D respectively (**Figure S1**). In total, 197 SSVs were exclusively found in subsoil (below 30 cm) vs 485 in topsoil. Richness and Shannon diversity values for each depth after normalization are presented in **table S2**. Community ordination shows a shift in community composition with depth (**Figure S3**). The effect of depth on community composition is significant (PERMANOVA, $F_{1,119}$= 3.21, P<0.001). Taxonomic assignment could only be carried at the phylum level due to technical issues and therefore, no distribution of AM fungal families can be presented yet.

Figure S1. Number of SSVs found exclusively at each depth using a non-normalized table (i.e. without accounting for different sampling efforts).

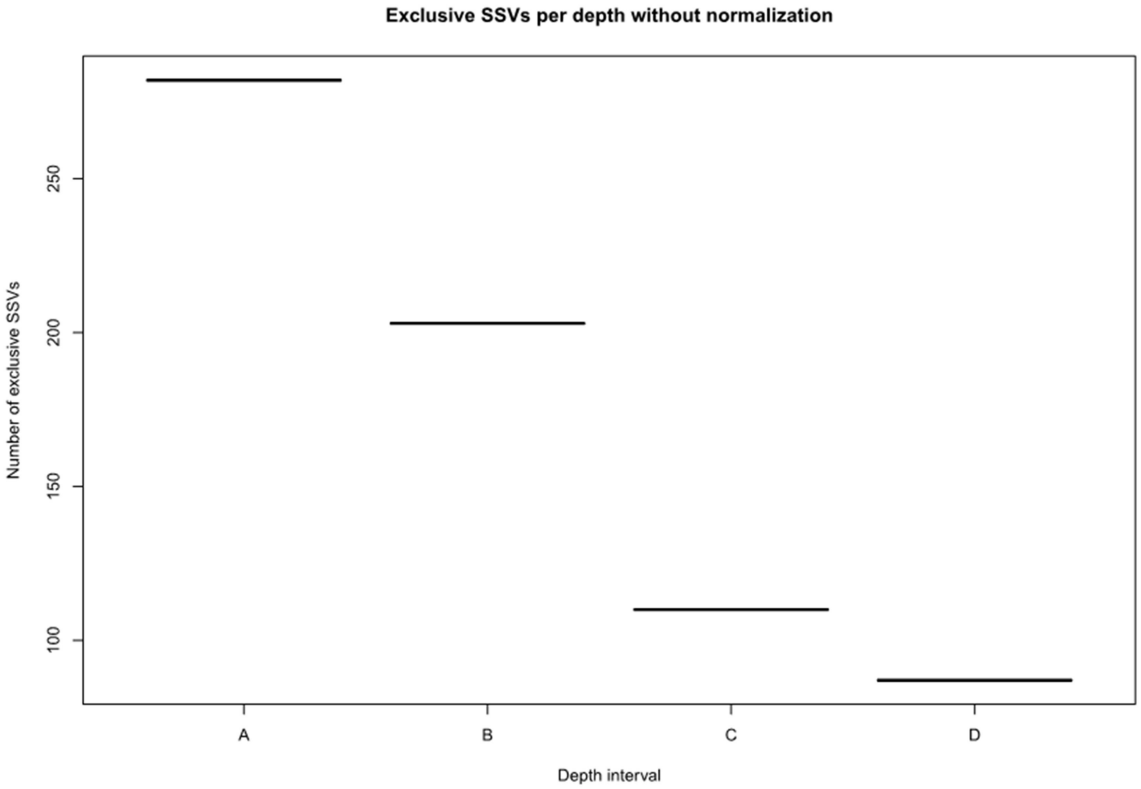
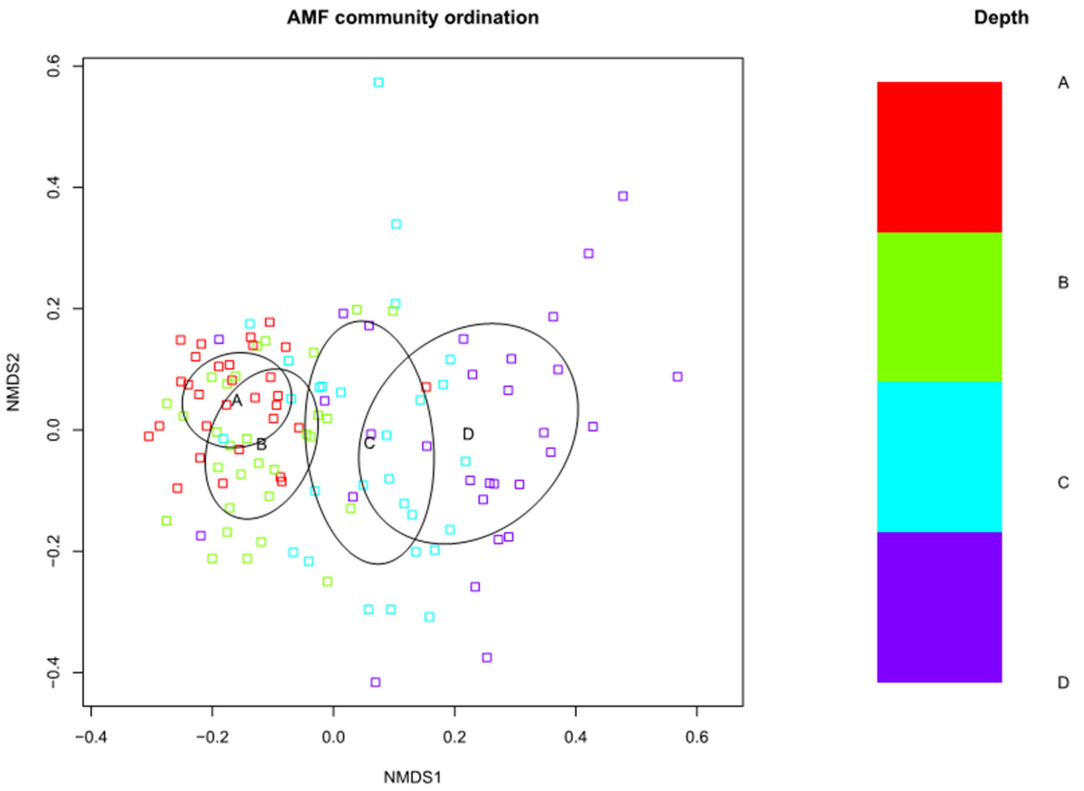


Table S2. Diversity indexes for various depths. Depth is given in cm, richness in number of SSVs, sd stands for standard deviation.

Depth	Mean richness	Richness sd	Mean Shannon	Shannon sd
A	134.6333333	38.92830132	3.830193314	0.396984709
B	96.06666667	26.83530013	3.366119875	0.429185837
C	61.46428571	26.20783335	2.401167122	0.802870813
D	41.53333333	24.38163997	1.903859566	0.759535747

Figure S3. Non metric multidimensional scaling (NMDS) of the AM fungal communities at different depths, based on Bray-Curtis dissimilarities.



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Appendix 3

Fig. S1. Rarefaction curves.

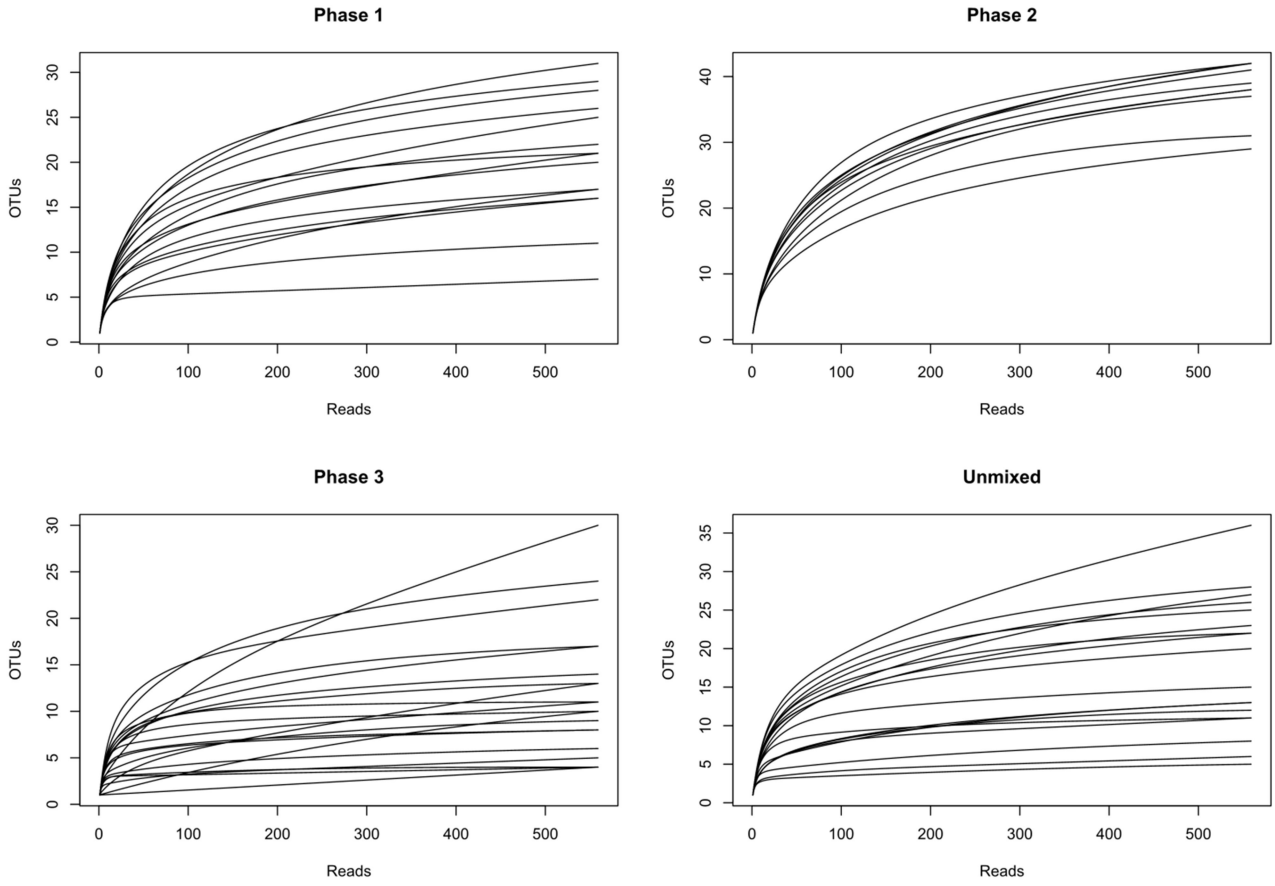

Fig. S2. OTU accumulation curves.

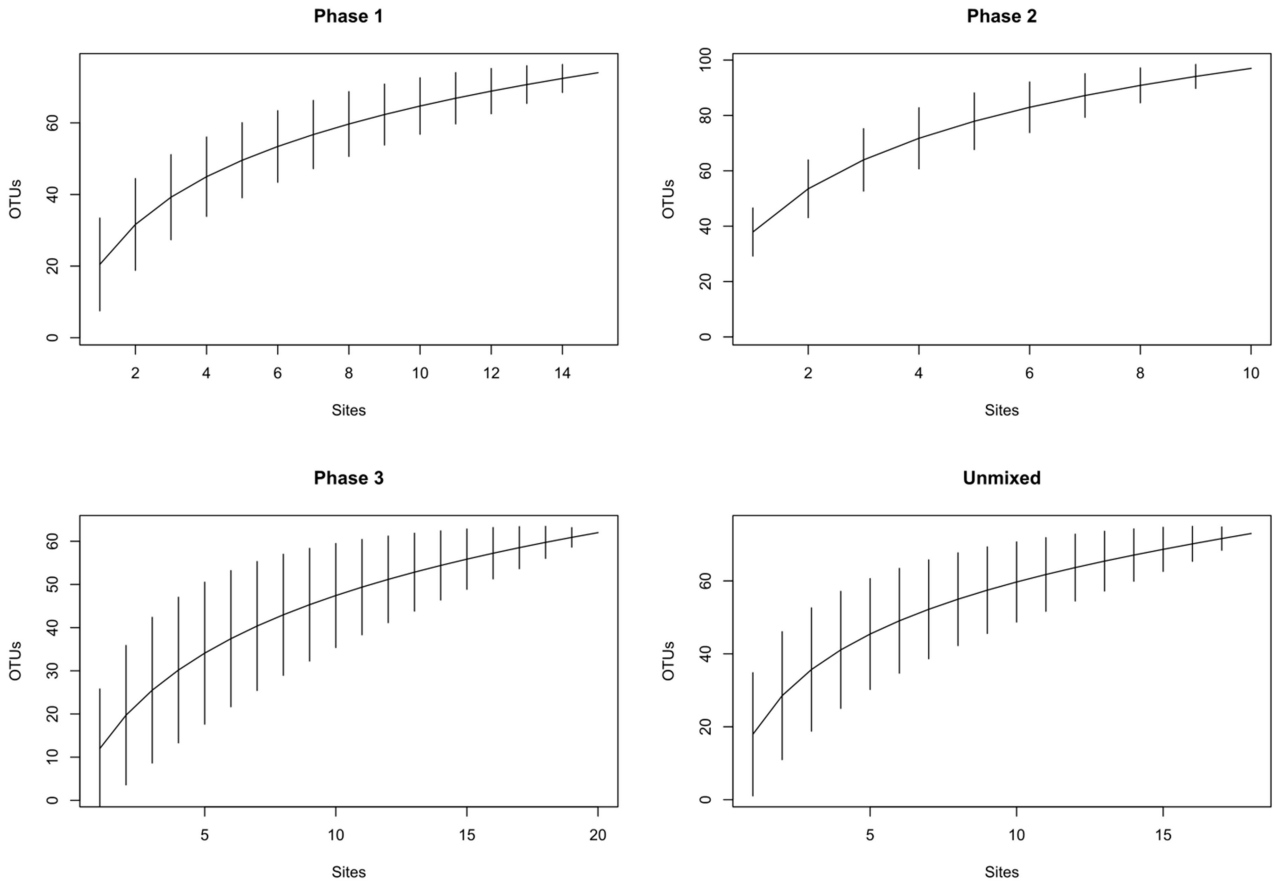

Fig. S3 Dissimilarities to Sub- and Topsoil over time. Jaccard distances (i.e. dissimilarities in community composition) between chronosequence fields and A) subsoil communities and B) topsoil communities. Dotted lines link the means, bars represent the standard error. Different phases are coded by color, significant differences between phases are represented by different letters. For details on the statistics consult Table S2. Phase 1: 1-3 years, n=135. Phase 2: 4-5 year, n=90. Phase 3: 10-52 years, n=180.

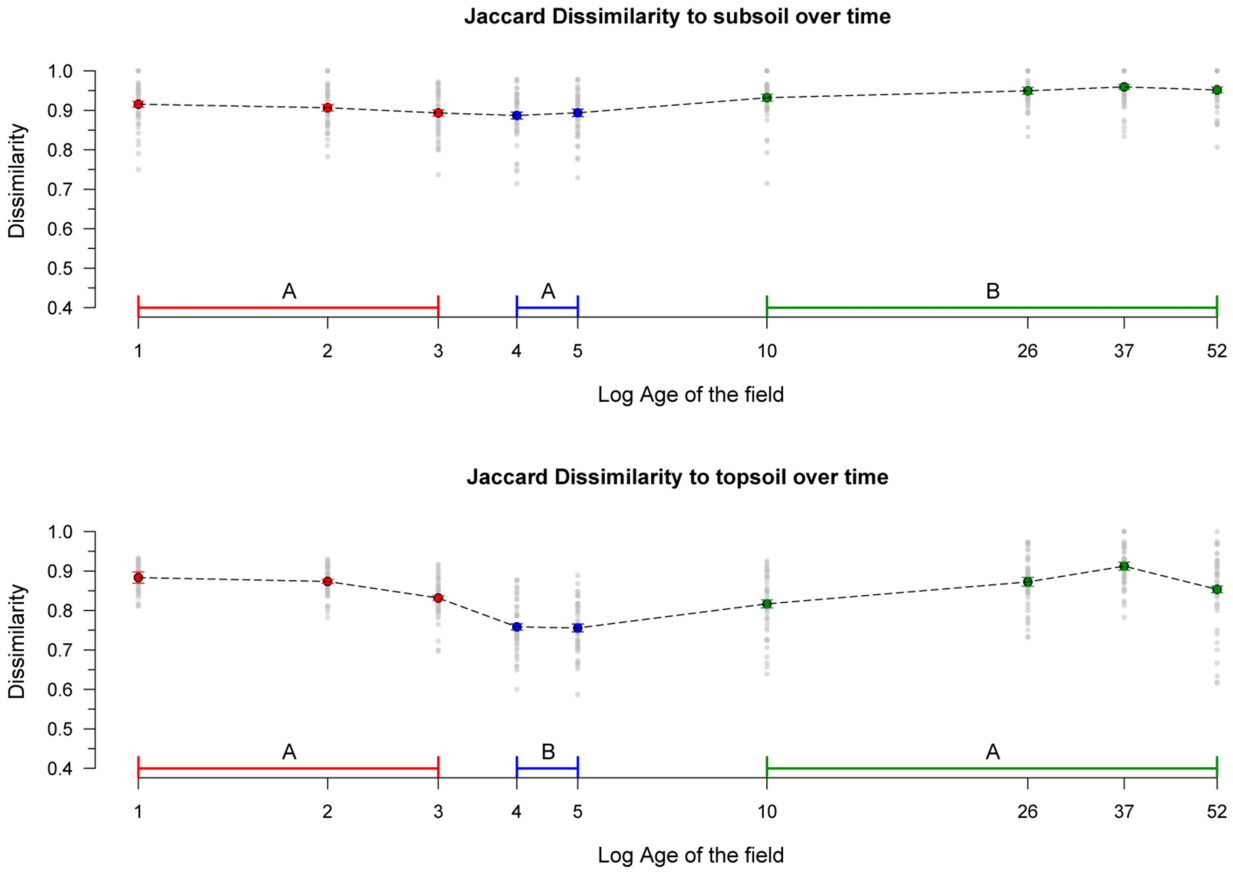

Table S1. Taxonomic assignment and relative abundance of detected OTUs

OTUs noted with "*" were identified as subsoil indicators and those noted with "**" were identified as topsoil indicators. Columns "Phase 1", "Phase 2", "Phase 3", "Subsoil" and "Topsoil" present the relative abundance of that OTU in the denoted portion of the dataset, ranging from 0 to 1.

Table S1. Taxonomic assignation and relative abundance of detected OTUs

OTU	Length in bp	Best Hit	HitName	% of identity	eValue	Assigned Taxon	Taxonomic Resolution	Phase 1	Phase 2	Phase 3	Subsoil	Topsoil
OTU_1*	409	Claroideoglomus luteum	FM876810_R	90.46	1.71E-154	Claroideoglomus sp.	Genus	0.037	0.014	0.000	0.306	0.004
OTU_3	408	Diversispora celata	AM713419_R	100.00	0.00E+00	Diversispora celata	Species	0.088	0.114	<0.001	0.031	0.128
OTU_4*	409	Claroideoglomus sp.	FR750076	91.02	7.92E-158	Claroideoglomus sp.	Genus	0.011	0.008	0.000	0.113	0.001
OTU_5**	409	Diversispora sp.	JF439148	94.39	0.00E+00	Diversispora sp.	Genus	<0.001	0.037	0.016	0.016	0.157
OTU_6	410	Claroideoglomus luteum	FM876810_R	89.35	3.75E-146	Claroideoglomeraceae	Family	<0.001	0.002	0.000	0.029	0.031
OTU_7*	393	Claroideoglomus microaggregatum	FJ461843	80.28	1.85E-69	Claroideoglomeraceae	Family	0.000	0.000	0.000	0.076	<0.001
OTU_9	406	Glomus macrocarpum	FR750526_R	98.03	0.00E+00	Glomus macrocarpum	Species	0.000	0.000	0.009	0.053	0.067
OTU_11	415	Funneliformis mosseae	FN547491_R	99.52	0.00E+00	Funneliformis mosseae	Species	0.232	0.030	<0.001	0.074	0.091
OTU_12**	408	Diversispora sp.	FR686952	94.63	0.00E+00	Diversispora sp.	Genus	0.003	0.008	0.029	0.016	0.034
OTU_13**	409	Claroideoglomus etunicatum	HM485693	90.31	7.98E-153	Claroideoglomus sp.	Genus	0.027	0.104	0.040	0.010	0.105
OTU_14**	416	Funneliformis caledonius	FN547496_R	98.32	0.00E+00	Funneliformis caledonius	Species	0.016	0.192	0.158	0.023	0.086
OTU_18**	418	Funneliformis constrictus	JF439180	97.13	0.00E+00	Funneliformis constrictus	Species	0.087	0.092	0.019	<0.001	0.038
OTU_21	373	Scutellospora calospora	EU252109	97.59	0.00E+00	Scutellospora calospora	Species	0.003	0.020	0.178	0.010	0.007
OTU_22	413	Rhizophagus irregularis	FM992386_R	99.52	0.00E+00	Rhizophagus irregularis	Species	0.083	0.012	<0.001	0.000	0.000
OTU_23	412	Rhizophagus irregularis	FR750067_R	94.92	0.00E+00	Rhizophagus sp.	Genus	0.015	0.050	0.005	0.002	0.007
OTU_26	416	Funneliformis caledonius	FN547499_R	98.56	0.00E+00	Funneliformis caledonius	Species	0.028	0.020	0.095	0.000	<0.001
OTU_27	417	Claroideoglomus etunicatum	HM485734	87.56	5.01E-135	Claroideoglomeraceae	Family	0.000	0.005	0.000	0.000	0.013
OTU_28	368	Archaeospora sp.	FR750038	95.99	1.94E-173	Archaeospora sp.	Genus	0.000	0.000	0.026	0.000	0.000
OTU_29	407	Claroideoglomus sp.	FM876806_R	99.51	0.00E+00	Claroideoglomus sp.	Genus	0.113	<0.001	<0.001	0.000	0.000
OTU_30	359	Paraglomus laccatum	FJ461809	94.35	9.09E-147	Paraglomus sp.	Genus	<0.001	0.003	0.151	0.013	0.016
OTU_32	418	Funneliformis constrictus	JF439180	97.13	0.00E+00	Funneliformis constrictus	Species	0.000	0.000	0.001	0.021	0.030
OTU_33	409	Claroideoglomus sp.	FR750077	99.27	0.00E+00	Claroideoglomus sp.	Genus	0.021	<0.001	<0.001	0.000	0.000
OTU_34	411	Funneliformis constrictus	JF439180	91.13	1.32E-160	Funneliformis sp.	Genus	0.004	0.009	0.061	0.000	0.000
OTU_35	358	Ambispora gerdemannii	JF439210	98.32	1.12E-180	Ambispora gerdemannii	Species	0.000	0.003	0.057	0.000	0.001
OTU_36	407	Glomus macrocarpum	FR750526_R	98.28	0.00E+00	Glomus macrocarpum	Species	0.000	<0.001	0.000	0.076	0.005
OTU_37	417	Funneliformis constrictus	JF439180	95.69	0.00E+00	Funneliformis sp.	Genus	0.000	0.000	0.000	0.028	0.001
OTU_39	412	Rhizophagus irregularis	FM992386_R	94.92	0.00E+00	Rhizophagus sp.	Genus	0.003	0.013	0.001	0.002	0.001
OTU_40	372	Archaeospora schenckii	FR750023	93.32	2.59E-157	Archaeospora sp.	Genus	0.000	0.001	0.031	0.000	0.000
OTU_41	409	Diversispora sp.	JF439136	95.61	0.00E+00	Diversispora sp.	Genus	0.018	0.003	<0.001	<0.001	0.003
OTU_42	429	Diversispora sp.	JF439137	91.38	2.30E-163	Diversispora sp.	Genus	0.005	0.008	0.002	0.000	0.000
OTU_44	416	Funneliformis sp.	FM876813_R	96.39	0.00E+00	Funneliformis sp.	Genus	0.020	0.048	0.017	0.000	0.000
OTU_45	409	Diversispora sp.	FR686952	94.15	5.95E-179	Diversispora sp.	Genus	0.000	0.009	0.000	0.000	0.004
OTU_46	409	Diversispora sp.	FR686952	97.31	0.00E+00	Diversispora sp.	Genus	0.000	0.019	0.000	<0.001	0.006
OTU_47	411	Funneliformis caledonius	FN547496_R	95.19	0.00E+00	Funneliformis sp.	Genus	0.002	0.002	<0.001	0.000	0.004
OTU_49	412	Funneliformis constrictus	JF439176	88.78	2.27E-143	Glomeraceae	Family	0.010	0.002	0.000	0.000	0.002
OTU_50	415	Funneliformis constrictus	JF439176	94.72	0.00E+00	Funneliformis sp.	Genus	0.000	0.000	0.000	0.000	0.002
OTU_51	409	Claroideoglomus etunicatum	AF145749	92.18	3.63E-166	Claroideoglomus sp.	Genus	0.020	<0.001	<0.001	0.000	0.000
OTU_54	402	Diversispora eburnea	AM713416_R	88.89	3.70E-141	Diversisporaceae	Family	0.000	0.000	0.000	0.000	0.003
OTU_56	407	Claroideoglomus claroideum	FR750058	97.80	0.00E+00	Claroideoglomus claroideum	Species	0.009	<0.001	<0.001	0.000	0.000
OTU_57	418	Funneliformis sp.	FM876813_R	98.56	0.00E+00	Funneliformis sp.	Genus	<0.001	0.012	0.023	0.000	0.000
OTU_58	415	Funneliformis constrictus	JF439167	99.52	0.00E+00	Funneliformis constrictus	Species	<0.001	0.000	0.004	0.000	0.002
OTU_59	412	Rhizophagus irregularis	FM992381_R	98.54	0.00E+00	Rhizophagus irregularis	Species	0.002	0.002	0.000	0.000	0.000
OTU_60	404	Diversispora eburnea	AM713416_R	93.69	4.58E-175	Diversispora sp.	Genus	0.003	<0.001	0.000	0.000	0.000
OTU_62	413	Rhizophagus irregularis	FM992381_R	96.61	0.00E+00	Rhizophagus sp.	Genus	<0.001	0.009	0.000	0.000	0.000
OTU_63	373	Archaeospora sp.	FR750038	92.84	1.56E-154	Archaeospora sp.	Genus	0.000	0.000	0.003	0.000	<0.001
OTU_64	409	Claroideoglomus sp.	FR750074	90.31	7.98E-153	Claroideoglomus sp.	Genus	<0.001	0.011	0.000	0.000	<0.001
OTU_65	413	Rhizophagus irregularis	FR750081_R	99.52	0.00E+00	Rhizophagus irregularis	Species	0.005	<0.001	0.000	0.000	0.000
OTU_66	410	Diversispora eburnea	AM713413_R	95.37	0.00E+00	Diversispora sp.	Genus	0.000	<0.001	0.003	0.000	<0.001
OTU_67	368	Archaeospora schenckii	FR750023	80.42	1.72E-74	Archaeosporaceae	Family	0.000	0.003	0.005	0.000	0.000
OTU_68	409	Claroideoglomus luteum	FM876810_R	91.20	1.70E-159	Claroideoglomus sp.	Genus	<0.001	0.008	0.000	0.000	0.000
OTU_70	412	Rhizophagus irregularis	FR750198	99.51	0.00E+00	Rhizophagus irregularis	Species	0.002	0.003	0.000	0.000	0.000

"*"Subsoil indicators, "**" Topsoil indicators

OTU	Length in bp	Best Hit	HitName	% of identity	eValue	Assigned Taxon	Taxonomic Resolution	Phase 1	Phase 2	Phase 3	Subsoil	Topsoil
OTU_71	409	Claroideoglomus claroideum	FJ461815	97.93	0.00E+00	Claroideoglomus claroideum	Species	0.001	<0.001	0.000	0.000	0.000
OTU_72	413	Funneliformis constrictus	JF439167	88.10	6.37E-139	Glomeraceae	Family	0.002	<0.001	0.000	0.000	<0.001
OTU_74	411	Funneliformis constrictus	JF439180	90.41	1.33E-155	Funneliformis sp.	Genus	<0.001	0.006	0.000	0.000	0.000
OTU_75	412	Rhizophagus sp.	FR750085	94.59	0.00E+00	Rhizophagus sp.	Genus	0.003	0.000	0.000	0.000	0.000
OTU_77	414	Rhizophagus sp.	FR750090	99.76	0.00E+00	Rhizophagus sp.	Genus	0.009	<0.001	0.000	0.000	0.000
OTU_79	414	Funneliformis mosseae	FR750024	94.22	0.00E+00	Funneliformis sp.	Genus	0.005	<0.001	0.000	<0.001	0.001
OTU_81	416	Funneliformis constrictus	JF439167	88.84	1.77E-144	Glomeraceae	Family	0.000	<0.001	0.000	0.000	0.000
OTU_82	409	Claroideoglomus sp.	FR750076	89.32	3.74E-146	Claroideoglomeraceae	Family	0.000	0.000	0.001	0.000	0.000
OTU_83	349	Ambispora gerdemannii	JF439210	86.87	4.31E-110	Ambisporaceae	Family	0.000	<0.001	<0.001	0.000	<0.001
OTU_84	359	Paraglomus laccatum	FJ461809	94.05	4.23E-145	Paraglomus sp.	Genus	0.000	0.000	0.001	0.000	0.000
OTU_92	415	Funneliformis caledonius	FN547495_R	96.40	0.00E+00	Funneliformis sp.	Genus	<0.001	<0.001	0.000	0.000	0.000
OTU_93	418	Funneliformis constrictus	JF439180	89.79	2.94E-152	Glomeraceae	Family	0.000	0.001	0.000	0.000	0.000
OTU_95	417	Claroideoglomus etunicatum	HM485734	87.80	1.08E-136	Claroideoglomeraceae	Family	0.000	<0.001	0.000	0.000	0.000
OTU_96**	408	Diversispora sp.	FR686952	94.13	5.94E-179	Diversispora sp.	Genus	0.000	<0.001	0.002	0.000	0.001
OTU_98	417	Funneliformis constrictus	JF439180	92.82	6.12E-174	Funneliformis sp.	Genus	0.000	0.000	0.001	0.000	0.000
OTU_99	410	Funneliformis constrictus	JF439176	90.87	7.94E-158	Funneliformis sp.	Genus	0.000	0.000	0.008	0.000	0.000
OTU_104	414	Funneliformis constrictus	JF439180	93.30	3.63E-176	Funneliformis sp.	Genus	0.000	<0.001	0.000	0.000	0.000
OTU_107	419	Funneliformis caledonius	FN547496_R	95.70	0.00E+00	Funneliformis sp.	Genus	0.000	<0.001	0.000	0.000	0.000
OTU_108	439	Diversispora sp.	JF439149	88.84	4.03E-146	Diversisporaceae	Family	0.000	0.000	0.000	0.000	<0.001
OTU_110	412	Rhizophagus irregularis	FM992381_R	96.12	0.00E+00	Rhizophagus sp.	Genus	<0.001	<0.001	0.000	0.000	0.000
OTU_113	410	Claroideoglomus sp.	FR750076	90.00	3.72E-151	Claroideoglomus sp.	Genus	0.000	0.000	0.000	0.000	<0.001
OTU_114	372	Archaeospora sp.	FR750037	96.26	3.25E-176	Archaeospora sp.	Genus	0.000	<0.001	<0.001	0.000	0.000
OTU_120	406	Funneliformis mosseae	FN547491_R	96.63	0.00E+00	Funneliformis sp.	Genus	0.011	<0.001	<0.001	0.000	0.000
OTU_122	368	Archaeospora schenckii	FR750023	92.51	3.33E-151	Archaeospora sp.	Genus	0.000	0.003	0.000	0.000	0.000
OTU_125	413	Rhizophagus irregularis	FR750189	98.06	0.00E+00	Rhizophagus irregularis	Species	<0.001	<0.001	0.000	0.000	0.000
OTU_129	417	Funneliformis constrictus	JF439180	93.54	6.08E-179	Funneliformis sp.	Genus	0.000	<0.001	0.000	0.000	0.000
OTU_130	394	Claroideoglomus microaggregatum	FJ461843	80.62	1.11E-71	Claroideoglomeraceae	Family	0.000	0.000	0.000	0.015	0.000
OTU_133	416	Funneliformis caledonius	FN547496_R	96.18	0.00E+00	Funneliformis sp.	Genus	<0.001	0.002	0.002	<0.001	0.004
OTU_135	416	Funneliformis sp.	FM876813_R	95.42	0.00E+00	Funneliformis sp.	Genus	0.000	0.010	<0.001	0.000	0.000
OTU_137	418	Funneliformis constrictus	JF439180	95.93	0.00E+00	Funneliformis sp.	Genus	<0.001	<0.001	0.000	0.000	0.002
OTU_138	409	Diversispora sp.	FR686952	97.07	0.00E+00	Diversispora sp.	Genus	0.001	0.001	0.000	0.000	0.000
OTU_143	415	Funneliformis caledonius	FN547495_R	96.40	0.00E+00	Funneliformis sp.	Genus	<0.001	0.000	0.000	0.000	0.000
OTU_149	404	Glomus macrocarpum	FR750526_R	95.07	0.00E+00	Glomus sp.	Genus	0.000	0.000	0.006	0.009	0.002
OTU_152	409	Claroideoglomus etunicatum	HM485693	88.86	8.09E-143	Claroideoglomeraceae	Family	0.004	0.024	0.017	<0.001	0.010
OTU_160	418	Funneliformis constrictus	JF439180	96.65	0.00E+00	Funneliformis sp.	Genus	0.000	0.000	0.001	0.000	0.016
OTU_164	418	Funneliformis constrictus	JF439180	94.99	0.00E+00	Funneliformis sp.	Genus	<0.001	<0.001	0.000	0.000	0.000
OTU_166	415	Funneliformis constrictus	JF439180	85.48	1.09E-121	Glomeraceae	Family	0.000	0.000	0.000	0.000	<0.001
OTU_167	418	Funneliformis constrictus	JF439180	94.99	0.00E+00	Funneliformis sp.	Genus	0.000	<0.001	0.000	<0.001	0.002
OTU_178	376	Scutellospora calospora	FJ461865	97.15	5.54E-169	Scutellospora calospora	Species	0.000	<0.001	<0.001	0.000	<0.001
OTU_182	410	Diversispora sp.	JF439137	95.38	0.00E+00	Diversispora sp.	Genus	0.000	0.002	<0.001	0.000	0.002
OTU_184	408	Diversispora sp.	JF439136	93.19	7.74E-173	Diversispora sp.	Genus	<0.001	<0.001	0.000	0.000	0.000
OTU_185	418	Funneliformis constrictus	JF439180	95.23	0.00E+00	Funneliformis sp.	Genus	<0.001	0.004	0.000	0.000	<0.001
OTU_188	414	Rhizophagus irregularis	FM865591	95.65	0.00E+00	Rhizophagus sp.	Genus	<0.001	0.000	0.000	0.000	0.000
OTU_190	415	Funneliformis mosseae	FR750033	97.35	0.00E+00	Funneliformis mosseae	Species	0.028	<0.001	0.000	0.000	<0.001
OTU_192	409	Claroideoglomus sp.	FR750077	89.49	8.04E-148	Claroideoglomeraceae	Family	0.002	0.000	0.000	0.000	0.000
OTU_195	408	Diversispora sp.	FR686952	94.39	1.28E-180	Diversispora sp.	Genus	0.004	0.008	<0.001	0.000	0.000
OTU_200	416	Funneliformis caledonius	FN547499_R	94.95	0.00E+00	Funneliformis sp.	Genus	0.000	0.000	<0.001	0.000	0.000
OTU_201	409	Claroideoglomus etunicatum	AF145749	90.78	3.69E-156	Claroideoglomus sp.	Genus	0.010	0.000	0.000	0.045	0.000
OTU_202	407	Diversispora celata	AM713419_R	97.07	0.00E+00	Diversispora celata	Species	0.001	0.001	0.000	<0.001	0.000
OTU_211	374	Scutellospora calospora	EU252109	96.84	4.38E-150	Scutellospora sp.	Genus	0.000	<0.001	0.000	0.000	0.000
OTU_215	409	Diversispora sp.	JF439137	94.88	0.00E+00	Diversispora sp.	Genus	<0.001	0.010	0.002	0.000	<0.001
OTU_217	409	Claroideoglomus luteum	FM876810_R	90.98	7.92E-158	Claroideoglomus sp.	Genus	0.001	0.002	0.000	<0.001	0.000

"*"Subsoil indicators, "**" Topsoil indicators

OTU	Length in bp	Best Hit	HitName	% of identity	eValue	Assigned Taxon	Taxonomic Resolution	Phase 1	Phase 2	Phase 3	Subsoil	Topsoil
OTU_223	406	Glomus macrocarpum	FR750526_R	93.61	1.65E-174	Glomus sp.	Genus	0.000	0.000	<0.001	<0.001	0.002
OTU_224**	410	Diversispora sp.	JF439137	94.89	0.00E+00	Diversispora sp.	Genus	0.000	<0.001	<0.001	0.000	0.004
OTU_225**	408	Diversispora sp.	JF439148	92.68	1.67E-169	Diversispora sp.	Genus	0.000	0.001	0.008	0.005	0.043
OTU_228	414	Rhizophagus sp.	FR750116	98.55	0.00E+00	Rhizophagus sp.	Genus	0.008	0.005	0.000	0.000	0.000
OTU_234	411	Rhizophagus irregularis	FM865591	93.22	1.68E-174	Rhizophagus sp.	Genus	0.000	0.002	0.000	0.000	0.000
OTU_237	416	Funneliformis sp.	FN547481_R	96.88	0.00E+00	Funneliformis sp.	Genus	0.000	<0.001	<0.001	0.000	0.000
OTU_239	411	Funneliformis constrictus	JF439176	89.93	1.04E-151	Glomeraceae	Family	0.000	0.000	0.002	0.000	0.000
OTU_242	414	Funneliformis constrictus	JF439167	93.29	1.31E-175	Funneliformis sp.	Genus	0.000	0.000	<0.001	0.000	0.000
OTU_246	418	Funneliformis constrictus	JF439180	95.47	0.00E+00	Funneliformis sp.	Genus	0.001	<0.001	0.000	0.000	<0.001
OTU_249	405	Claroideoglomus sp.	FM876805_R	83.25	8.48E-103	Claroideoglomeraceae	Family	0.000	0.000	0.000	<0.001	0.000
OTU_251	414	Funneliformis mosseae	FN547491_R	95.90	0.00E+00	Funneliformis sp.	Genus	<0.001	0.000	0.000	0.000	0.000
OTU_252	374	Scutellospora calospora	EU252109	95.45	3.29E-171	Scutellospora sp.	Genus	0.000	<0.001	0.006	<0.001	<0.001
OTU_258	413	Acaulospora lacunosa	FR750116	97.82	0.00E+00	Acaulospora lacunosa	Species	<0.001	0.000	0.000	0.000	0.000
OTU_260	409	Rhizophagus sp.	FR750076	90.27	7.98E-153	Rhizophagus sp.	Genus	0.010	0.000	0.000	0.013	0.000
OTU_264	415	Funneliformis caledonius	FN547496_R	95.19	0.00E+00	Funneliformis sp.	Genus	0.000	<0.001	0.000	0.000	0.000
OTU_268	415	Funneliformis caledonius	FN547499_R	97.36	0.00E+00	Funneliformis caledonius	Species	<0.001	<0.001	0.001	0.000	0.000
OTU_273	409	Claroideoglomus luteum	FM876810_R	92.18	3.63E-166	Claroideoglomus sp.	Genus	0.000	0.004	0.000	0.000	0.000
OTU_280	407	Claroideoglomus sp.	FR750074	96.33	0.00E+00	Claroideoglomus sp.	Genus	<0.001	0.000	0.000	0.000	0.000
OTU_282	417	Funneliformis caledonius	FN547499_R	95.44	0.00E+00	Funneliformis sp.	Genus	0.000	0.005	0.000	0.000	0.000
OTU_284	401	Diversispora eburnea	AM713415_R	89.08	2.85E-142	Diversisporaceae	Family	0.000	0.002	0.000	0.000	0.002
OTU_287	409	Claroideoglomus claroideum	AF235009	90.02	2.22E-153	Claroideoglomus sp.	Genus	0.005	0.006	0.000	<0.001	0.000
OTU_289	411	Funneliformis constrictus	JF439180	89.69	1.34E-150	Glomeraceae	Family	0.002	0.001	0.000	0.000	0.000
OTU_291	409	Diversispora eburnea	AM713416_R	94.89	0.00E+00	Diversispora sp.	Genus	<0.001	0.000	0.000	0.000	<0.001
OTU_292	409	Claroideoglomus claroideum	AF396785	97.55	0.00E+00	Claroideoglomus claroideum	Species	0.013	<0.001	<0.001	0.000	0.000
OTU_301	410	Diversispora sp.	FR686952	94.88	0.00E+00	Diversispora sp.	Genus	<0.001	0.003	<0.001	0.000	0.001
OTU_307	376	Scutellospora calospora	EU252109	94.95	1.99E-168	Scutellospora sp.	Genus	0.000	0.000	<0.001	0.000	0.000
OTU_314	407	Glomus macrocarpum	FR750526_R	96.81	0.00E+00	Glomus sp.	Genus	0.000	0.000	0.000	0.003	0.000
OTU_316**	409	Diversispora sp.	JF439148	94.15	1.66E-179	Diversispora sp.	Genus	0.002	0.004	0.002	0.005	0.049
OTU_317	414	Funneliformis constrictus	JF439180	94.50	0.00E+00	Funneliformis sp.	Genus	0.000	0.000	0.000	<0.001	0.000
OTU_321	408	Diversispora celata	AM713418_R	97.07	0.00E+00	Diversispora celata	Species	0.000	<0.001	0.000	0.000	<0.001
OTU_324	406	Diversispora sp.	JF439148	92.46	2.79E-167	Diversispora sp.	Genus	0.000	0.000	0.000	<0.001	0.000
OTU_325	413	Diversispora sp.	JF439137	92.51	6.10E-169	Diversispora sp.	Genus	0.000	0.000	0.000	0.000	<0.001
OTU_329	409	Claroideoglomus luteum	FM876812_R	89.29	3.74E-146	Claroideoglomeraceae	Family	0.000	<0.001	0.000	<0.001	0.000

“*”Subsoil indicators, “**” Topsoil indicators

Table S2. Pairwise Mann–Whitney tests

Bray Curtis Topsoil (**Fig. 3A**)

	Phase 1		**Phase 2**	
	W	p Value	W	p Value
Phase 2	10272	< 2E-16	"---"	"---"
Phase 3	6783	< 2E-11	909	< 2E-16

Jaccard Topsoil (**Fig. S3A**)

	Phase 1		**Phase 2**	
	W	p Value	W	p Value
Phase 2	10926	< 2E-16	"---"	"---"
Phase 3	11255	0.26	2461.5	< 2E-16

Bray Curtis Suboil (**Fig. 3B)**

	Phase 1		**Phase 2**	
	W	p Value	W	p Value
Phase 2	6621.5	0.25	"---"	"---"
Phase 3	6353.5	6.00E-13	3272	2.8E-15

Jaccard Suboil (**Fig. S3B**)

	Phase 1		**Phase 2**	
	W	p Value	W	p Value
Phase 2	6842.5	0.11	"---"	"---"
Phase 3	6742.5	2.20E-11	3272	6.10E-14

Phase 1: 1-3 years, n=135. **Phase** 2: 4-5 year, n=90. **Phase 3**: 10-52 years, n=180.