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Community ecology of arbuscular mycorrhizal fungi in the molecular era

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by

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Foreword

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

I. **Vályi, K.**, Rillig, M.C. & Hempel, S. (2015). Land-use intensity and host plant identity interactively shape communities of arbuscular mycorrhizal fungi in roots of grassland plants. *New Phytol.*, 205, 1577–1586. DOI: 10.1111/nph.13236

II. **Vályi, K.**, Rillig, M.C., Joana, Bergmann & Hempel, S. (201X). Host and environmental control in arbuscular mycorrhizal fungal communities and the impact on phylogenetic clustering. *To be submitted.*

III. **Vályi, K.**, Mardhiah, U., Rillig, M.C. & Hempel, S. (2016). Community assembly and coexistence in communities of arbuscular mycorrhizal fungi. *ISME J.,* **10**, 2341–2351. *DOI: 10.1038/ismej.2016.46*

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Chapter 1: General introduction

In the era of affordable, culture independent, high throughput molecular methods, investigations of cryptic organisms that live in complex habitats and defy conventional analyses are becoming attainable. One such rising field is the community ecology of root symbiotic arbuscular mycorrhizal (AM) fungi, one of the most widely distributed, ecologically and economically important fungal groups.

Arbuscular mycorrhizal fungi and their communities

A mycorrhiza is a symbiotic association between filamentous fungi and the roots of plants, in which the fungal partner delivers soil nutrients to the plant and, usually, receives carbohydrates in exchange (Smith & Read 2008). It is now generally accepted that mycorrhiza, and not simply roots are the primary organs through which the vast majority of land plants acquire nutrients. There is ample evidence that supports that the first plants on land did not have true roots, and were colonized by filamentous fungi, which formed symbiotic structures similar to extant AM fungi. With the help of these symbionts, these plants could access nutrients that were otherwise unavailable.

Arbuscular mycorrhizal fungi are the members of their own monophyletic clade, the Glomeromycota phylum (Schüβler *et al.* 2001). They form multispecies symbiosis with on average 75% of land plants species and crops in a wide range of biomes (Treseder & Cross 2006). An arbuscular mycorrhiza has three distinct compartments: the root itself, the fungal structures inside the root, and an extraradical hyphal network (mycelium) in the soil. The nutrient exchange between the plant and the fungi occurs inside the cortical root cells through distinctive symbiotic structures, called arbuscules, after which the group has been named. The symbiosis is obligate for the fungi, as they cannot complete their life cycle in the absence of a host plant. They completely rely on the host for carbon, but through their mycelium they forage for other nutrients (predominantly phosphate and nitrogen) in the soil, that they offer in exchange. This unique dual interaction with both the host and the soil determines their community ecology.

AM fungi can provide a range of services to the host, including improving its supply of water, nutrients (e.g. phosporous, nitrogen (van der Heijden *et al.* 2006)), and tolerance to drought (Augé 2001) and pathogens (Veresoglou & Rillig 2012). Thus they have important roles in the

maintenance of plant diversity (Klironomos *et al.* 2000; O'Connor *et al.* 2002) and ecosystem processes (Rillig 2004). In exchange, up to 20% of photosynthates can be allocated to the fungi (Jakobsen & Rosendahl 1990; Bago *et al.* 2000; Johnson *et al.* 2002).

AM fungi can colonize a taxonomically diverse range of plants, and plants that develop AM symbioses can be associated with AM fungi from different taxa. As there are more potential host plant species than described AM fungal morphologically defined species, the AM symbiosis has been considered nonspecific on the species level. However, there is a variation in the extent of colonization by different fungal species on different plant species, which can be termed as "ecological specificity" or preference. Specificity may occur at the level of ecological or functional groups: specialist AM fungal taxa tend to associate with habitat specialist plants, and generalists with generalists, both in forests (Öpik *et al.* 2009) and in grasslands (Chapter 2, Vályi *et al.* 2015). Plant species identity has an effect on the composition of root AM fungal communities in the field (Gollotte *et al.* 2004).

The mutual benefit in the AM symbiosis is dependent not only on the identity of the partners, but also on abiotic environmental factors and soil conditions (Walder & van der Heijden 2015), for example the availability of phosphorous and nitrate (reviewed by Johnson 2010). Observational studies have shown the adaptation of AM fungi to certain soil conditions, such as P levels, soil micronutrient levels, aridity, salinity, pH, toxic levels of metals, and temperature (Brundrett 1991). Another factor shown to influence AM fungal communities, especially diversity, is heavy anthropogenic disturbance (see details and references in chapter **4**). However, moderate land use is not necessarily detrimental for AM fungal communities (Verbruggen *et al.* 2010, chapter **2**), or shifts the community in a predictable manner (Lekberg *et al.* 2012).

Molecular methods in community ecology of arbuscular mycorrhizal fungi

As this thesis includes research that was carried out with extensive DNA sequencing, a brief introduction to the methods and the justification for their choice is given below.

In spite of the profound importance of AM fungi in maintaining plant diversity in different ecosystems, there has been a lack of information on community level processes affecting them. This knowledge gap has been caused by the difficulty of culturing (Helgason *et al.* 2002) and distinguishing AM fungi, especially in roots, on a sufficient taxonomic level (Merryweather & Fitter 1998). Traditionally the taxonomy of AM fungi has been based on spore morphology. However, spore abundance can be poorly correlated with AM fungal colonization and mycorrhiza

formation. Firstly, sporulation depends on environmental conditions, and fungi may be active in the roots even though they do not sporulate. Spores that based on morphological assessment belong to two different genera have been observed to be formed by the same hypha (Morton *et al.* 1997). Thus, the identification of active root-colonizing AM fungi is currently only possible by molecular methods (Redecker 2002).

A single AM fungus can be identified based on its DNA sequence with classical Sanger sequencing (Sanger *et al.* 1977), which yields one sequence read per run, and had been the dominating sequencing method for 30 years. But as AM fungi occur as multispecies communities in roots, the sequencing step has to be preceded by cloning, to enable different DNA molecules to be sequenced separately from each other, making the process extremely time consuming, expensive and limited in the detection of infrequent organisms. In opposite to this, high-throughput sequencing methods are massively parallelized, avoid the cloning step and create millions of sequences in one run. Therefore, these methods (also termed "next-generation sequencing" methods) allow a more thorough characterization of AM fungal communities in a large number of samples. The different samples can be pooled and are identified by short DNA tags that are ligated to the target fragments in the preparatory steps

At the time of the planning of this research, 454 pyrosequencing (Margulies *et al.* 2005), which was used in **chapters 2** and **3**, already reached a read length of 450 base pairs, which made it the first high-throughput method suitable for the needs of AM fungal sequencing. Even though there had been generally accepted pipelines for the molecular and bioinformatics work for the pyrosequencing analysis of bacterial communities (Schloss *et al.* 2009), a universally accepted pipeline did not (and still does not really) exist for fungi, especially not for AM fungi.

Several primers are used to amplify AM fungi, mostly targeting the small subunit (SSU), the internal transcribed spaces (ITS) or the large subunit of the ribosomal DNA (rDNA). The SSU is one of the most widely sequenced region because it is the focal region of the first curated AM fungal sequence database (MaarjAM, Öpik *et al.* 2010), which makes matching AM fungal OTU-s between different studies possible by describing "virtual taxa" based on phylogenetic analysis of collected and submitted sequences. The suitability of the SSU region is further supported by its level of intra- versus interspecific nucleotide variation (Thiéry *et al.* 2016).

The Biodiversity Exploratories project

The Biodiversity Exploratories are three large-scale and long-term research sites set up in 2006 to understand the role of land use and management for biodiversity of different taxa (Fischer *et al.* 2010). The three Exploratories are located in North, Central and South Germany; the Schorfheide-Chorin Biosphere Reserve in the state of Brandenburg, the Hainich National Park and its surroundings in the state of Thuringia and Schwäbische Alb Biosphere Reserve in the state of Baden-Württemberg. In each Exploratory 50 forest and 50 grassland experimental plots were selected to represent a gradient of land use intensity ranging from near-natural, protected sites to intensively used ecosystems. On these plots (and further, in total 1000 study points) the number and abundance of plant species as well as land use types and intensity were recorded. A standardized land use intensity index combining effects of mowing, grazing and fertilization is calculated for each plot. The project provides central data management through the Biodiversity Exploratories Information System (BExIS).

Introduction to chapters and aims

The thesis in general is focusing on the different possibilities and knowledge gaps in the community ecology research of AM fungi using state-of-the-art molecular methods. In the data chapters we harnessed these possibilities in order to investigate intraradical AM fungal communities that actively interact with their hosts in the field using an unprecedented number of samples which had been made possible by the high throughput provided by pyrosequencing.

Chapter 2 presents the effects of host plant identity and land use intensity and their interaction on intraradical AM fungal communities in temperate grasslands. The aspects that we focused on were AM fungal community composition and structure (OTU richness, dominance structure, nestedness, indicator species for different plants and land use categories) in the Hainich Exploratory.

After we have shown that host plant identity has an effect on the AM fungal communities, we wanted to examine whether host plant traits and/or phylogeny are responsible for the host influence on AM fungal communities. To allow for a more in-depth analysis, **in chapter 3** we extended the number of sampling sites and included all three Exploratories (sampling regions). Root, leaf and ecological traits as well as phylogenetic distance between the host plants, as well as land use intensity of the Biodiversity Exploratories grassland sites were used as explanatory variables. In this chapter we carried out the analyses in the theoretical framework of community

phylogenetics (Webb *et al.* 2002), which explains phylogenetic patterns as the outcomes of ecological processes (e.g. environmental filtering and competitive exclusion), and therefore we focused on the effects on AM fungal community phylogenetic structure in the roots of grassland plants.

C**hapter 4** focuses on the community assembly and coexistence of AM fungi by reviewing recent developments and evidence in the field accumulated by using molecular methods. In addition to highlighting problems and knowledge gaps, the adaption of a hierarchical scale-dependent community system is proposed to resolve the idiosyncratic response of AM fungal communities to abiotic and biotic variables.

The **general discussion** (Chapter 5) presents the results of the previous chapters in the framework of AM fungal and in general symbiont ecology, and provide outlook for future potential research questions and applications.

Finally the thesis is summarized in **chapters 6 and 7** in English and German.

Chapter 2: Land-use intensity and host plant identity interactively shape communities of arbuscular mycorrhizal fungi in roots of grassland plants

Summary

- We studied the effect of host plant identity and land-use intensity (LUI) on arbuscular mycorrhizal fungi (AMF, Glomeromycota) communities in roots of grassland plants. These are relevant factors for intraradical AMF communities in temperate grasslands, which are habitats where AMF are present in high abundance and diversity. In order to focus on fungi that directly interact with the plant at the time, we investigated root-colonizing communities.
- Our study sites represent an LUI gradient with different combinations of grazing, mowing, and fertilization. We used massively parallel multitag pyrosequencing to investigate AMF communities in a large number of root samples, while being able to track the identity of the host.
- We showed that host plants significantly differed in AMF community composition, while land use modified this effect in a plant species-specific manner. Communities in medium and low land-use sites were subsets of high land-use communities, suggesting a differential effect of land use on the dispersal of AMF species with different abundances and competitive abilities.
- We demonstrate that in these grasslands, there is a small group of highly abundant, generalist fungi which represent the dominating species in the AMF community.

Introduction

According to modern coexistence theory, members of the regional species pool have to pass through environmental and biotic filters to colonize a particular habitat (HilleRisLambers *et al.* 2012). In the case of symbionts, an important component of the biotic filter is the presence and identity of the suitable partner with which they interact (e.g. Schöttner *et al.* 2013). Arbuscular mycorrhizal fungi (AMF) are ubiquitous, obligate symbiotic fungi from the phylum Glomeromycota (Schüβler *et al.* 2001) that form a multispecies (i.e. > one fungal species in one host plant) symbiosis with the majority of land plants (Smith & Read 2008). In the arbuscular mycorrhiza, the fungal partner relies on carbon (C) received from the root of the plant in exchange for other nutrients, mainly phosphorus (P) and nitrogen (N), for which mycelia forage in the soil. Because of this dual dependency on the plant and soil, their intraradical communities are influenced by both host traits and abiotic habitat characteristics.

Early AMF researchers concluded that there is very low species-level specificity (Smith & Read 2008) between the two partners, as there are much more potential host species than AMF species. However, this fact does not rule out effects of plant identity on the structure and composition of AMF communities in their roots. In addition, partner specificity might occur on a level of ecological groups (e.g. habitat generalists vs specialists, Öpik *et al.* 2009) or ecosystems (Veresoglou & Rillig 2014) instead of the level of species. As different AMF taxa have a differential effect on the growth of plant species coexisting in the same habitat (Sanders 2002), the study of AMF communities in planta is of substantial importance in grasslands.

With the emergence of molecular methods, evidence started to accumulate as to the effect of plant identity on AMF communities. Gollotte *et al.* (2004) were able to show that roots of *Agrostis capillaris* and *Lolium perenne* harbor different AMF communities, and Vandenkoornhuyse *et al.* (2003) showed that even coexisting grass species can have significantly different AMF communities. Unfortunately, the molecular methods applied in these studies one decade ago are strongly constrained in sample throughput and sequencing depth per sample and therefore also in the power of subsequent statistical analyses. Next-generation sequencing methods, such as pyrosequencing, offer a possible solution to these problems, as its sequencing depth allows a more thorough characterization of AM fungal communities in a large number of samples (e.g. Camenzind *et al.* 2014; Horn *et al.* 2014).

Plants can control the initiation and degree of AM colonization by signaling compounds (Schmitz & Harrison 2014), and the strength of this control was also shown to be dependent on plant identity; that is, plant species differ in the degree to which they adjust allocation to AMF in P-rich conditions (Grman 2012). Some of these signaling compounds, for example certain flavonoids, can even have a species-specific effect on the AM fungal partner (Scervino *et al.* 2005).

Another important group of factors influencing communities are habitat characteristics. Several components of land use have an impact on habitat characteristics, with potential effects on plants and fungi (Douds & Millner 1999). In this paper we investigate mowing, grazing, and fertilization; these are land-use elements in grasslands for which effects on AMF communities have been shown to be inconsistent and dependent of many other environmental parameters. Šmilauer (2001) found only a limited effect of mowing and no effect of fertilization on AMF morphotype diversity and composition in the roots of three grassland species. Herbivory and grazing are usually thought to reduce mycorrhizal colonization, but to a biologically meaningful degree this is true only in a limited subset of systems (Barto & Rillig 2010). Defoliation can result in a shift of mycorrhizal community composition, because while some species are limited by the reduced carbohydrate input from the plant, others seem to thrive. Grazing can also reduce species richness of AMF (Bethlenfalvay & Dakessian 1984). Fertilization can change the richness and composition of plant (Wilson & Tilman 1991) and fungal communities, but for AMF these results seem to be inconsistent and dependent on context and the choice of fertilizer or N : P ratio of the substrate (Rillig *et al.* 2002; Johnson 2010).

The study of the effect of plant identity, land-use characteristics, and their interaction in managed grasslands allows the placement of the patterns mentioned earlier into an ecologically relevant context, as grasslands are mostly under agricultural use to a variable degree in central Europe(Henwood 1998). However, large-scale studies addressing these questions usually focus on agricultural systems that are typically poorer in AMF richness (Helgason *et al.* 1998) and represent the most extreme end of land use, thereby leaving a gap of knowledge in AMF ecology research for grasslands, where AMF are most abundant (Treseder & Cross 2006).

In this study we aimed to investigate the effect of host plant identity and land-use intensity (LUI), and their interaction on AMF community structure and composition in roots of grassland plants. Our study plots were selected to represent an LUI gradient with different combinations of grazing, mowing, and fertilization, which represent actual real-life management practices. AMF

communities in soil and roots represent different parts of the AMF community, with a significantly greater diversity in the former (Hempel *et al.* 2007), which probably reflects the fact that some AM fungi, although present in the soil, are not at that time of the year active in the roots of the sampled plant (Vandenkoornhuyse *et al.* 2007) and can represent dormant or even dead organisms. Because our focus is on AMF that interact with the plant directly, we investigated communities in the root. High-throughput pyrosequencing data from a total of 250 samples allowed us to address the interactive effect of host plants and a wide range of land-use scenarios more generally.

Materials and Methods

Sampling region

The Hainich-Dün is a study region of c. 1300 km2 in the German Biodiversity Exploratories project (Fischer *et al.* 2010) located in the hilly lands of central Germany, and it consists of the Hainich National park and its surroundings (Appendix A, Fig. S1a). According to the framework of the project, we used the 50 grassland plots (Appendix A, Fig. S1b), which represent a land-use gradient typical of Germany, from grasslands with low input to fertilized, grazed, and mown meadows and pastures. The Hainich's dominant geological substrate is loess over Triassic limestone, and the most dominant soil groups on our sites are Cambisols and Stagnosols. Grazing animals are cattle and sheep.

Sample preparation

As part of a larger sampling campaign of several projects within the German Biodiversity Exploratories, in each of the 50 grassland plots (50 × 50 m size) in the Hainich-Dün region of Germany, 14 soil cores along two orthogonal transects were taken and the top 10 cm of all cores per plot were pooled. The center points of the plots are permanently marked by a subterranean metal tie (Fischer *et al.* 2010) and their exact position was recorded in 2008 with a Trimble precision GPS. At the soil sampling, we found the metal tie with a metal detector and set up the transects with the help of a compass and a measuring tape. The two transects were 18 m long and were sampled from north to south and from west to east at 3 m intervals. Sampling was done within 2 weeks in May of 2011.

Soil pH was measured in a 10 g subsample of sieved and air-dried soil with a WTW bench pH meter (Weilheim, Germany) (Schöning *et al.* 2012). Five randomly selected healthy-looking root

fragments with a length of 4 cm and diameter < 1 mm per plot were subsampled from these pooled soil samples and handled separately in all downstream processes and treated as within-plot replicates (see later). This random sampling strategy was chosen in order to obtain root fragments unbiased by the above-ground plant status (dormant, vegetative growth, flowering, etc.) within a predefined land-use background. More targeted approaches using a predefined set of plant species usually require the species to be flowering for identification, which inevitably limits the generalizability of the results obtained. Roots were washed thoroughly with distilled water, freeze-dried and pulverized using metallic beads. Afterwards, total DNA from the powder was extracted with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). DNA was amplified by a nested PCR approach, first with GlomWT0/Glomer1536, then with NS31/AM1a + b primer pairs (Morris *et al.* 2013), using 25 different Multiplex Identifier Adaptors (MID-s; Roche Diagnostics) in order to label sequences belonging to different samples. AM1a and AM1b are modified AM1 primers designed to capture AMF families not captured by the original AM1, which excludes some taxa (Daniell *et al.* 2001). The primers target the small subunit (SSU) of the nuclear-encoded ribosomal DNA (rDNA). Identification of Glomeromycota based on SSU sequences is also becoming more and more popular, because of the existence of a curated AMF sequence database (MaarjAM, Öpik *et al.* 2010), which enables fast operational taxonomic unit (OTU) classification and further ecological analysis by making comparisons with other studies possible. A detailed protocol of the PCR conditions is given in Methods S1.

Equal amounts of DNA were mixed into pools of 25 samples based on DNA content quantification by the image-analysis software GelQuant.NET (v. 1.8.2, www.BiochemLabSolutions.com). Pools were purified via agarose gel extraction with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and were 454 pyrosequenced on a Roche GS FLX+ system with titanium chemistry at the Göttingen Genomics Laboratory at the Georg-August University of Göttingen.

Bioinformatic analysis

Processing of flowgram data from pyrosequencing was done by Mothur (Schloss *et al.* 2009), using sffinfo, trim.flows (reads with < 300 flows were discarded), shhh.flows, and trim.seqs (reads shorter than 200 bp discarded) commands to unpack, screen, denoise and trim the sequences. Sequences with more than one base difference in the barcode or two bases in the forward primer were discarded. Glomeromycota sequences were extracted by comparing the sequences against the nucleotide collection of NCBI with MEGAN (Huson *et al.* 2011).

To define sequence clusters, we used a closed reference OTU picking approach (sensu Bik *et al.* 2012), because the identity of the OTUs is relevant to many of the analyses, using the MaarjAM Glomeromycota database's virtual taxa (VT), which correspond to a sequence similarity ≥ 97%, (Öpik *et al.* 2010). To categorize our reads to VT, Glomeromycota sequences were BLAST-ed against the VT-type sequences of the MaarjAM Glomeromycota database using FungalITSPipeline (Nilsson *et al.* 2009), using MAFFT as an aligner and a slightly modified blastall code to include DUST filtering. Only those OTUs in which the best BLAST result had at least 97% 'coverage percent' (maximum identity) similarity with a VT-type sequence in the MaarjAM database were included in further analyses. FungalITSPipeline was used only to BLAST effectively; the internal transcribed spacer (ITS) specific functions were not used. As the OTUs defined by this method are the same as the virtual taxa of the MaarjAM database, we use the terms OTU and VT interchangeably.

Multitag pyrosequencing might introduce bias to community composition as a result of the preferential amplification of certain barcoded primers during PCR (Berry *et al.* 2011). As such unequal representation of samples will lead to a differential sampling intensity, we resampled our dataset to an equal number of reads per sample. As the amplicons in our study were short and about the same length, read numbers in the resampled dataset could be used as a proxy for relative abundance of the OTUs (Ihrmark *et al.* 2012).

The OTU table was randomly resampled to 250 Glomeromycota sequences per sample without replacement by the function rrarefy in the vegan package (version 2.0-10, (Oksanen *et al.* 2013)) of R 3.0.2 (R Core Team 2013). With this method, the variance of rarefied communities is related to rarefaction proportion rather than to the size of the sample. Samples consisting of < 250 sequences were removed, and downstream data analysis was performed on a data table consisting of 190 root communities.

Host plant identity and land use

The vegetation of the plots was assessed by cover estimates of a 4 × 4 m core area by visual estimation (Schmitt *et al.* 2011). Plant identity of the root samples was assigned based on the sequence of the trnL-intron, which is suitable for plant species identification, as it is widely represented in the GenBank database and is quite variable between plant taxa (Borsch & Quandt 2009). In combination with coverage data from vegetation relevées, this method allowed us to identify plants with high confidence. Plant DNA from the root extract was amplified with trnL c/d

primers (Taberlet *et al.* 1991), cleaned, and Sanger-sequenced. Data were BLASTed against NCBI nucleotide collection and best hits based on maximum scores were matched with the vegetation of the plot. Sequences with more equally good hits that were all present in the field plot were grouped into plant identity units and analyzed as one unit. Equally good hits, where the trnL marker did not allow species-scale resolution, were only found in the case of grasses known to hybridize with each other, for example in the case of the *Lolium*/*Festuca* complex.

For a detailed analysis of the interaction of LUI and plant identity, we used the three most common plant identity units (*Arrhenatherum elatius*, *Festuca pratensis*/*Lolium perenne*/*Lolium multiflorum* and *Poa pratensis*), all from the Poaceae family. These plants are all competitors according to their strategy type using the system of Grime (Grime 2001; based on entries in the BiolFlor database, Klotz *et al.* 2002), and common in grasslands. They differ in the time of flowering, as *P. pratensis* and *L. perenne* start flowering in May, while the other plants flower later. *Arrhenatherum elatius* is taller and more sensitive to trampling and grazing than the other plants, and it prefers less intensively used grasslands as habitats.

Land-use intensity was measured by the LUI index (Blüthgen *et al.* 2012), which is an additive index summarizing the standardized intensity of fertilization (organic or inorganic N-fertilizer applied by farmers measured in kg N ha–1), frequency of mowing, and the intensity of livestock grazing (reflected by density of livestock) on the grassland sites of the Biodiversity Exploratories project, with larger values indicating higher intensity of land use. It was calculated for the years between 2006 and 2010 and then averaged (Klaus & Blüthgen 2013). The LUI provides a single unidirectional intensity gradient in our study region, which accounts for quantitative variation in the intensity of multiple management types with regimes that vary both spatially and temporally in real-life, heterogeneous landscapes. The grassland plots in our study were either mown for hay or silage production (meadows), or grazed by livestock (pastures), or both (mown pastures), and were either unfertilized or fertilized to varying degree. Because higher land use also requires higher nutrient inputs, all land-use components are correlated to a varying degree (see Blüthgen *et al.* 2012),. In the majority of plots, the type and/or intensity of the land-use components changed over the years, which would hamper categorical analyses or analyses using the components separately, just as the fact that the land-use components are often substituted by each other (nongrazed plots are usually heavily mown and fertilized). In contrast to the individual land-use components, the compound LUI index was shown to be significantly related to land-use response

variables in the Hainich-Dün study region, for example Ellenberg's N indicator (Ellenberg 1974), plant P, and soil C : N ratio by Blüthgen *et al.* (2012). Land-use data were obtained from interviews with farmers and land owners. The LUI index is the sum of standardized fertilization, standardized mowing, and standardized grazing, square-root-transformed for more even distribution and to reduce the effect of outliers in regressions. Standardization is carried out by dividing each individual land-use component by its mean in the respective study region.

For the nonmetric multidimensional scaling (NMDS) and the nestedness analyses (see later), LUI classes were created by dividing the distribution of the LUI values into three equal parts: low (lower third of the distribution; LUI = 0.66–1.33; 17 plots), medium (medium third of the distribution; LUI = 1.37–1.94; 16 plots) and high (highest third of the distribution; LUI = 1.99–3.08; 17 plots) land use.

Statistical analyses

Spatial structure of the AMF communities was characterized by redundancy analysis (RDA) and principal coordinates of neighborhood matrix (PCNM) in the spacemakeR package (Dray 2013). The best spatial model was selected by Akaike information criterion calculated by the ortho.AIC function in spacemakeR. To test the effect of LUI and plant identity on the AMF community composition, the community dataset was subsequently analyzed by permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) by the adonis function in vegan using 9999 permutations, including spatial descriptors, plant identity and LUI, and variance partitioning (Legendre & Legendre 2012). To confirm the output, we applied the varpart function in vegan using the same model structure and subsequent significance testing by RDA and ANOVA. To assess the potential interaction between LUI and plant identity on community composition, we also created a more balanced data set using the three most common plant identity units (*A. elatius*, *F. pratensis*/*L. perenne*/*L. multiflorum* and *P. pratensis*), and performed PERMANOVA. We visualized the communities of the three most common plant identity units by NMDS with the metaMDS function in vegan, which uses several (at most 20) random starts to reach a stable ordination solution. We plotted ellipses representing communities belonging to the different LUI classes using the ordiellipse function in vegan using the standard deviations of weighted averages.

To explore the effect of LUI on the rarefied OTU richness, we first fitted a smoothed curve through the scatterplot with local polynomial regression fitting by the scatter.smooth function in the stats package of R (R Core Team 2013). Then, based on the smoothed curve, we computed orthogonal

polynomials of second degree with the poly function and fitted the resulting model with a linear regression.

To test whether the AMF communities that are poor in distinct OTUs are a subset of communities of OTU-rich root samples, nestedness analysis was conducted on the community dataset using BINMATNEST (Rodriguez-Girones & Santamaria 2006), as implemented in the nestedness function in the bipartite package of R (Dormann *et al.* 2008). This function calculates matrix temperature, a commonly used nestedness measure developed by Atmar & Patterson (1993). To estimate the probability that chance alone is responsible for the nested pattern, this implementation can generate random matrices based on three different types of null model, and reports the number of cases where the matrix temperature was lower than that of the analyzed matrix. We generated 100 matrices of each null model. The position of the root samples representing different LUI classes in the stacked minimum temperature matrix was compared by a Kruskal–Wallis rank sum test followed by Wilcoxon rank sum tests with continuity correction.

To test if any of the AMF virtual taxa were indicative of LUI classes or plant groups, we conducted a Dufrene-Legendre indicator species analysis (Dufrêne & Legendre 1997) implemented in the indval function of the labdsv package of R (Roberts 2013). The analysis calculates the indicator value based on fidelity and relative abundances. VT were considered indicative if the indicator value was ± 0.25, as suggested by Dufrêne & Legendre (1997).

Results

Pyrosequencing

Pyrosequencing was carried out in pools of 25 samples, which resulted in 1 301 668 filtered reads for the 250 samples altogether, ranging from 323 to 22 508 reads per sample. We have found 399 603 Glomeromycota reads ranging from 0 to 12 294 reads per sample. To keep 90% of the samples, we calculated the 10th percentile of read abundance per sample, which was 243.6. Then we resampled without replacement to the lowest read number, which was 250. We have found 74 VT in our resampled dataset, from the families Archaeosporaceae, Claroideoglomeraceae, Diversisporaceae, Glomeraceae and Paraglomeraceae, indicating a good coverage of the Glomeromycota. Available taxonomic information of the VT acquired from the MaarjAM database and information on morphologically described species included in some VT can be found in Appendix A, Table S1.

Community composition

Using PERMANOVA, we found that AMF communities significantly differed between host plants and along the LUI gradient, even after taking spatial autocorrelation into account (Table 2.1), as also confirmed by variance partitioning (Appendix A, Fig. S2). To address the importance of the interaction between LUI and plant identity, we investigated the composition of AMF communities in the three most common plant identity units (*A. elatius, F. pratensis/L. perenne/L. multiflorum,* and *P. pratensis*), which were well represented along the LUI gradient (Table 2.2). For those, the PERMANOVA confirmed that the host plant effect on AMF communities was significantly modified by the intensity of land use (P = 0.046). This finding is further illustrated in the NMDS plots (using land-use classes instead of the continuous land-use index), where Fig. 2.1 shows that in different plants, the AMF community shifts in a different direction as LUI increases, reflecting interaction between the two components, while Fig. 2.2 shows the differential host plant effects on AMF communities in the same LUI intensity class, which represents the same interaction from a different point of view. Both plots clearly indicate that in the three most abundant plant species, the increase in LUI does not affect AMF communities in the same way. The addition of other abiotic variables (pH, soil type) did not change the PERMANOVA model outcome regarding the significance and interaction of our focal factors, LUI, and plant identity (data not shown).

Table 2.1: Permutational multivariate analysis of variance of AMF community matrix in the roots of grassland plants along a land use intensity gradient using all 190 samples.

	df	**Sums of squares**	**Mean Squares**	**F**	**R^2**	**P**
Longitude	1	0.39	0.39	1.64	0.01	0.09
Latitude	**1**	**0.51**	**0.51**	**2.14**	**0.01**	**0.02**
Additional spatial vectors	**3**	**2.26**	**0.75**	**3.16**	**0.04**	**<0.001**
Plant identity	**21**	**7.42**	**0.35**	**1.48**	**0.15**	**<0.001**
Land use intensity	**1**	**1.20**	**1.19**	**5.01**	**0.02**	**<0.001**
Plant identity: land use intensity	13	3.05	0.23	0.98	0.06	0.54
Residuals	149	35.56	0.24		0.71	
Total	209	50.39			1.00	

Table 2.2: Permutational multivariate analysis of variance of AMF community matrix in the roots of the three most common plant identity units on grasslands along a land use intensity gradient.

	df	Sums of squares	Mean Squares	F	R^2	P
Longitude	1	0.35	0.35	1.41	0.02	0.16
Latitude	1	0.38	0.38	1.55	0.02	0.10
Additional spatial vectors	1	0.39	0.39	1.60	0.02	0.08
Plant identity	**2**	**0.92**	**0.46**	**1.89**	**0.04**	**0.01**
Land use intensity	**1**	**0.85**	**0.85**	**3.46**	**0.04**	**<0.001**
Plant identity: land use intensity	**2**	**0.75**	**0.38**	**1.54**	**0.03**	**0.046**
Residuals	77	18.82	0.24		0.84	
Total	85	22.46			1.00	

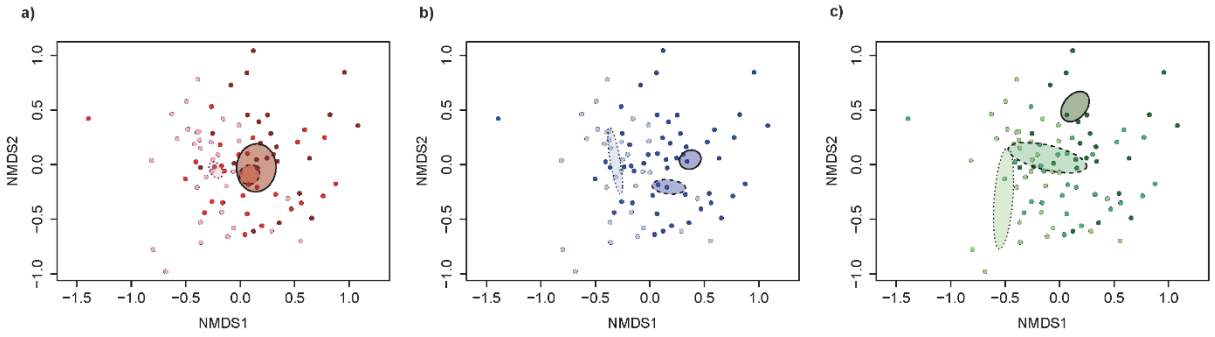

Figure *2.1: Interaction of host plant identity and land-use intensity on the arbuscular mycorrhizal fungi (AMF) communities in roots. Dots are sites from a nonmetric multidimensional scaling (NMDS) ordination of community data in the three most common plant taxa: (a)* Arrhenatherum elatius*; (b)* Festuca pratensis/Lolium perenne/L. multiflorum*; (c)* Poa pratensis*. Ellipses are dispersion ellipses using the standard error of the weighted average of NMDS scores. Land-use intensity is coded by the darkness of the dots and ellipses: a darker color depicts lower land-use intensity.*

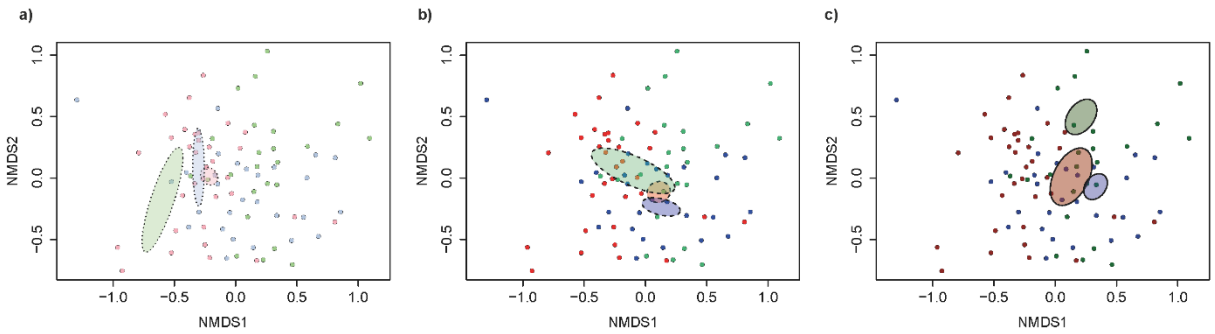

Figure 2.2: *Differences of host plant effect on arbuscular mycorrhizal fungi (AMF) communities in the same land-use intensity class: (a) low land-use intensity; (b) medium land-use intensity; (c) high land-use intensity. Dots are sites from a nonmetric multidimensional scaling (NMDS) ordination of community data in the three most common plant taxa:* Arrhenatherum elatius *(red),* Festuca pratensis/Lolium perenne/L. multiflorum *(blue),* Poa pratensis *(green).*

Community structure

OTU richness

Based on the appearance of the smoothed curve on the plot of rarified per sample OTU richness with rising LUI, we chose to fit a second-order polynomial regression model ($y = 10.27 - 3.24x + 1.48x^2$, multiple $R^2 = 0.08$, $P < 0.001$). The plot and the model (Fig. 2.3) indicate that LUI does not have an effect on OTU richness until it reaches LUI 1.5 (representing medium land use), from which the OTU richness per sample starts to rise. This shows that, contrary to expectations, higher land use does not reduce richness in root AMF communities.

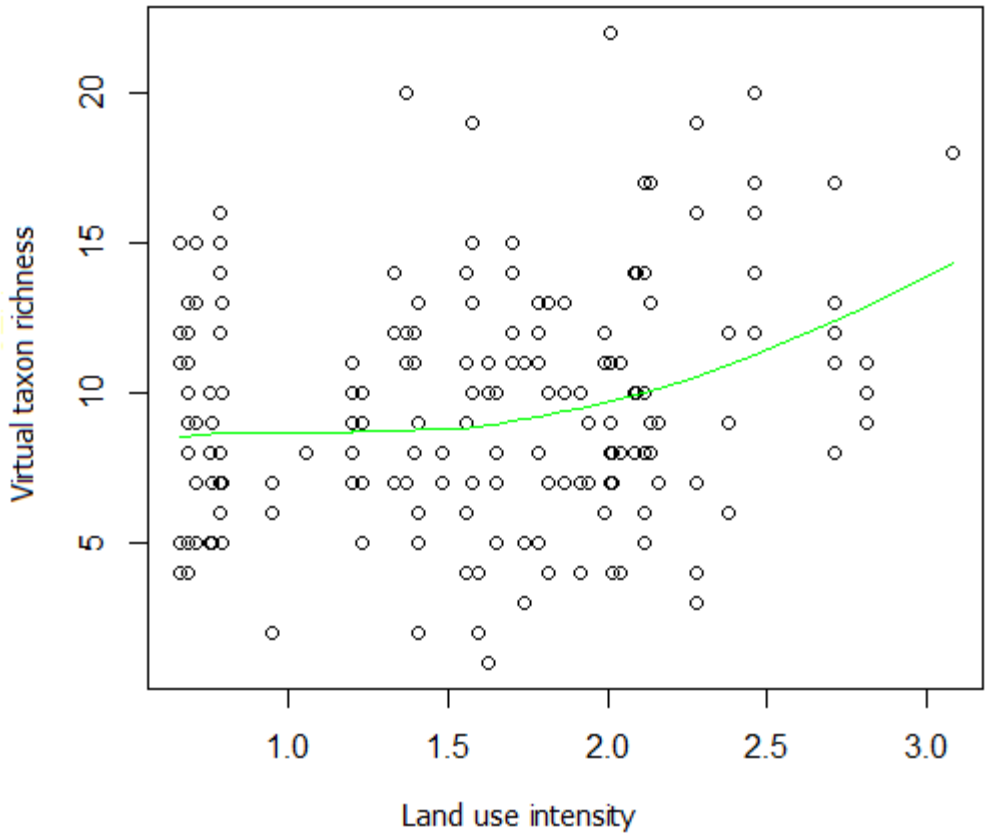

Figure 2.3: *The effect of land-use intensity on the operational taxonomic unit (OTU) richness of the arbuscular mycorrhizal fungi (AMF) community in plant roots. Second-order polynomial regression:* $y = 10.27 - 3.24x + 1.48x^2$. *Multiple* R^2*: 0.08. For coefficients, see Appendix A, Table S2.*

Dominance structure

The most abundant VT in the individual root samples were on average four times more abundant than the second most abundant ones. There was a set of 29 different VT which were most abundant within a single sample at least once. In 55 out of 190 root AMF communities, the most abundant taxon was Glomus VTX00113.

Nestedness

The distribution of AMF virtual taxa showed significant nestedness as indicated by a matrix temperature of 9.78, which was lower than the temperatures of all randomly generated null model

matrices (Appendix A, Fig. S3). The position of roots from plots with different LUI categories significantly differed from each other in the stacked minimum temperature matrix (Kruskal–Wallis rank sum test, $\chi^2 = 17.83$, df = 2, P-value < 0.001). More specifically, roots from high LUI were positioned significantly higher than those from medium LUI (Wilcoxon rank sum test, W = 2478, P = 0.003) and low LUI (W = 2981, P < 0.001), while the latter two were not significantly different. Consequently, medium and low land-use root communities can be considered nested within the high land-use communities, that is, medium and low land-use communities are subsets of high land-use communities.

Indicator species analysis

Four VT (*Glomus* VTX00072, *Glomus* VTX00153, *Glomus* VTX00163, and *Glomus* VTX00417) were importantly (indicator value > 0.25) and significantly (P < 0.05) indicative of low LUI in the whole dataset. Similarly, *Glomus* VTX00114 (containing the species *Rhizophagus irregularis* and *Rhizophagus intraradices*) and *Glomus* VTX00130 were indicators of high LUI (Table 2.3).

We assessed indicators for plant taxa in the dataset including the three most common plant identity units, as these had a good coverage of different LUI. *Claroideoglomus* VTX00056 and *Glomus* VTX00065 (containing the species *Funneliformis geosporum, Funneliformis fragilistratum, Funneliformis verruculosum* and *Funneliformis caledonium*) were associated as an indicator with the plant identity unit F. *pratensis*/*L. perenne*/*L. multiflorum*.

Table 2.3: Indicator value analysis of AMF VTX on different land use intensity classes and plant identities. Taxonomic information on the type sequences of the VTX can be found in the supporting information (Appendix A, Table S1).

OTU ID	LUI category	Indicator value	p
Glomus VTX00153	low	0.57	0.001
Glomus VTX00072	low	0.36	0.002
Glomus VTX00417	low	0.28	0.003
Glomus VTX00163	low	0.28	0.011
Glomus VTX00114	high	0.29	0.001

Glomus VTX00130	high	0.28	0.001

OTU ID	**Plant identity**	**Indicator value**	**p**
Glomus VTX00065	*Festuca pratensis/Lolium perenne/L. multiflorum*	0.35	0.001
Claroideoglomus VTX00056	*Festuca pratensis/Lolium perenne/L. multiflorum*	0.31	0.039

Discussion

In this study, we intended to systematically investigate the effect of host plant identity, LUI and their interactions on AMF community composition and structure in the roots sampled in grasslands based on an unprecedented number of individual samples. Rather than aiming at comprehensively disentangling all factors shaping AMF communities in roots, we chose these two factors because they are highly relevant in grasslands, as most grasslands are managed in central Europe and, in contrast to most agricultural fields, harbor several host plants. In temperate grasslands, AMF reach high abundance (Treseder & Cross 2006) and richness (Öpik *et al.* 2006), making them important ecosystems in which to study this association.

The results showed that in our study system intraradical AMF community composition was strongly shaped by host plant identity, even after taking spatial and land-use effects into account. This supports the hypothesis that AMF communities in roots are not random draws of the regional pool (Davison *et al.* 2011), but that host plant identity plays a role in defining its root community, probably reflecting host plant-specific traits shaping the community of their symbionts. Likewise, in alpine habitats, host identity explained an important proportion of rhizosphere fungal communities, especially for AMF species (Becklin *et al.* 2012).

Land-use intensity clearly modified the effect of host plant identity. Even though very intensive land use can be detrimental to AMF communities(Douds *et al.* 1995; Oehl *et al.* 2004), relatively moderate land use does not necessarily have an effect on AMF community composition and structure (Verbruggen *et al.* 2010). In agricultural fields, host plant species were found to be more important in determining the AM community than fertilization (measured as soil P) at low and

medium P, but at the highest P concentration, soil P overrode host identity in determining the communities (Gosling *et al.* 2013). From these earlier studies and our results on the effect of LUI on OTU richness (Fig. 2.3), we suggest that as LUI decreases, its effect is gradually overridden by other factors, such as host identity. As the land-use index applied in our study is correlated with plant and soil nutrient status (Blüthgen *et al.* 2012), plant species-specific responses to increased nutrient status towards their mycorrhizal symbionts, as observed in Grman (2012), might be the driving force, to some extent, behind the observed interactive effect of host plant and LUI.

We have shown that land-use identity and host identity have interacting effects and that a portion of the variance that they explain is shared (Figs 2.1, 2.2, Appendix A S2; Table 2.2). The plant species forming our subset of the most commonly sampled plant identity units, namely *A. elatius, F. pratensis*/*L. perenne*/*L. multiflorum,* and *P. pratensis*, are very similar plants from the same family (Poaceae) with competitive strategy type (Grime 2001). Even though there are some known differences in their above-ground traits (e.g., that *A. elatius* has a larger biomass and flowers earlier), much less is known about their below-ground traits, which might have been more useful for explaining why their root AMF communities differ. There is a difference in their tolerance to mowing, trampling, and grazing: *A. elatius* is sensitive to trampling and grazing (BiolFlor database, Klotz *et al.* 2002), moderately tolerant to mowing and prefers non intensively used grasslands, while *P. pratensis* is common on commercial grasslands and very tolerant to mowing, and quite tolerant to grazing and trampling. While *L. perenne* is quite tolerant to mowing, grazing, and tramping, *F. pratensis* is only moderately tolerant, so their hybrids may vary in tolerance. These differences of the host plants in tolerance to the disturbance-related aspects of land use likely explains a portion of the interaction effect: increasing LUI affects plants with lower tolerance differently from plants with higher tolerance, and our results show that they shift their root AMF communities accordingly (Fig. 2.1).

We did not include soil variables in our final model, as the two variables of soil pH and soil type did not change our results (data not shown) and also because these variables were not the focus of our study. Many other studies have shown the effects of soil parameters, such as pH (e.g. Dumbrell *et al.* 2010) and soil nutrient status (e.g. Camenzind *et al.* 2014), and we are aware that some of the unexplained variation in the PERMANOVA will be a result of these factors.

By using indicator species analysis, we have found that *Claroideoglomus* VTX00056 and *Glomus* VTX00065 were indicators of the plant identity unit *F. pratensis*/*L. perenne*/*L. multiflorum*. Both

VT are reported from several continents and biomes, including forests (e.g. (Moora *et al.* 2011; Öpik *et al.* 2013) and grasslands (e.g. (Santos-González *et al.* 2007; Liu *et al.* 2012) in the MaarjAM database. Their most common host in our study belongs to the *Lolium*/*Festuca* complex in which both interspecific and intergeneric hybrids can be produced and which is common in many farming systems (Stammers *et al.* 1995). The species are common both in our dataset along the whole LUI gradient and overall in Germany (see http://www.floraweb.de), occurring in a variety of grasslands and field margins (see the BiolFlor database; Klotz *et al.* 2002). Such an association of a generalist plant with a generalist fungus was also found in forests (Öpik *et al.* 2009; Davison *et al.* 2011) and supports the hypothesis that the specificity between plant and fungus can occur on the level of ecological groups (Öpik *et al.* 2009).

Regarding the structure of root AMF communities, we have consistently found heavy overdominance of the most abundant OTU. This community pattern is a typical feature of AMF communities, as shown in a meta-analysis of different habitats (Dumbrell *et al.* 2010a). These authors suggest that this pattern can be explained by strong biotic interactions between nondominant species (most likely competition for plant C), minimizing abundance differences and increasing the disproportionate advantage of the dominant fungus with an exceptionally high recruitment rate that is able to colonize as yet uncolonized roots.

We have observed that some taxa are repeatedly the most dominant ones in root communities; for example, *Glomus* VTX00113 was dominant in 55 out of 190 root communities. This taxon is the most frequent one in our dataset, as well as in the MaarjAM database, and some of its entries are identified as *G. intraradices* (syn. *R. intraradices*), a widespread mycorrhizal fungus. It is reported from different ecosystems and continents, from arable fields to grasslands and forests. In forests, it was found to be an indicator of a generalist plant species(Davison *et al.* 2011). Dumbrell *et al.* (2010) argue that the identity of the dominant fungus depends on stochastic processes ('the species in the right place in the right time'). We would like to complement this model by suggesting that there is indeed a small group of regionally or even globally highly abundant, generalist taxa, which, by having a high propagule abundance in the local habitat, have a higher chance being the most dominant taxon in local communities.

Arbuscular mycorrhizal fungal communities have been repeatedly described by nestedness in different ecosystems (Verbruggen *et al.* 2012; Camenzind *et al.* 2014), indicating that taxa from a larger metacommunity are filtered out under specific conditions. In our study, we observed that

low and medium land-use communities were nested within high land-use communities. High land-use sites are characterized by more disturbance (e.g. trampling as a result of mowing and grazing, farm machinery use for fertilization). We suggest that a possible reason is the differential effect of land use on the dispersal of AMF species with different regional abundances and competitive abilities (Wilson 1984). Regionally more abundant and more competitive species are abundant in every sample. In habitats with low land use, regionally rare and less competitive AMF species can only rarely colonize roots. In habitats with higher land use, dispersal of fungal inoculum, especially of large spored taxa (which are more limited by wind dispersal; Renker *et al.* 2004) might be increased by soil mixing and homogenization of soil by grazing animals, zoochory on hooves and fur or by accidental ingestion and dung deposition (Allen 1987; Frank *et al.* 2003) and by agochory (i.e. sticking to farm machinery and boots; (Bowyer 1999). Thus, because of the increased dispersal of their propagules, regionally rarer species have a higher chance of colonizing roots, but still remain less abundant than the dominant species because of their inferior competitive abilities in these systems.

Our results emphasize that LUI has complex effects, as demonstrated by the nestedness pattern, and highlight the need for more experimental studies to disentangle these effects, and which complement the high external validity and realism of large-scale observational projects such as ours.

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Chapter 3: Host traits significantly influence phylogenetic structure of symbionts in communities of intraradical arbuscular mycorrhizal fungi

Abstract

The arbuscular mycorrhiza (AM) is a multispecies symbiosis between plant roots and Glomeromycota, in which AM fungi are obligate endosymbionts and completely rely on the plant partner for carbon, and forage in the soil for other nutrients. Since AM fungi always occur as communities, they are a compelling target for symbiont community ecology. We used a community phylogenetics and host trait based framework to explore how host phylogeny, root and leaf traits, host ecological preferences, and land use influence the phylogenetic structure of intraradical AM fungal communities. We carried out this analysis in 150 grassland plots using pyrosequencing. AM fungal communities in the root, as opposed to previous results from soil, were consistently phylogenetically clustered, a possible sign of more "filtering" in the root niche than competitive exclusion among the AM fungal co-colonizers. The ability of plants to selectively reward their symbionts was previously shown to vary not only with plant identity and phylogeny, but with environmental conditions as well. Less host filtering and the resulting increased competition could explain the observed decrease in phylogenetic clustering under certain environmental conditions or host traits, like shade tolerance or root characteristics. Taking these results together we propose a heterarchically structured system of influence between the symbiont community, the host and the environment, where the relative effect of the elements on each other varies in different situations. Environmental conditions, in relation to host plant preferences, define nutrient allocation to roots and host reward ("filtering") of AM fungi. This interplay between competition and host control then determines AM fungal community structure.

Introduction

The simple theoretical framework of community phylogenetics (Webb *et al.* 2002) states that the relative importance of competitive exclusion (and in some cases other species interactions: Vamosi *et al.* 2009; Mayfield & Levine 2010) and environmental filtering causes communities to exhibit nonrandom phylogenetic structure. Phylogenetic patterns in communities of macroorganisms have been studied at least since Darwin (1859), who stated that species of the same genus are expected to be more similar in traits and therefore compete more severely.

Compared to macrobes, microbial interactions with the abiotic and biotic environment have some unique features, aside from scale, prompting the question whether the influence of environmental filtering and competitive exclusion on microbial communities is similar. For example, while unicellular microorganisms and their direct physical interactions with the environment are usually on the micron scale, microbial assemblages can metabolically interact and create chemical gradients over many meters, making it hard to delineate a relevant scale of environment and a community with tight interactions (Konopka 2009). Several mechanisms unique to microbial groups might affect the importance of competitive exclusion. In bacteria, horizontal gene transfer can promote cooperation as opposed to competition (Dimitriu *et al.* 2014). In filamentous fungi, the mycelium can span different habitats and resource patches through indeterminate modular growth, re-absorbing mycelium parts in resource depleted patches, which could have consequences on competition for resources. Therefore, it is not trivial to ask if abiotic habitat filtering and local competitive exclusion have the same importance in the assembly and coexistence of microbial communities as in macroorganisms.

In comparison with free living microorganisms, the complications in revealing community ecology patterns are heightened in endosymbiotic microbial communities. The interaction between a host and its symbiont community can be so critical that the symbiosis becomes obligate; either the symbiont or the host is unable to survive without the other. While this underlines the importance of endosymbiotic communities, it also makes *in situ* quantification of their life history traits technically difficult. In addition to abiotic habitat filtering and local competitive exclusion, the host becomes another significant determinant of endosymbiont communities. Host traits can be interpreted as niche axes, or as an additional "habitat" filter, because hosts, just as the abiotic environment, may select symbionts with a certain trait complex from the local species pool.

Recent technical advancements in molecular techniques that allowed researchers to collect DNA-based data even from these "cryptic" communities, coupled with the interest in exploring the peculiar interactions of microbial communities, have been prompting investigation of phylogenetic patterns. Due to the lack of species level trait data and sometimes even taxonomic description, phylogeny is often used as a proxy of ecologically relevant functional traits (Martiny *et al.* 2015), for instance in bacteria (Horner-Devine & Bohannan 2006), or in AM fungi (Montesinos-Navarro *et al.* 2016) a prominent group of obligate endosymbionts associated with the vast majority of plant species.

The importance of host effects on obligate multispecies symbiont communities is underlined by the number of studies that report consistent phylogenetic clustering in host samples as opposed to the variety of outcomes from the environmental matrix, which suggests host selection or symbiont preference. In adult fleas, phylogenetic clustering was observed in mammalian fur (Krasnov *et al.* 2014, 2016). In AM fungi, Horn *et al.*, (2014) and Saks *et al.*, (2014) found phylogenetic clustering in roots when studying natural habitats, but clustered or random structure in soils.

AM fungi (Glomeromycota) are ideal to study the interplay of host and habitat effects, because while they completely rely on the plant partner for carbon, they also must forage in the soil for nutrients they offer in exchange, therefore not only the host, but also the abiotic environment has an effect on their community structure. Soil P (Krüger *et al.* 2015), elevated CO_2 (Mueller & Bohannan 2015) and shade (Liu *et al.* 2014) were shown to influence AM fungal phylogenetic structure. In addition, the framework of community phylogenetics is well suited to AM fungal community analyses, because AM fungal traits related to competition for space are conserved (Maherali & Klironomos 2007) and thus the connection between phylogenetic relatedness and functional trait similarity has been established.

However, studies that take host traits into account as potential defining factors for AM fungal community structure in addition to environmental characteristics are missing, even though plant roots are hypothesized to have evolved as a habitat for mycorrhizal fungi (Brundrett 2002). Therefore, among host characteristics, root traits have a great potential importance for defining AM fungal communities.

Root trait variation among species is immense (Bardgett *et al.* 2014). The framework of the resource economic spectrum can be used to explain this variation by ordering traits ranging from

resource acquisitive to resource conservative. Resource-acquisitive plant species are hypothesized to have roots with high specific root length (SRL, root length divided by root biomass) with elevated mycorrhizal colonization, higher N and lower C content and uptake, high root respiration and low root life span, in contrary to resource-conservative plants (Roumet *et al.* 2006).

A high SRL can arise either from low root tissue density or high root fineness. Fine roots are similar to mycorrhizae in function (soil exploration and absorption). Highly mycorrhizal plants tend to have coarse, long lived, relatively thick roots, which maximize cortex area available for AM fungi while low mycorrhizal plants need fine roots for nutrient acquisition. There is a trade-off in maintaining high mycorrhizal colonization and a fine root system; facultatively mycorrhizal plants have lower root cortex volume in order to invest in greater surface area (Brundrett 2002).

Specific root volume (SRV, the inverse of tissue density) is expressed as the ratio of volume and dry mass, and it is also considered to be an important predictor of plant strategies. Roots with high specific volume have a short turnover and can grow quickly with low investment in dry matter, and therefore are typical of fast growing plant species. While for AM fungi relatively slow growing roots are optimal, root longevity has to be balanced with suitably high volume of the intercellular air channels in the cortex, where AM fungal hyphae can rapidly spread by linear growth (Brundrett & Kendrick 1990; Brundrett 2002).

In this study we combined for the first time host and symbiont pylogeny, intrinsic and extrinsic host traits and environmental characteristics to explain root AM fungal phylogenetic structure. Host related niche axes were described by leaf traits and morphological root traits (intrinsic traits) and extrinsic ecological preference (extrinsic traits). A combined index of fertilization, grazing and mowing was used to describe the environment in a series of grasslands representing a land use intensity gradient. We formulated the following hypotheses:

1. The distribution of AM fungi among root communities is nonrandom with respect to phylogeny

2. Phylogenetic structure of root AM fungal communities changes with increasing land use intensity

3. Phylogenetically related plants have more phylogenetically similar AM fungal communities

4. Host plant traits (a. root traits b. combination of morphological and ecological traits) explain the phylogenetic structure of root AMF communities

Methods

Sampling sites

Soil sampling was carried out in the 150 grassland plots (50 × 50 m of size) of the three sampling regions of the German Biodiversity Exploratories representing a land use gradient of typical grassland use in Central Europe (Table 3.1., Fischer *et al.* (2010)), including grasslands with low input and also fertilized, grazed and mown meadows and pastures.

Table 3.1: Sampling regions of the German Biodiversity Exploratories used in this study, from Fischer et al. 2010

	Schorfheide-Chorin	**Hainich-Dün**	**Schwäbische Alb**
Location	NE Germany	Central Germany	SW Germany
Size	~1300 km²	~1300 km²	~422 km²
Geology	Young glacial landscape	Calcareous bedrock	Calcareous bedrock with karst phenomena
Human population density	23 km⁻¹	116 km⁻¹	258 km⁻¹
Altitude a.s.l.	3–140 m	285–550 m	460–860 m
Annual mean temperature	8–8.5 ◦C	6.5–8 ◦C	6–7 ◦C
Annual mean precipitation	500–600 mm	500–800 mm	700–1000 mm

Sample preparation

As part of a larger sampling campaign of several projects within the German Biodiversity Exploratories, in each of the 150 grassland plots 14 soil cores along two orthogonal transects were taken and the top 10 cm of all cores per site were pooled; sampling was done within two weeks in May of 2011. Five randomly selected healthy looking root fragments with a length of 4 cm and diameter less than 1 mm per plot were subsampled from these pooled soil samples, resulting in 750 root samples which were used for AM fungal and plant molecular analyses. This random sampling strategy was chosen in order to obtain root fragments in a way that was not biased by the aboveground plant status (dormant, vegetative growth, flowering etc.) within a predefined land use background. Roots were washed thoroughly with distilled water, freeze dried and pulverized using metallic beads. Afterwards, total DNA from the powder was extracted with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, USA). DNA was amplified by a nested PCR approach, first with GlomWT0/Glomer1536, then with NS31/AM1a+b primer pairs (Morris *et al.* 2013), using 25 different Multiplex Identifier Adaptors (Roche Diagnostics GmbH, Germany) in order to label sequences belonging to different samples. AM1a and AM1b are modified AM1 primers designed to capture AMF families not captured by the original AM1, which excludes some taxa (Daniell *et al.* 2001). The primers target the small subunit (SSU) of the nuclear encoded ribosomal DNA (rDNA). A detailed protocol of the PCR conditions is given in the Supporting Information (Appendix A, Methods S1).

Equal amounts of DNA were mixed into pools of 25 samples based on DNA content quantification by the image-analysis software GelQuant.NET (v. 1.8.2, BiochemLabSolutions.com). Pools were purified via agarose gel extraction with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and were 454 pyrosequenced on a Roche GS FLX+ system with Titanium chemistry. Raw sequencing data from one of the three sampling sites (Hainich) was previously used in Vályi *et al.*, 2015 for testing different hypotheses, but was bioinformatically re-analyzed.

Bioinformatics analysis

Processing of flowgram data from pyrosequencing was done by Mothur (Schloss *et al.* 2009), using the sff.multiple command, discarding reads with less than 300 flows and reads shorter than 200 bp. Sequences were unpacked, screened, denoised and trimmed. Reads with more than 1 base

difference in the barcode or 2 bases in the forward primer were discarded. Afterwards, the 750 samples had an average of 4217.62 reads per sample (SD: 3192.51).

As we were interested in the structure of the AM fungal communities rather than their identity, we used an open reference OTU picking approach (sensu Bik *et al.* (2012)), to define sequence clusters using the CROP clustering tool (Hao *et al.* 2011), which performs an unsupervised Bayesian clustering. This method works with a Gaussian mixture model with a flexible OTU cutoff threshold.

The processed sequences were BLASTed against the nucleotide collection of NCBI. Based on the BLAST results, Glomeromycota sequences were extracted with MEGAN (Huson *et al.* 2011). Average Glomeromycota reads per sample were 994.89 reads (SD=1451.22).

Multi-tag pyrosequencing might introduce a bias to community composition due to the preferential amplification of certain barcoded primers during PCR (Berry *et al.* 2011). Since such unequal representation of samples will lead to a differential sampling intensity, we resampled our dataset to an equal number of reads per sample. As the amplicons in our study were short and about the same length, we believe that read numbers in the resampled dataset could be used as a proxy for relative abundance of the OTUs (Ihrmark *et al.* 2012).

The OTU table was randomly resampled to 500 Glomeromycota reads per sample with replacement by bootstrapping, repeated 100 times, and then averaged. To avoid excessive upsampling, samples consisting of less than 400 sequences were removed prior to resampling, as were singleton OTUs. Downstream data analysis was performed on a data table consisting of 495 root communities and 144 Glomeromycota OTUs. In this resampled dataset the average OTU richness per sample was 10.94 (SD=7.17).

Phylogenetic structure analysis

We calculated pairwise genetic distances between the center sequences of each Glomeromycota OTU with ESPRIT (Sun *et al.* 2009), that uses the Needleman–Wunsch algorithm (Needleman & Wunsch 1970).

To test hypothesis 1, we addressed the phylogenetic structure of AMF root communities by calculating the standardized effect size of mean pairwise distances (SES-MPD), an equivalent to the additive inverse of the nearest relative index (Webb *et al.* 2002).

SES-MPD was calculated by the ses.mpd function in picante package (version 1.6-2, (Kembel *et al.* 2010)) of R, using the "taxa.label" algorithm with 999 randomized null communities, which uses null model randomization of distance matrix labels across all taxa to calculate effect sizes of deviations from mean phylogenetic distance between samples (i.e. AMF community of each individual root sample). Negative SES-MPD values are correlated with clustering, positive values with overdispersion.

Phylogenetic beta diversity was addressed by calculating inter-community mean pairwise distance (IC-MPD) by the comdist function in picante. Both ses.mpd and comdist are adapted to R from Phylocom (Webb *et al.* 2008).

Land use

In order to test hypothesis 2, we included land use information in our analyses. Land use intensity was measured by the LUI index (Blüthgen *et al.* 2012), which is an additive index summarizing the standardized intensity of fertilization (organic or inorganic N-fertilizer applied by farmers measured in kg nitrogen per hectare), frequency of mowing, and the intensity of livestock grazing (reflected by density of livestock) on the grassland sites of the Biodiversity Exploratories project. It was calculated for the years between 2006 and 2010 and then averaged (Klaus & Blüthgen 2013).

Host plant identity

For testing hypothesis 3, detailed information on host plant identity and phylogeny was required. The vegetation of the plots was assessed by cover estimates of a 4x4 m core area by visual estimation (Schmitt *et al.* 2011). Plant identity of the root samples were assigned based on the sequence of the trnL-intron, which is suitable for plant species identification, as it is widely represented in the GenBank database and is quite variable between plant taxa (Borsch & Quandt 2009). In combination with coverage data from vegetation relevées, this method allowed us to identify plants with high confidence. Plant DNA from the root extract was amplified with trnL c/d primers (Taberlet *et al.* 1991), cleaned and Sanger-sequenced. Data was BLASTed against NCBI nucleotide collection and best hits based on maximum scores were matched with the vegetation of the plot, which allowed us to identify 52 different host plants (Appendix B, Table S1).

A phylogenetic tree of the detected plant species was created by pruning the Daphne phylogeny (Durka & Michalski 2012) to include only our plant taxa. Phylogenetic distance was calculated by the cophenetic.phylo function from the ape package of R (Paradis *et al.* 2004).

Intrinsic (root and leaf) traits

The most common plant species found in our sampling sites were grown in 2012 in a greenhouse in Muri, Switzerland under uniform conditions. Seeds from commercial seed suppliers and botanical gardens in Germany were surface sterilized and germinated in Petri dishes on an autoclaved substrate consisting of an 80:20 % mixture of washed sand and commercial soil (Rasentragschicht AarGround, AareKies Brienz AG, Switzerland). The substrate had a pH of 6.5, a carbon concentration of 9.69 mg/g ± 2.04 (mean ± SD, n = 3) and a nitrogen concentration of 0.88 ± 0.15.

After germination seeds were transplanted into flower pots containing the same substrate and inoculated with a microbial wash from field soil filtered through a 250 µm sieve to allow colonization by AM fungi. After 4-6 weeks (depending on the species specific growth) plants were harvested before the roots were pot bound. Roots were separated from the aboveground plant parts, washed, dried and weighed by a precision balance. Root length, root surface area and root volume were determined using the WinRhizo scanner bases system (version 2007d, Regent Instruments Inc., Québec, Canada). Fine roots, the absorptive part of the root system, were defined as roots thinner than 0.2 mm in diameter, and constituted 78.5 % of scanned root length. We measured root length and surface area separately for all roots and fine roots. Subsequently, specific root variables were calculated by dividing the total values by root dry weight. Out of the 52 plant species identified by root DNA, 43 plant species were included in the root trait analysis (Appendix B, Table S1).

Leaf anatomy traits were acquired from the BIOLFLOR database (Klotz *et al.* 2002; Kühn *et al.* 2004). Plant species are characterized as having either scleromorphic, mesomorphic, hygromorphic or helomorphic leaves. Scleromorphic leaves are firm and stiff with thick epidermis and cuticula. The plants have extensive root system and mechanisms to promote water transport under beneficial conditions. Plants with hygromorphic leaves are characterized as delicate plants of shade and semi-shade and relatively high humidity. Their root system is not very extensive and ensures no quick resupply of water to the plant. Mesomorphic leaves are between scleromorphic and hygromorphic. Plants with helomorphic leaves have many stomata and vascular bundles and aeration tissue in the root as adaptation to oxygen deficiency in swampy soils. Their root system is mostly flat and extensive.

Plant ecological traits (extrinsic traits)

Ecological strategy types (following the system of Grime (2001)) were acquired from the BIOLFLOR database. Plant species are characterized either as competitors, stress-tolerators, ruderals or one of the intermediate combinations.

Ellenberg indicator values (Ellenberg *et al.* 1992) for light and moisture preference reflect the realized environmental optima of plant species of Central Europe and are expressed as ordinal numbers. Light preference is characterized as low (1–3), medium (4–6), high (7–9) or indifferent. Moisture preference as expressed as low (1–4), medium (5–8) or high (9–12). Ellenberg values were acquired from FLORKART (hosted as floraweb by the German Federal Agency for Nature, available under http://www.floraweb.de).

Statistical analyses

All statistical analyses were performed using the statistical software R version 3.2.1 (R Core Team 2016).

To test whether the magnitude of AM fungal clustering changes with increasing land use intensity (Hypothesis 2), we fitted a linear model using the lm command in the package stats with LUI as an explanatory and SES-MPD as a response variable. Linear model assumptions here and in further models were checked visually using plot diagnostics in the package stats.

To test the relationship between host plant relatedness and AM fungal phylogenetic community distance directly (Hypothesis 3), we fitted a linear model using host cophenetic distance and inter-community mean pairwise phylogenetic distance of AM fungi found in the roots.

To explore the potentially different effects of different root traits on phylogenetic clustering (SES-MPD) of the AM fungal communities in one root piece (Hypothesis 4a) we fitted separate linear models. We checked correlation between root traits using Pearson's correlation.

To analyze the effect of the combination of intrinsic (root and leaf) and extrinsic (ecological) traits in relation to the magnitude of clustering (Hypothesis 4b), PCA-s were performed with the rda function in package vegan version 2.3-5 (Oksanen *et al.* 2016) to reduce dimensionality of the root, leaf, and ecological host traits separately. Entries with a missing value in any trait were deleted. We combined the PC scores of these variables (The first 4 axes per trait type for leaf and ecological traits, and 2 axes for root traits, that accounted for >90% variability) and the identity of sampling site (region), and fitted a linear model with SES-MPD as a response variable.

Results

Hypothesis 1: The distribution of AM fungi among root communities is nonrandom with respect to phylogeny

SES-MPD values were negative in almost all (99.4%) root communities (on average: -2.2873 ±0.8667), which means that the communities were more phylogenetically clustered than expected by chance. Three communities consisted of only one taxon so the SES-MPD could not be calculated.

Hypothesis 2: Phylogenetic structure of root AM fungal communities changes with increasing land use intensity

Land use intensity had a significant (p= 0.0496), but small (Multiple R-squared: 0.0078, adjusted R-squared: 0.0058) positive effect on SES-MPD (Figure 3.1), which means that as the land use intensified, the AM fungal communities became less clustered.

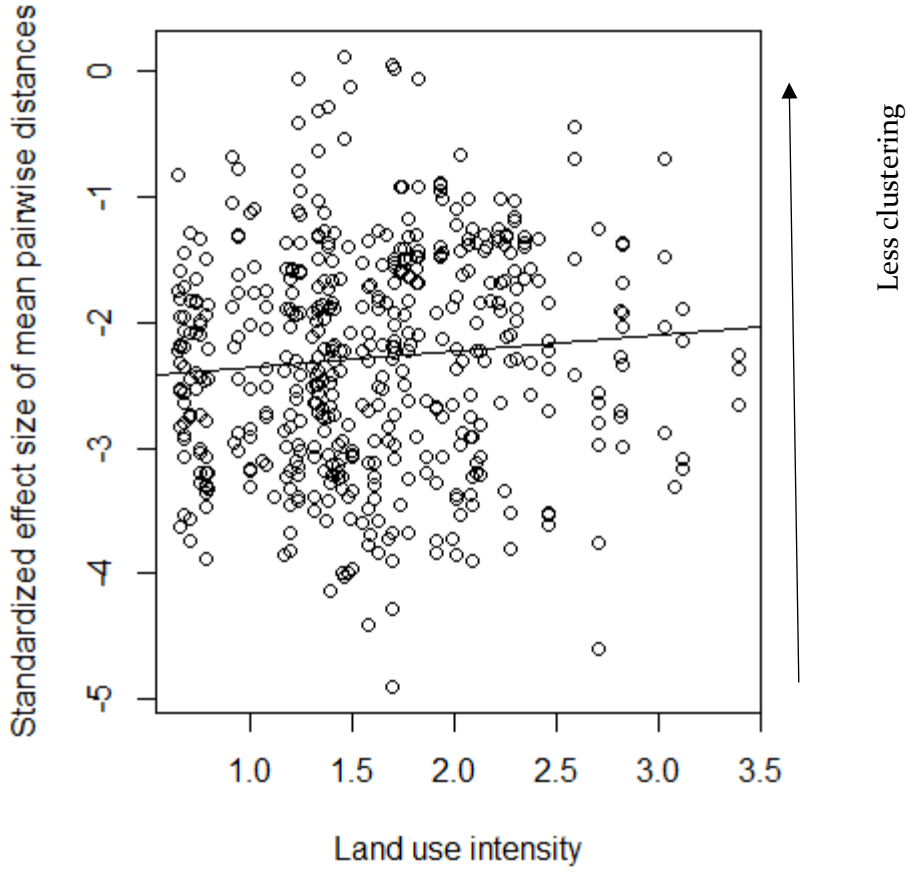

Figure 3.1: A linear model of the magnitude of AM fungal community phylogenetic clustering as a response to increasing land use intensity. As land use intensifies, AM fungal communities in plant roots become less phylogenetically clustered.

Hypothesis 3: Phylogenetically related plants have more phylogenetically similar AM fungal communities

Host plant phylogenetic distance had a small, but significant positive effect (p=0.0022, Multiple R-squared: 6.813e-05, Adjusted R-squared: 6.082e-05) on inter-community mean pairwise phylogenetic distance of AM fungi.

Hypothesis 4: Host plant traits explain the phylogenetic structure of root AMF communities

a. Root traits

Root traits were strongly correlated with each other (Pearson's r between 0.92 and 0.99), except for specific root volume, which correlated weakly with the rest of the root variables (Pearson's r

between 0.38 and 0.67). All specific root variables had a small, significantly negative effect on the magnitude of clustering (positive on the value SES-MPD). The higher the specific length, area or volume of the root, the less phylogenetically clustered were the AM fungal communities in it (Table 3.2).

Table 3.2: Linear model parameters of the effect of root traits on the magnitude of phylogenetic clustering (SES-MPD) of AM fungal communities in roots. Bold letters signify significant effect ($p < 0.05$).

	p	Multiple R^2
Specific root length	**0.0155**	0.0154
Specific fine root length	**0.0211**	0.0140
Specific root surface area	**0.0275**	0.0128
Specific fine root surface area	**0.0347**	0.0117
Specific root volume	**0.0229**	0.1125

b. Morphological and ecological traits

To include ecological and aboveground (leaf) traits in addition to the root traits measured by us, we created a linear model of the PCA scores. This model had a multiple R^2 of 0.1761, and a p value < 0.0001. Significant factors were: Sampling region (p= 1.738e-08), root principal component (p=6.992e-05), ecological principal components which were correlated with medium soil moisture preference, light preference of well-lit environment or partial shade (p=0.0098), and a leaf principal component correlated with hygromorphic leaves (p=0.037).

Discussion

Root AM fungal communities are phylogenetically clustered

We showed that AM fungal phylogenetic structure was significantly different from random, which confirmed hypothesis 1, specifically it was phylogenetically clustered. Given conserved traits (which was shown for spatial niche use of AM fungi by Maherali & Klironomos (2007)), phylogenetic clustering is understood as a sign of habitat filtering influencing community

assembly more than local competitive exclusion (Webb *et al.* 2002). As we examined communities from within the root, this "habitat" filtering effect can be attributed to both the host plant and its environment, including land use and other anthropogenic influences.

Effect of land use in dissolving phylogenetic clusters can be less drastic on root communities

More intensive land use (mowing, grazing and fertilization) was correlated with less clustering in accordance with hypothesis 2. This is in line with previous research that has shown that elements of land use intensity, for example high level fertilizer treatments can shift AM fungal phylogenetic structure even to overdispersion in soil (Liu *et al.* 2015). Even though the phylogenetic structure of AM fungal communities in the root did exhibit a modest shift in the same direction, it just became less clustered, not overdispersed. This difference can be explained by host effects which interact with land use intensity (Vályi *et al.* 2015) in shaping AM fungal communities.

Hosts effects can be phylogenetically conserved

Hosts effects can be phylogenetically conserved. More related plants had more phylogenetically similar AM fungal communities, as expected in hypothesis 3, but the phylogeny of the plants explained only a small part of the variation. Even though originating from the same soil pool, different AM fungal OTUs associate with plants of different species (Gosling *et al.* 2013), but more closely related plants surprisingly do not have more similar AM fungal communities in terms of composition (Veresoglou & Rillig 2014). Taking this together with our results, we can conclude that host phylogeny does not have an effect on the actual composition of the AM fungal communities but instead selects AM fungal OTUs from specific higher level clades, which is congruent with a functional conservation on a deeper phylogenetic level relevant to host-symbiont interactions (Powell *et al.* 2009).

Root traits are important determinants of AM fungal community structure

Out of all host traits that definitely contribute to the interaction between plant and symbionts, root traits influence mycorrhizae most directly. Morphological root traits appeared to be multidimensional. While specific root length and surface area were strongly correlated for both the whole root system and fine roots, specific root volume was less correlated with them. However, the effect for all root traits was similar: the higher the specific length, surface or volume of the root, the less phylogenetically clustered the AM fungal communities (hypothesis 4a).

Specific root volume explained the most variation in the phylogenetic structure of the fungi. High specific root volume can be attributed to less dense tissue, e.g. because of higher volume of air channels in the cortex, the habitat of AM fungi in the plant (Brundrett 2002). Growth rate of AM fungi within roots is reported to be faster in plant species with air channels in the cortex than in species where hyphae spread by intracellular growth (Brundrett & Kendrick 1990). Faster growth could have led to less clustering through increased competition. In contrast, plants with lower SRL or SRV are on the resource conservative end of the spectrum, and can be characterized by a root system with few fine roots. These plant species might have to recruit specific AM fungal communities to ensure sufficient water and nutrient input (Kong *et al.* 2014). The more clustered structure of the communities we have shown in these plants might signal this.

Host traits interact with abiotic environmental conditions to influence symbiont communities through direct filtering or influencing competition

The host filter and the habitat filter are not independent: for example, the plant's resource exchange with their symbionts varies not only according to plant identity but with environmental conditions as well (Bever 2015; Walder & van der Heijden 2015), suggesting a complex system of interactions with the symbiont community. Plants with different intrinsic and extrinsic (ecological) traits respond differently to environmental conditions. This is a possible explanation for why plants with different moisture and light preferences had different AM fungal phylogenetic structures (hypothesis 4b). Plants with hygromorphic leaves and plants associated with shade habitats had less clustered AM communities. It was experimentally shown that shade decreased the ability of plants to selectively reward beneficial AM fungi due to decreased nutrient allocation to roots (Zheng *et al.* 2015). Plants of shade that have hygromorhic leaves and prefer more humid soil have less extensive root system (Klotz *et al.* 2002). The lack of spatial separation in the root system also decreases preferential allocation of carbon (Bever *et al.* 2009). Decreased preferential allocation, the resulting less host filtering and stronger competition between co-occurring AM fungal species can explain the decrease in clustering we have shown in this study.

As another example of the interaction of the host and environmental filter, increasing soil fertility was shown to result in less nutrient allocation to roots and thus to AM fungi (Liu *et al.* 2012). Less clustering (in this study) or outright overdispersion (Liu *et al.* 2014) with increasing land use intensity could signify not only a direct environmental effect, but also an interaction between host

and environment: plants not investing in filtering AM fungal species due to sufficient nutrient input.

Taking these results together, it emerges that if the nutrient allocation from host to AM fungi decreases, because of outside environmental conditions, it leads to increased competition and less clustering. In soil communities outside the plant this can even lead to overdispersion (Figure 3.2).

Research on other obligately symbiotic organism groups that form multispecies communities frequently explains symbiont community structure by host phylogeny and traits that have either direct effects or influence community structure through influencing competition between co-colonizing symbionts. For example, host phylogeny explained the structure of microbial communities of marine sponges: although the identity of specific microbial OTUs varied substantially among the hosts, more closely related sponge species tended to harbor microbial communities with more similar relative abundance and dominance structure (Easson & Thacker 2014). In a variety of parasites, clustering was explained by host mediated effects: facilitation mediated by immunosuppression(protozoa, helminths, bacteria, viruses: Cox 2001; fleas: Krasnov *et al.* 2006). It was also shown that developmental and life history differences between different symbiont organism groups might cause subtle differences in the relative importance of the host trait-symbiont interaction. Mites, as opposed to fleas had a tighter association between host traits and parasite diversity, probably because of the dependence of both imago and preimaginal stages on the host body (Korallo *et al.* 2007). Spatial structure and historical events might be more important in defining the phylogenetic structure of symbionts of mobile hosts than those of sessile ones (Krasnov *et al.* 2013), or when examining patterns at a global scale (Kivlin *et al.* 2011).

Symbiont community ecology in the light of global change

The relative lack of information in coexistence of symbiotic assemblages, especially in non-culturable microbial taxa, reflects the challenges in studying these organisms. We propose that phylogenetic *and* trait-based frameworks, including diverse host and symbiont traits, environmental and sequence data would help to further explore the complex effects of environmental, host-related and local competitive factors on symbiont coexistence and may be useful for predicting shifts in symbiont assemblages and functioning in the face of global change. As symbiont communities are critical both to the world's ecosystems and to the function of plants, animals and humans, searching for similarities in community structure across taxonomic domains despite life history variation might reveal common patterns that could have consequences even for human or animal health.

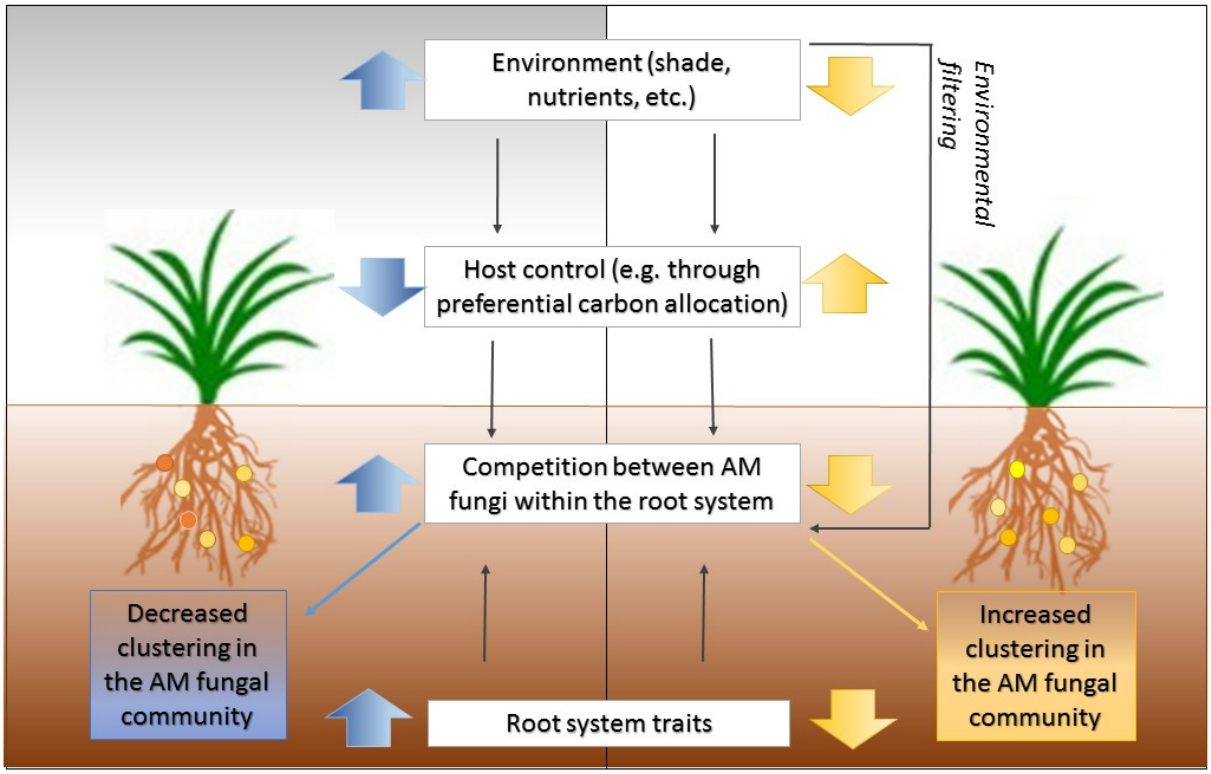

Figure 3.2: proposed framework of factors influencing AM fungal phylogenetic community structure in roots

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Chapter 4: Community assembly and coexistence in communities of arbuscular mycorrhizal fungi

Abstract

Arbuscular mycorrhizal fungi are asexual, obligately symbiotic fungi with unique morphology and genomic structure, which occupy a dual niche, that is, the soil and the host root. Consequently, the direct adoption of models for community assembly developed for other organism groups is not evident. In this paper we adapted modern coexistence and assembly theory to arbuscular mycorrhizal fungi. We review research on the elements of community assembly and coexistence of arbuscular mycorrhizal fungi, highlighting recent studies using molecular methods. By addressing several points from the individual to the community level where the application of modern community ecology terms runs into problems when arbuscular mycorrhizal fungi are concerned, we aim to account for these special circumstances from a mycocentric point of view. We suggest that hierarchical spatial structure of arbuscular mycorrhizal fungal communities should be explicitly taken into account in future studies. The conceptual framework we develop here for arbuscular mycorrhizal fungi is also adaptable for other host-associated microbial communities.

Introduction: applying models of community assembly and contemporary coexistence theory to communities of arbuscular mycorrhizal fungi: knowledge gaps and difficulties

How communities assemble and which species can coexist in the same locale has been a central question of ecology. The recent advancement of high-throughput molecular barcoding methods has enabled researchers to obtain information on the composition and structure of natural microbial communities more easily than ever. This is especially important for organisms that are difficult to culture, such as arbuscular mycorrhizal (AM) fungi, which are obligate symbionts of plants where they form multispecies symbiont communities in the same root. Given the growing number of molecular studies in the field of AM fungal community research, it is timely to review the progress on understanding the processes that underlie AM fungal community assembly, and highlight the knowledge gaps.

The theoretical framework of community assembly and coexistence (HilleRisLambers *et al.* 2012) combines a classic filter model of community assembly with modern coexistence theory (Chesson 2000). The filter model describes a regional pool of species from which the members of local communities are selected by passing through environmental and biotic filters. Modern coexistence theory (Chesson 2000) addresses interactions on the local scale, which arise from niche differences and fitness similarities. The combined approach therefore acknowledges factors on a wide spatiotemporal scale. Regional and local processes are connected by the neutral process of dispersal. The filter model is further nuanced by taking into account feedbacks, when organisms are not only influenced by but also have an impact on their environmental and biotic filters (Figure 4.1).

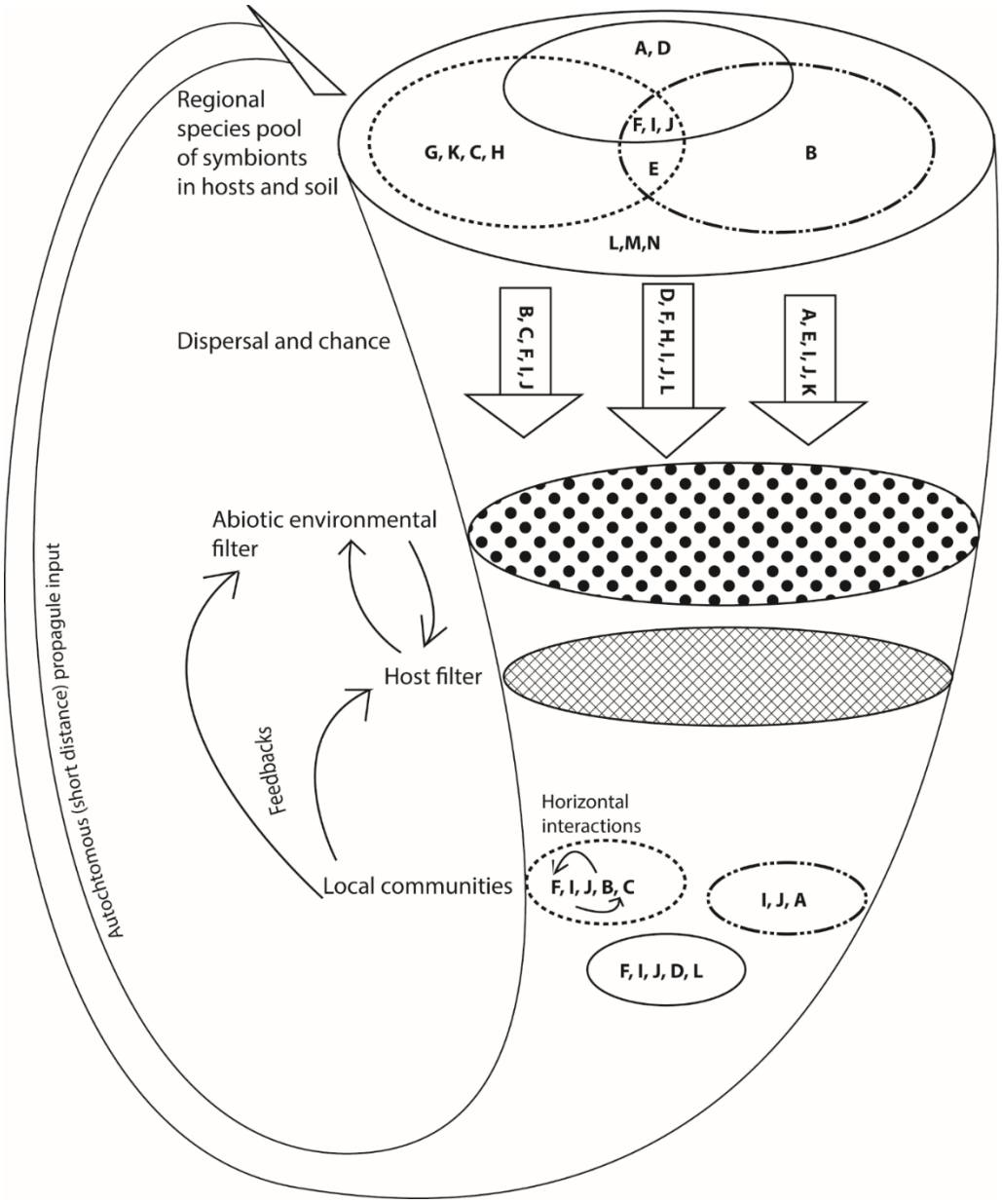

Figure 4.1: Applying the combination of a filter model of community assembly and neutral processes for AM fungi. The regional pool of AM fungi consists of species present in the soil and in the roots of the host community. Through local or long-distance dispersal and chance, species reach local habitats. The environmental filter prevents species whose environmental tolerances do not overlap with local conditions from entering the community. The host filter allows colonization only for compatible fungal partners, thus further removing species. The local community reflects the cumulative effects of these processes, and in turn influences them through feedbacks. Horizontal interactions within the symbiotic community and with other non-host species also affect local communities. Local communities in turn contribute to the regional species pools with autochtonous propagule input. The capital letters refer to different AM fungal species. Ellipses with different lines depict different root system communities. Details of the depicted community assembly and coexistence model elements can be found in the section 'Factors affecting AM fungal community assembly: review of the elements of the proposed model' in the main text.

AM fungi are a monophyletic group (phylum Glomeromycota) of asexual, obligately symbiotic fungi with a unique combination of traits regarding morphology, genomic structure and ecology. Within their coenocytic mycelia and spores, multiple, potentially genetically divergent nuclei coexist, making it difficult to delineate an individual even with molecular methods (Table 4.1). Through intraradical and extraradical mycelia, they occupy a dual niche, the plant root and soil. Both the soil environment and plant root can be described according to a simple filter model as species filters preventing certain AM fungal species from entering the local community. However, the simple filter analogy ends when taking into account that host plants and soil do not remain unchanged during community assembly: AM fungi interact with their hosts through hormonal crosstalk and actively shape soil as ecosystem engineers (See section: *'Feedbacks: AM fungi as ecosystem engineers'*). Taking into account these developmental, genetic and ecological angles, the direct adoption of models for community assembly developed for other organism groups is not evident. There are several points from the individual to the community level where the application of modern community ecology terms runs into problems when AM fungi are concerned (Table 4.1). Especially in the area of coexistence, even for the definitions of such fundamental concepts as 'fitness' further research and discussion are needed (Table 4.2).

Table 4.1: Fundamental questions in defining levels of biological organization for AM fungi. Abbreviations: AM, arbuscular mycorrhizal; CMN, common mycorrhizal network; OTU, operational taxonomic unit.

Level of biological organisation	**General definition**	**Problem with usage for AM fungi**	**Possible solutions**
Individual	In modular organisms, an individual can be defined: •as a physically continuous unit, which is separated from other such units (ramet) •a unit with uniform genetic composition (genet)	In AM fungi, these two definitions do not delineate the same parts of the mycelium (Rosendahl 2008): •genetically different AM fungi are able to anastomose with each other (Chagnon 2014) and might form a continuous mycelium where nuclei of different genetic compositions mingle (Young 2009) •a genetically uniform mycelium might be physically disrupted •because of asexual reproduction with no recombination, different mycelia with the same genetic composition can be found in large geographical distance from each other •'ramet' and 'genet' are used by some researchers; however, many use the terms 'clone', 'strain' and 'isolate' as with other microbes to grasp different aspects of the concept of an individual	•DNA profiling of individuals using mitochondrial markers, because, in contrast to nuclear DNA, the mitochondrial genome appears to be genetically identical within mycelia (de la Providencia *et al.* 2013; Daubois *et al.* 2016)
Species	Some commonly applied species concepts for fungi are (Moore *et al.* 2011): •morphological: based on morphological similarity •biological: based on reproductive isolation	•Morphological: many AM fungal species are unculturable and their appearance in roots varies with the host •Biological: no evidence of sexual reproduction, so mating tests are not possible •Phylogenetic: It is not clear what level, if any, of genetic difference is a suitable proxy for species or other levels of biologically interacting units(Hao *et al.* 2011; Caruso *et al.* 2012b; Powell 2012)	•Morphological: traditional taxonomy of AM fungal morphotypes is based on the characteristics of spores •phylogenetic: –Fixed and named OTUs are available for the sake of comparability between environmental studies, based on the small subunit of the

	•phylogenetic: defining OTUs based on genetic similarity		ribosomal DNA (Öpik *et al.* 2010) –Efforts are made to create a unified sequence-based species delimitation of Glomeromycota using multiple loci (Öpik *et al.* 2014)
Community	Species with similar ecology that coexist in the same spatial region (Chesson 2000). Definitions often include that community members must be able to interact (for example, Whittaker 1975)	At which spatial scale should the AM fungal community be defined?	Adapting a spatially explicit, hierarchical community system from parasitology (Figure 4.2, see also section 'Scale dependency: different assembly rules for different spatial scales? An analogy borrowed from parasite communities' in the main text): •AM fungi in a root fragment •infracommunity: AM fungi in an entire root system of one host •component community: AM fungi in the root systems of a population of one host species •compound community: AM fungi in the root systems of the host community (mixed root samples from a sampling site)
Metacommunity	Metacommunities are spatially divided species assemblages, where dispersal among communities is limited (Morin 2011)	The assemblage of AM fungal communities living in the root systems of a plant community cannot be easily described by the metacommunity theory: •instead of only dispersing between hosts by propagules, AM fungi in	Application of metacommunity theory would require modifications

		different hosts might interact or even be physically continuous with AM fungi living in other root systems, forming CMNs (Selosse *et al.* 2006), which are large, interconnected networks of fungal hyphae that are simultaneously connecting multiple hosts •hosts are not passive islands: –AM fungi can preferentially allocate nutrients to high-quality hosts connected to the same CMN (Fellbaum *et al.* 2014) and CMNs can provide means of infochemical transport between connected plants (Barto *et al.* 2011) –AM fungi can modify the fitness of their hosts depending on their identity. Furthermore, responsiveness of hosts to AM fungal colonization can change over time after the initial colonization (Mihaljevic 2012a, b; Veresoglou *et al.* 2012)	

Table 4.2: Problems and solution attempts in applying community ecology terms to AM fungi. Abbreviations: AM, arbuscular mycorrhizal; PCR, polymerase chain reaction.

Community ecology term	**Problems in using it in AM fungal community ecology**	**Current solution attempts and their issues**
Fitness	The definition of fitness in other organisms usually includes a measure of reproduction. •As AM fungi are asexual organisms, how can their fitness be defined? •It is difficult to use a proxy for AM fungal fitness, which could be used to compare species, as: (1) higher propagule abundance does not necessarily translate to higher colonization (2) there are significant allocation differences among species in growth of spores versus hyphal network (Veresoglou & Halley 2012) • AM fungal fitness always depends on plant carbon, as they do not have independent ways to take up carbon (Johnson 2010)	•Spore production and root colonization rates are possible fitness measures •Marker gene copy numbers can be used as a proxy of root colonization (Thonar *et al.* 2014). Distinguishing some AM fungal species in co-colonized roots is now possible with species-specific quantitative real-time PCR.
Traits	How to study AM fungal traits?	•Traits in culture: traits are assigned to strains and might not be representative of a species •Transcriptomes of a single species: the study of the transcriptomes of species

		(Tisserant *et al.* 2012) might explain perceived functional redundancy (Peay *et al.* 2008) and provide mechanical understanding of community assembly, but it suffers from the same problem •Metatranscriptomes: solving the annotation problem in the emerging field of metatranscriptomics might enable us to study traits in field communities
Niche	Dual niche of AM fungi in root and soil	•AM fungi are obligate symbionts, but not only are they required to colonize a root system to complete their life cycle, but also to forage in the soil for nutrients and water •Thus, they are affected by factors both within and outside the root system at the same time •The composition of AM fungal communities is different in the two compartments (Hempel *et al.* 2007), and it is likely that forces governing soil and root communities are different (Liu *et al.* 2012). •AM fungal species differ in functional traits regarding spatial niches (for example, to what extent do they colonize roots or soil), and these traits are also conserved (Hart & Reader 2002; Powell *et al.* 2009)

Bipartite networks	How does the network theory describe host–AM fungal interactions (Chagnon *et al.* 2012)?	•AM fungal–plant networks regularly show nestedness (species interact with a subset of the species generalists interact with) and modularity (species tend to group into modules in which interactions are more frequent than with the rest of the community (Öpik & Moora 2012; Verbruggen *et al.* 2012). •These network characteristics may derive from overdominance of the founder AM fungus (Dumbrell *et al.* 2010a), habitat heterogeneity, specific selectivity in plant–AM fungal associations, plant–AM fungal overlapped phenology or AM fungal competition within the root (Montesinos-Navarro *et al.* 2012) •However, in order to correctly apply network theory to AM fungal–plant interactions, basic assumptions need to be verified, that is, detected co-occurence must imply interactions (Caruso *et al.* 2012b)

Here we introduce the elements of a community assembly and coexistence model by highlighting recent research on AM fungal communities. As examples for each element, we included studies that used DNA-based methods (preferentially, high-throughput sequencing approaches) to investigate AM fungal communities.

Factors affecting AM fungal community assembly: review of the elements of the proposed model

Regional pool

AM fungi have species pools with distinct composition according to paleocontinents, although endemic species are rare (Kivlin *et al.* 2011; Davison *et al.* 2015). Regionally, observed AM fungal communities are spatially heterogenous, but temporally stable, suggesting a fairly constant soil species pool from which mycorrhizae form during the season (Davison *et al.* 2012).

Dispersal and chance (neutral processes)

Propagules and vectors of dispersal in AM fungi AM fungi disperse by autochthonous (local mycelium spread) and allochthonous propagules (spores and other inoculum, such as hyphal fragments or colonized root fragments from outside), with the allochthonous propagules being less important locally (Jumpponen & Egerton-Warburton 2005). AM fungi often have large spores, and many species are distributed by zoochory (for example, through the guts of rats (Janos *et al.* 1995), earthworms (Shapiro *et al.* 1993) and collembolans (Klironomos & Moutoglis 1999) or on the hooves of bison (Lekberg *et al.* 2011) as opposed to wind, where their spores are detected rarely (Egan *et al.* 2014). Thus, AM fungal species are mostly limited to short-distance dispersal. However, over long timespans, these limited dispersal capabilities allow for a surprisingly efficient spread of taxa (Davison *et al.* 2015).

Spatial community structure, dispersal limitation and other stochastic processes AM fungal communities are spatially structured, patchily distributed even in relatively homogenous local environments (Rosendahl & Stukenbrock 2004; Mummey & Rillig 2008), which suggests that there are other processes beyond environmental filtering that contribute to the structure of AM fungal communities, for example, dispersal limitation. The relative importance of dispersal to environmental filtering is scale-dependent and varies (soil type and dispersal ability, Lekberg *et al.* 2007; soil pH, C/N ratio, phosphorus and dispersal, Dumbrell *et al.* 2010b; soil temperature, plant biomes and dispersal, Kivlin *et al.* 2011). Dispersal and other neutral processes thus exhibit an effect size spectrum that can be completely masked by extreme environmental heterogeneity or anthropogenic disturbance, resulting in communities more dissimilar than expected under the assumptions of neutral theory. On the other hand, stochastic effects are also limited under very homogeneous environmental conditions because of niche effects (Caruso *et al.* 2012a).

Environmental filter

Niche partitioning along environmental gradients

The assembly process and the coexistence of AM fungi are influenced by various soil environmental variables, such as pH, soil type, soil chemistry and nutrient availability. As nutrient transport is a function of AM fungi, the effect of nutrient availability is well studied (reviewed in Johnson 2010). The filtering role of the environment, when some species from the regional pool are not present under certain soil conditions, was shown in fertilizer addition experiments: AM fungal phylotype diversity decreased with increasing N and P availability and some AM fungi were only found in specific soil nutrient conditions (for example, (Liu *et al.* 2012; Camenzind *et al.* 2014; Liu *et al.* 2015).

Seasonality

AM fungi show temporal niche partitioning over the course of the year. Previously rare types might replace the dominant species (Husband *et al.* 2002). As possible explanations for this shift, both changing environment, for example, changes in temperature and sunshine hours (which influence the soil carbon pool, Dumbrell *et al.* 2011), and the seasonal cycle of the plant community and phenology were suggested.

Disturbance

Increasing agricultural land-use intensity selectively removes rare AM fungal species from the local community (Helgason *et al.* 1998; Verbruggen *et al.* 2012). Heavy anthropogenic disturbance, such as plowing, tillage and fungicide treatment, can lower the number of AM fungal species, abundance and root colonization while favoring generalist species (Helgason *et al.* 1998, 2007; Hijri *et al.* 2006; Schnoor *et al.* 2011). However, disturbance does not always shift communities in a predictable way (Lekberg *et al.* 2012) probably because of the dominance of stochastic effects (Caruso *et al.* 2012a).

Host filter

One of the particular features of AM fungal community assembly is the importance of the host filter compared with free-living or facultatively symbiotic organisms. Plants restrict AM fungal diversity in roots (Johnson *et al.* 2004) and they also differentially influence sporulation (Eom *et al.* 2000). Given the obligatory symbiotic AM fungal lifestyle, the existence of host effects could be obvious. The non-evident detail in the host–AM fungal relationship is the apparent lack of

species-level specificity (there are many fewer AM fungal species than plant species, even though most land plants are mycorrhizal). As an explanation, it was proposed that being able to colonize and to be colonized by a wider range of partners has an evolutionary benefit, and that environmental conditions affect the ability of plants to differentially reward their symbionts (reviewed in Walder & van der Heijden 2015). In the field, different plant species, and even plants of the same species at different growth stages, associate with different fungal communities from the same soil (Gollotte *et al.* 2004; Sýkorová *et al.* 2007; Gosling *et al.* 2013) and some AM fungi do not colonize certain plants (Helgason *et al.* 2007). AM fungi differ regarding how beneficial they are for hosts (Helgason *et al.* 2007), and plants are able to reward better fungal partners with photosynthates (Bever *et al.* 2009; Kiers *et al.* 2011). The solution might be that host specificity does not happen at the species level, but on an ecological level, where generalist AM fungi interact with generalist plants while specialists tend to occur in the roots of specialist plants (Öpik *et al.* 2010; Davison *et al.* 2011). Furthermore, pairings of hosts and symbionts with similar life history strategies (competitive, stress tolerant, ruderal, as described for AM fungi in (Chagnon *et al.* 2013) are likely more beneficial. The functional traits defining these strategies are often conserved at a higher taxonomic level (Maherali & Klironomos 2007; Chagnon *et al.* 2013). Not only the host itself but also neighboring plants (Hausmann & Hawkes 2009) and plant species richness (Burrows & Pfleger 2002; Engelmoer & Kiers 2015) influence fungal communities. In addition, AM fungal preference regarding hosts also exists (Davison *et al.* 2011).

Non-host biotic interactions and feedbacks

Horizontal interactions between members of local AM fungal communities

Past work has found intense competition for root space (Cano & Bago 2005; Engelmoer *et al.* 2014) and even competitive exclusion (Hepper *et al.* 1988). As opposed to root colonization, the ability of AM fungal species to colonize soil did not influence coexistence (Maherali & Klironomos 2012). Phylogenetic overdispersion promotes coexistence: communities of more distantly related and functionally different species showed higher realized species richness (Maherali & Klironomos 2007). Conserved differences in other functional traits, such as timing of spore production and hyphal growth rate, metabolism of photosynthates, P and N uptake, might alleviate competition as well.

Despite the potential importance for commercial use of fungal inocula, the effect of arrival order in AM fungi is not well understood. Priority effects were shown (Mummey *et al.* 2009); however,

it was recently observed that the resident AM fungi did not suffer from reduced growth despite being invaded, which makes competition for space an unlikely explanation, and suggesting downregulation by the host instead (Werner & Kiers 2015).

Interactions with other non-host organisms

Negative interactions with consumers (fungal grazers), pathogens and parasites could reduce competition between AM fungi. However, collembola feeding on AM fungi had no effect on the community composition (Gange 2000), and parasitism has not yet been conclusively shown to exist in AM fungi (Purin & Rillig 2008). Either these interactions are really not important for AM fungal communities or we are limited by data.

AM fungi harbor bacteria associated with their spores. These bacteria promote hyphal growth and stimulate nutrient biodynamics. They might facilitate not only the fungus, but the whole mycorrhizal system by contributing to the suppression of soil-borne plant pathogens and by adding nitrogen fixation to the benefits of the plant (Cruz & Ishii 2011).

Feedbacks: AM fungi as ecosystem engineers

AM fungi significantly modify their habitat both in the soil and in the plant in a way that influences their own communities. In the soil they increase soil aggregation and the water stability of the aggregates by a variety of mechanisms, including hyphal enmeshment (Rillig *et al.* 2015). Greater particle size and pore space may in turn benefit hyphal growth (Rillig & Steinberg 2002).

They affect plant diversity and composition by improving the nutrient status of their host plants and by facilitating their hosts, which was shown to induce shifts in plant communities (van der Heijden *et al.* 1998). To harness this effect, enhancing natural AM fungal communities is suggested as an environmentally friendly weed-control option in agricultural ecosystems (Cameron 2010). On the other hand, plant community composition also has an effect on AM fungal communities, completing the feedback loop.

Relative importance of different elements: possible explanations for the idiosyncratic response of AM fungi to biotic and abiotic variables

Despite the considerable literature that exists on the host, abiotic environmental and neutral factors influencing AM fungal community composition, there is no consensus on their relative importance. AM fungi have an idiosyncratic response to these variables. We propose two hypotheses to explain this pattern.

'Law of the minimum': an idea from plant nutrition

In agricultural science, the 'law of the minimum' is an idea that the scarcest essential nutrient (the most limiting factor) is the most important in determining plant growth (Gorban *et al.* 2011). Similarly, but stepping away from only thinking about resources, the relative importance of assembly factors would depend on the most restrictive component, and the most limiting factor would explain the most variability. Under non-filtering environmental conditions, in an abiotically homogenous sampling area, host effects would be relatively more important. A strong environmental gradient that includes harsh conditions unsuitable for certain species would result in environmental filtering as the dominant structuring force.

Scale dependency: different assembly rules for different spatial scales? An analogy borrowed from parasite communities

Studies on AM fungal communities vary strongly in the spatial scale being addressed. Definitions range from AM fungi found in a root piece through an entire root system to a mixed root sample of an entire site. As different assembly factors act on different scales, explicitly considering the spatial structure of AM fungal communities could lead to a synthesis between contrasting responses to assembly factors (for example, host versus abiotic environmental filter). Parasitology defines a hierarchical, host-based, scale-dependent community system (Figure 4.2 and Table 4.1). In infra- and compound communities of fleas, which also have varying levels of host specificity, the relative importance of environmental and host effects depends on the spatial scale (Linardi & Krasnov 2013; Krasnov *et al.* 2015). AM fungal communities have a similar host-based hierarchical spatial structure (Figure 4.2); therefore, it is a compelling idea that the relative importance of assembly factors depends on the spatial scale in AM fungi too. Maherali & Klironomos (2012)

hypothesized that subplot-scale interactions (infracommunity) such as competition could determine coexistence, whereas the AM fungal composition of a whole site (compound community) would mostly depend on niche requirements or climate (environmental filter). Consequently, AM fungal communities are found to show phylogenetic clustering within study sites (Kivlin *et al.* 2011), with sometimes negligible effects of the environment (Horn *et al.* 2014), which might indicate facilitation between species. At a global scale, the AM fungal community composition was shown to be best predicted by spatial distance, edaphic and climatic factors, and plant community type (Kivlin *et al.* 2011; Davison *et al.* 2015). To sum up, the scale dependency of the relative importance of the elements of community assembly and coexistence is well established in many organisms; however, explicit consideration of spatial scale in AM fungal community studies is still rare. An example of how the relative importance of the assembly processes might change with spatial scales is shown in Figure 4.2.

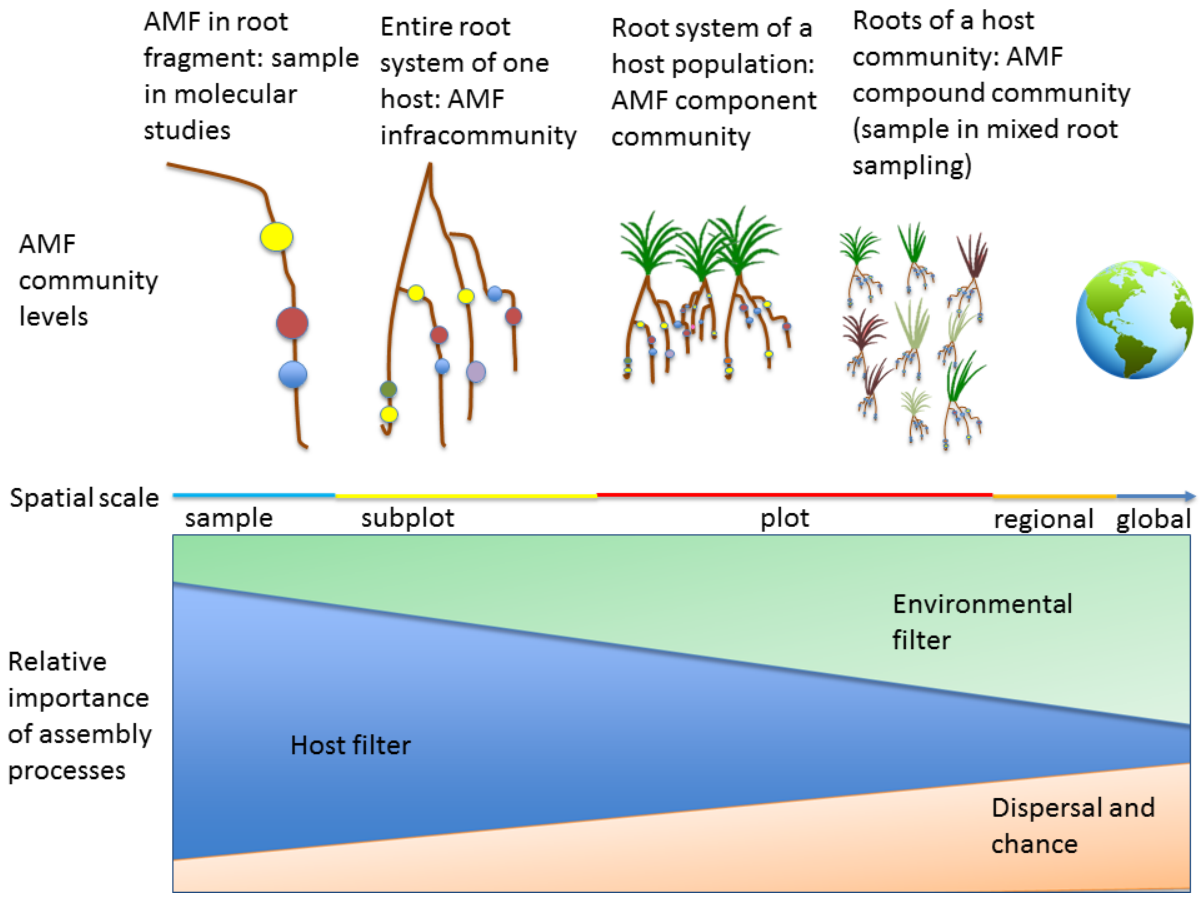

Figure 4.2: *Hierarchical scale-dependent community system in AM fungi. At a given spatial (or temporal) scale, multiple processes influence the assembly of AM fungal communities. Relative importance of assembly processes changes with spatial scale, causing idiosyncrasy in response to different assembly factors, when the hierarchical spatial structure is not explicitly considered.*

Conclusion: community ecology from the viewpoint of a microbial symbiont

We presented a conceptual framework of community assembly and coexistence adapted to a microbial symbiont group with a unique combination of characteristics. The importance of factors influencing obligate symbionts differs from those affecting free-living organisms, or even facultative symbionts (Linardi & Krasnov 2013). The host–AM fungal relationship, similarly to parasites, exhibits a hierarchical spatial structure, which should be explicitly incorporated into future studies, to enable the study of the scale dependency of the relative importance of elements of community assembly. Adapting a symbiont-centered point of view in addition to considering how the host community is affected would help to fill the knowledge gaps of coexistence research, especially in the field of non-host interactions.

Outlook: how further research on AM fungal communities could advance the field of community assembly and coexistence theory

Owing to the advance of high-throughput molecular methods, researchers gained insight into the communities of specialized organisms, for example, the AM fungal communities in plant roots. With the number of AM fungal community studies rising, it is now possible to start to piece together the mechanisms influencing community assembly and coexistence. By considering the unique combination of characteristics in genetic makeup, physiology, niche and dispersal of AM fungi, and highlighting problems in applying community ecology concepts stemming from these, we are getting closer to adapting community assembly and coexistence models to them.

Taking levels of community organization related to the host into account (infracommunities, component communities and compound communities, see Figure 4.2) can help reconcile contrasting results regarding the relative importance of assembly factors.

In AM fungi, where the effect of the host filter is so significant, non-host biotic interactions, although they might not be able to act as a filter in community assembly, are still influencing

community structure, and future studies in this currently neglected field might reveal more interesting relations.

Examining different assembly and coexistence factors in a multitude of specialized microbial groups would help advance the field of community ecology by increasing the external validity of its models and theories. Although it is important to transfer concepts from general ecology, it is critical that these concepts be carefully evaluated before application (Table 4.1): two examples are the application of metacommunity concepts to symbiotic systems (Veresoglou *et al.* 2012) and the use of network theory in mycorrhizal ecology(Caruso *et al.* 2012b); in both cases it is important to verify the validity of assumptions lest analyses be misleading. Emerging concepts in community ecology, like metrics for quantifying intransitive competition (Soliveres *et al.* 2015) or community coalescence (Rillig *et al.* 2015), will require similar validation to apply them to specific microbial communities. Doing so can lead to new hypotheses in the AM fungal and broader community ecology, as in applying community phylogenetics (Webb *et al.* 2002; Vamosi *et al.* 2009) to AM fungi: after carefully proving that AM fungal traits related to spatial niche use are conserved at a higher taxonomic level (Maherali & Klironomos 2007), this was used to generate hypotheses and a theoretical framework on the coupling of plant and AM fungal life history strategies (Chagnon *et al.* 2013).

Answers to the questions of community assembly and coexistence in AM fungi are increasingly required in order to more successfully manage AM fungi for application. Community composition influences ecosystem services, which is true also for AM fungi (van der Heijden *et al.* 1998). Better understanding of AM fungal communities could be a powerful tool in mitigating the effects of global change, for example, in agriculture and habitat restoration.

Chapter 5: General Discussion

Molecular methods in the study of AM fungal communities

Molecular studies enable the examination of AM fungal communities that are actively interacting with their plant partners. In this work, we studied AM fungal community composition and structure with the intention to infer ecological processes that generate these patterns. To achieve this goal, we made use of the only recently available high-throughput nature of molecular methods both by analyzing a large number of root communities (chapters 2 and 3), and by synthetizing knowledge from studies using similar methods (chapter 4).

Since the completion of these studies, the developments in the technology of high throughput DNA sequencing have not stopped. The manufacturing of 454 pyrosequencing platforms has been discontinued, which restarted the process of standardizing AM fungal metabarcoding. Pipelines for general fungal metabarcoding (Lindahl *et al.* 2013) including one using the ITS primers on the Illumina MiSeq platform have been published (Bálint *et al.* 2014). To sum up the advantages and disadvantages for studies specifically concentrating only on AM fungi, the Illumina platform recommended by the above pipelines allows for high read numbers, further improving the resolution of community surveys. However, currently the longest read length possible is 300 base pairs, which is shorter than the last incarnation of 454 pyrosequencing (GS FLX Titanium XL+) which provided a read length up to 1000 base pairs. Even though it is possible to achieve slightly longer reads by reducing the overlap between paired reads and adding sample distinguishing barcodes by tagmentation, there is a trade-off between read length and read quality. This is a problem for sequence-based AM fungal community surveys, the majority of which uses SSU markers around 600 bp long (Öpik *et al.* 2014). Perhaps the way of the future is further perfecting developing new platforms, like the single-molecule real time sequencing system of PacBio (Schloss *et al.* 2016), which has been suffering from high error rate.

The importance of host effects in AM fungal communities

For AM fungi, as they are obligate symbionts, the host has a unique dual importance both as a host filter in the community assembly (**chapter 4**) and as part of their habitat, where host traits can be understood as niche axes. After showing in **chapter 2** that plant identity influences AM fungal community composition and structure, it was a logical step to investigate whether plant

traits and plant phylogeny can be linked to these effects, for which a community phylogenetics framework was used. **Chapter 3** showed the importance of intrinsic and extrinsic plant traits, such as root and leaf morphology and ecological preference. The study of root traits, including morphological traits are increasingly in the focus of researchers and plant breeders alike (Bishopp & Lynch 2015), not only because of the direct effects of traits on resource uptake, but also due to of their influence on soil structure and microbiota, including symbionts (Bergmann *et al.* 2016). Taking into account the spatial configuration of AM fungal communities in root systems by the adaptation of a hierarchical community system similar to those used in parasite ecology is a promising solution to reconcile often contradictory responses of AM fungi to biotic and abiotic variables (**chapter 4**).

AM fungal communities and land use

Land use intensification is a major driver of changes in biodiversity and possibly, as a functional consequence, ecosystem services. Land use intensity is constantly increasing on a global scale, causing land degradation on approximately 23% of the globe's terrestrial area (Stavi & Lal 2015). Despite this, land use effects are only well documented for a few select taxa. By being included in such a large-scale and long term framework as the Biodiversity Exploratories, we advanced the knowledge on the effects of land use intensity on AM fungal communities with a systematic study of a high number of Central European grasslands. Grasslands, where AM fungi are abundant and diverse, cover approximately 40% of the world's continental surface and provide important ecological services, including providing an important carbon sink (Scurlock & Hall 1998). We have shown that land use influences AM fungal community composition and structure, in a complex, host plant specific manner (**chapter 2**). As opposed to very intensive agricultural disturbance, moderate land use is not necessarily detrimental to the richness of AM fungal communities. However, the nestedness of moderate and low land use communities within high land use communities, and the dissolution of phylogenetic clusters with increasing land use intensity (**chapter 3**) suggests a negative effect on beta diversity on a landscape level.

The above results underline the importance of sustainable land management techniques in preventing the degradation of soil biodiversity. Due to their manifold services to plants, mycorrhizal technology, including engineering communities of AM fungi and associated microbes has great potential in the sustainable intensification of agriculture (Rillig *et al.* 2016). Application of mycorrhizal inoculum should not be the exclusive focus of next generation mycorrhizal

technology, and even more subtle ways of soil microbiome management should be accompanied by careful monitoring of potential biogeochemical, exotoxicological and soil biodiversity effects (de Souza Machado *et al.,* in press.).

Interaction of the host and the abiotic environment

The heterarchically structured (Cumming 2016) system of influence and feedback of the host, the abiotic environment and the symbiotic communities on each other, where none of the components remains unchanged is becoming more and more evident: AM fungi which significantly modify their habitat both in the soil and in the plant in a way which in turn influences their communities are a good example (see **chapter 4, Feedbacks: AM fungi as ecosystem engineers**). Several exciting question around the edges of this "interaction triangle" exist (Fig. 5.1).

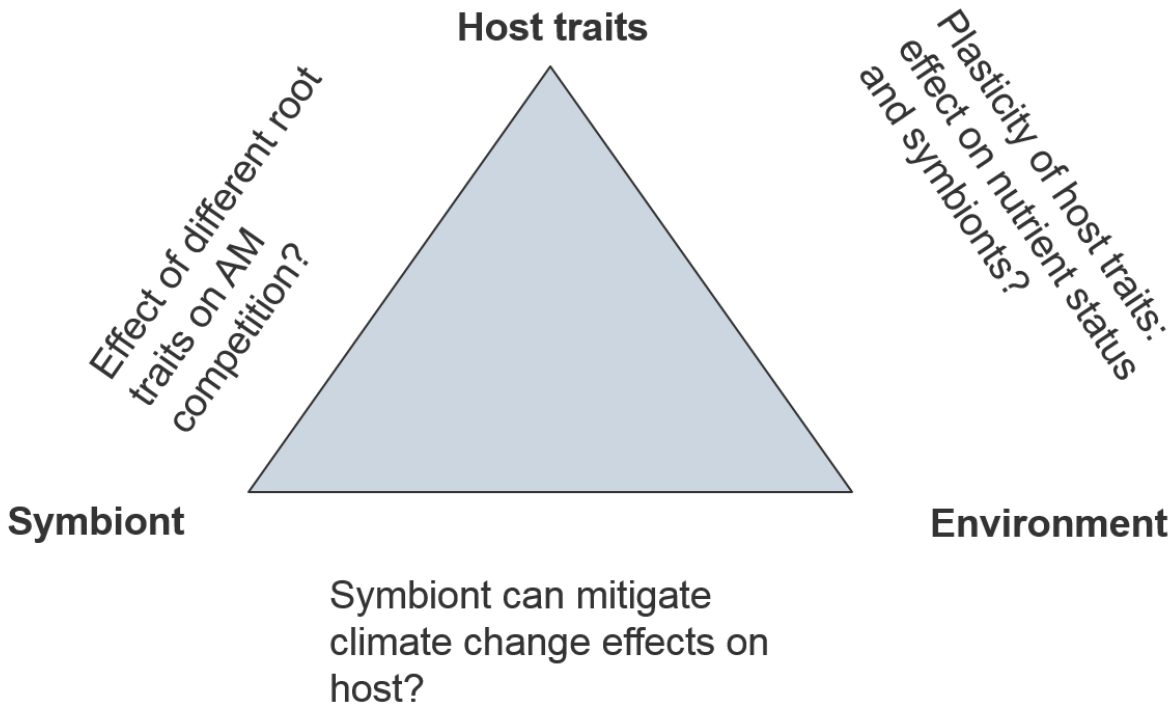

Figure 5.1 Host-symbiont-environment interaction triangle and future research questions.

For example, for the arbuscular mycorrhiza, along the edge of host-symbiont interaction, a further examination of the effects of root traits on symbiotic communities and competition among them would be of interest. As most morphological root traits were correlated, other architectural and physiological root traits, like cortical structure and physicochemical characteristics of root

exudates should be considered in addition (Bardgett *et al.* 2014). Regarding the environment's effect on host traits, certain root traits were shown to be plastic in response to environmental conditions (de Vries *et al.* 2016) with possible consequences to soil nutrient status and the symbiont communities. For example, elevated CO_2 promoted a significant response across several root traits, including an increase in root length, diameter, mycorrhizal colonization and root C (Nie *et al.* 2013). Inoculation with AM fungi was able to change root morphology and protect crop plants from environmental (salt) stress (Sheng *et al.* 2009; Fan *et al.* 2011), completing the interaction triangle.

Future directions for AM fungal community ecology in the light of global change

Modeling host trait – symbiont community – environment interactions would be useful even in the efforts of mitigating climate change impacts. Such an ecosystem model was already introduced for corals and dinoflagellate algae (Ortiz *et al.* 2014).

Better understanding of AM fungal communities could be a powerful tool in mitigating the effects of global change, for example, in agriculture and habitat restoration, as AM fungi may play a key role in regulating ecosystem responses to environmental change at local to global scales. Despite increasing knowledge on their specific functions, many classical models, for example soil food web models, do not take the diversity of mycorrhizal fungi into account, including the important distinct functions of AM and ectomycorrhizal fungi in the decomposition and transportation of organic C (de Vries & Caruso 2016). A detailed understanding of AM fungal traits and those of other functional groups is urgently needed to revise these models. Even though this is a challenging task, metabolomics, compound-specific isotope analyses, metatranscriptomics (as suggested in **chapter 3**) and improved high-throughput sequencing and barcoding approaches are increasingly available to facilitate it. Incorporating the synthesis of growing numbers of studies from different habitats will increase understanding of the functioning of AM fungi and other symbiotic groups under environmental change.

This work aimed to unify high-throughput molecular methods with the conceptual advances of modern community ecology in order to shed light on the patterns and processes influencing a group of important, but previously cryptic organisms. As an ambition for the future, it would be most advantageous to join forces with ecologists focusing on trait based or modeling methods to continue this exciting quest and to advance the field of symbiont ecology.

Chapter 6: Summary

In this dissertation I present studies on the community ecology of arbuscular mycorrhizal (AM) fungi, a group of formerly cryptic plant symbionts of formidable importance. Such studies have been made possible to a formerly unprecedented extent by the use of state-of-the-art, high throughput DNA sequencing methods. The chapters discuss the composition, structure, assembly and coexistence of AM fungal communities and the influence of both host and environmental factors on them.

Chapter 1 provides a brief introduction to the importance of AM fungi, to molecular methods employed in studying their communities, the Biodiversity Exploratories project (the framework in which the research in chapters 2 and 3 were carried out) and outlines the aims of the following chapters.

Chapter 2 is a study of intraradical AM fungal communities in the Hainich sampling area of the Biodiversity Exploratories, which represents a gradient of different combinations of grazing, mowing, and fertilization typical of Central Europe. Using 454 pyrosequencing, it shows that host plants significantly differed in AMF community composition, while land use modified this effect in a plant species-specific manner. Communities in medium and low land-use sites were subsets of high land-use communities, suggesting a differential effect of land use on the dispersal of AMF species with different abundances and competitive abilities. The chapter demonstrates that in these grasslands, there is a small group of highly abundant, generalist fungi which represent the dominating species in the AM fungal community.

In **chapter 3** the number of samples and sampling areas was increased to enable an in-depth examination of AM fungal communities in the roots of different host plants using a community phylogenetics and host trait based framework. At the same time, the land use intensity framework and the molecular methods were kept the same. The influence of host phylogeny and land use, but especially host plant traits (root and leaf morphology, ecological preference) are shown in this chapter. The observed phylogenetic structure of root communities, which is different from previously published results from soil, suggests the influence of the root environment in limiting the effect of land use intensification, and at the same time emphasizes the effect of host filtering

in relation to competitive exclusion between co-colonizers in determining AM fungal communities.

While chapters 2 and 3 focused on particular factors that are important in the specific context of Central European grasslands, **chapter 4** aimed at synthetizing results from research employing contemporary molecular methods. It reviews elements of community assembly and coexistence of AM fungi, addresses the problems of the application of modern community ecology to the special characteristics of AM fungi, and suggests that the hierarchical spatial structure of these communities might account for different assembly patterns for different spatial scales.

Finally, **chapter 5** gives a general evaluation of the results, and discusses them in the light of the most recent developments in molecular methods and AM fungal ecology, with some outlook to the most exciting potential future research directions.

Chapter 7: Zusammenfassung

In dieser vorliegenden Dissertation präsentiere ich meine Studien zur Gemeinschaftsökologie arbuskulärer Mykorrhizapilze (AM Pilze) – eine Gruppe dereinst kryptischer Pflanzensymbionten mit eindrucksvoller Bedeutsamkeit. Diese Studien wurden ermöglicht durch bisher beispiellose, moderne Hochdurchsatz-DNA-Sequenziermethoden. In den vorliegenden Kapiteln wird diskutiert wie der Pflanzenwirt und Umweltfaktoren die Zusammensetzung, Struktur und Koexistenz von AM Pilzlebensgemeinschaften beeinflussen.

Kapitel 1 bietet eine kurze Einführung zur Bedeutung von AM Pilzen und molekularen Techniken, die entwickelt wurden um deren Gemeinschaft zu untersuchen sowie Informationen zum Projekt der Biodiversitätsexploratorien, in dessen Rahmen die Studien für Kapitel 2 und 3 durchgeführt wurden. Dieses Kapitel schließt mit einer Aussicht auf die nachfolgenden Kapitel.

Kapitel 2 ist eine Studie über intraradikale AM Pilzlebensgemeinschaften in Hainich – einem der Beprobungsareale der Biodiversitätsexploratorien, welches einen Landnutzungsgradienten verschiedener, typischer Kombinationen aus Beweidung, Mähen, Düngung aufweist, die typisch sind für Zentraleuropa. Mittels der Technik der 454 Pyrosequenzierung konnte gezeigt werden, dass Wirtspflanzen signifikant in der AM Pilzlebensgemeinschaftszusammensetzung variieren, während der Landnutzungsgradient diesen Effekt weiter in Art-spezifischer Weise beeinflusst. Lebensgemeinschaften in Flächen mit niedriger und mittlerer Nutzungsintensität waren Untergruppen der Lebensgemeinschaften aus den intensiv genutzten Flächen. Dies legt einen differenzierten Effekt der Landnutzung auf die Ausbreitung von AM Pilzen mit unterschiedlicher Abundanz und kompetitiven Fähigkeiten nahe. Dieses Kapitel verdeutlicht, dass es in diesen Grasländern kleine Gruppen hochabundanter, generalistischer Pilze gibt, welche die dominanten Arten in den AM Pilzlebensgemeinschaften darstellen.

In **Kapitel 3** wurde die Beprobungsanzahl und -fläche erhöht, um eine tiefergreifende Untersuchung der AM Pilzlebensgemeinschaften in Wurzeln verschiedener Wirtspflanzen mittels Lebensgemeinschaftsphylogenie und Wirtsmerkmalen zu ermöglichen. Der Landnutzungsgradient sowie die molekularen Techniken wurden aus der vorherigen Studie übernommen. Der Einfluss der Wirtsphylogenie, des Landnutzungsgradienten, aber im Besonderen der der Wirtsmerkmale (z.B. Wurzel- und Blattmorphologie, ökologische

Präferenzen) werden in diesem Kapitel präsentiert. Die beobachtete phylogenetische Struktur der Wurzellebensgemeinschaften, welche sich von bisher publizierten Ergebnissen in Boden unterscheiden, legt nahe, dass die Wurzelumgebung die Intensität des Landnutzungsgradienten limitieren kann. Zugleich kann der Wirt als Filter agieren und co-kolonisierende Arten, die unter Konkurrenz stehen, beeinflussen und somit die AM Pilzlebensgemeinschaft formen.

Während **Kapitel 2** und **3** sich auf bestimmte Faktoren, die bedeutsam sind für den spezifischen Kontext zentraleuropäischer Grasländer, fokussieren, zielt Kapitel 4 auf die Synthese von Ergebnissen der Forschung zu zeitgenössischen molekularen Methoden ab. Hier werden die Elemente der Lebensgemeinschaftszusammensetzung und Koexistenz von AM Pilzen zusammengetragen und bewertet sowie die Probleme der Anwendung moderner Lebensgemeinschaftszusammenfügungen auf die besonderen Merkmale der AM Pilze dargestellt. Schließlich wird vorgeschlagen, dass die hierarchische, räumliche Struktur dieser Lebensgemeinschaften die verschiedenen Zusammensetzungsmuster auf verschiedenen räumlichen Skalen erklären kann.

Abschließend in **Kapitel 5** wird eine generelle Bewertung der Ergebnisse gegeben und diskutiert diese im Licht der neusten Entwicklungen im Bereich der molekularen Methoden und AM Pilzökologie mit weiterem Ausblick auf die interessantesten und vielversprechendsten zukünftigen Forschungsrichtungen.

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Contribution to the publications

I. **Vályi, K**., Rillig, M.C. & Hempel, S. (2015). Land-use intensity and host plant identity interactively shape communities of arbuscular mycorrhizal fungi in roots of grassland plants. *New Phytol.*, 205, 1577–1586.

Own contributions: I lead the field sampling campaign of the grasslands in the Schorfheide Exploratory during the joint soil sampling of the Biodiversity Exploratories. I performed molecular laboratory work (library preparation for pyrosequencing) and subsequent bioinformatical analysis. I conducted the statistical analyses and wrote the manuscript.

II. **Vályi, K**., Rillig, M.C., Joana, Bergmann & Hempel, S. (201X). Host and environmental control in arbuscular mycorrhizal fungal communities and the impact on phylogenetic clustering. *To be submitted.*

Own contributions: I lead the field sampling campaign of the grasslands in the Schorfheide Exploratory during the joint soil sampling of the Biodiversity Exploratories. I performed molecular laboratory work (library preparation for pyrosequencing) and subsequent bioinformatical analysis. I conducted the statistical analyses and wrote the manuscript.

III. **Vályi, K**., Mardhiah, U., Rillig, M.C. & Hempel, S. (2016). Community assembly and coexistence in communities of arbuscular mycorrhizal fungi. *ISME J.,* **10**, 2341–2351.

Own contributions: I conceived the ideas of the paper, performed literature research, wrote the manuscript.

Curriculum Vitae

For reasons of data protection,
the curriculum vitae is not included in the online version

Publications

de Souza Machado, AA, **Vályi, K** and Rillig, MC. (201x). Potential environmental impacts of an "underground revolution". *Trends in Ecology & Evolution* (in press).
Rillig, MC, Sosa-Hernandez, M, Roy, J, Aguilar-Trigueros, CA, **Vályi, K** and Lehmann, A: Towards an integrated mycorrhizal technology: Harnessing mycorrhiza for sustainable intensification in Agriculture (201x). *Frontiers in Plant Science*, doi: 10.3389/fpls.2016.01625 (*in press*)
Vályi, K, Mardhiah, U, Rillig, MC and Hempel, S (2016): Community assembly and coexistence in communities of arbuscular mycorrhizal fungi. *ISME J.*, **10**, 2341–2351.
Rillig, MC, Lehmann, A, Aguilar-Trigueros, CA, Antonovics, J, Caruso, T, Hempel, S, Lehmann, J, **Vályi**, K, *et al.* (2016). Soil microbes and community coalescence. *Pedobiologia (Jena)*, **59**, 37–40.
Vályi, K, Rillig, MC and Hempel, S (2015): Land-use intensity and host plant identity interactively shape communities of arbuscular mycorrhizal fungi in roots of grassland plants. *New Phytologist*, **205** (4), 1577-1586.
Vályi, K, Szécsy, O, Dombos, M and Anton, A (2013): Sampling design optimization on arable lands for integrated soil monitoring for sustainable production. *Communications in Soil Science and Plant Analysis*, 44(1-4), 178-194.
Vályi, K, Szécsy, O, Dombos, M and Anton, A (2010): Sampling optimization for complex soil monitoring. In: MONTABIO IV: *Complex monitoring system for analytical detection and biological evaluation of soil micropollutants for a sustainable environment*, Publisher: Plant Protection Institute, Hungarian Academy of Sciences, Ed.: Székács, A. pp.7-13. [Hungarian with English Summary]
Forró, L, Dombos, M, **Vályi, K**, Gubányi, A (2010): Temporal dynamics of microcrustacean assemblages in the Szigetköz, NW Hungary. In: *A Szigetköz állattani értékei [Zoological significance of the Szigetköz, NW Hungary]*, Publisher: Magyar Természettudományi Múzeum, Eds.: Gubányi, A, Mészáros,F pp.147-154. [Hungarian with English Summary]

International conference contributions

Host and environmental control in arbuscular mycorrhizal fungal communities and the impact on phylogenetic clustering. Talk. *Mycological Society of America*, Berkeley, CA, USA, 2016.

Land use intensity shapes the effect of plant identity on communities of arbuscular mycorrhizal fungi in roots of grassland plants. Poster. *33rd New Phytologist Symposium*, Zürich, Switzerland, 2014.

Effects of land-use and plant biodiversity on arbuscular mycorrhizal communities in roots;

problems and preliminary results. Poster. *7th International Conference on Mycorrhiza*, New Delhi, India, 2013.

Appendix A: Supplementary Information to Chapter 2

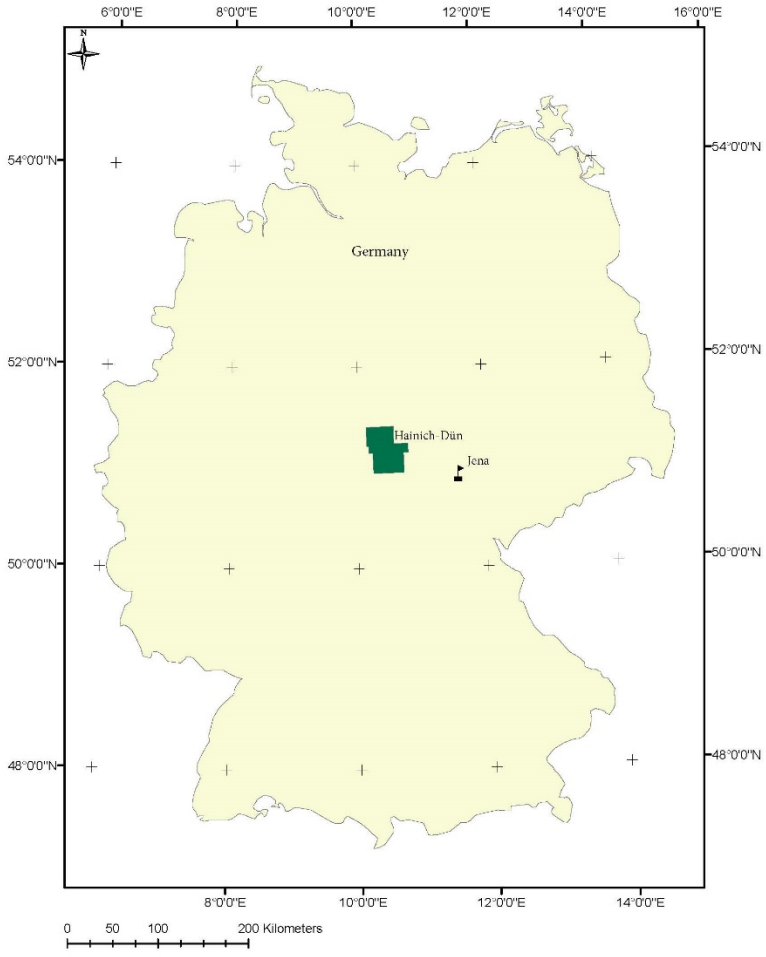

Figure S1a: Location of the sampling area (Hainich-Dün) in Germany, Europe. Modified from the map of the Biodiversity Exploratories sampling areas, by Jens Nieschulze, central data management, from the Biodiversity Exploratories Information System (BEXIS).

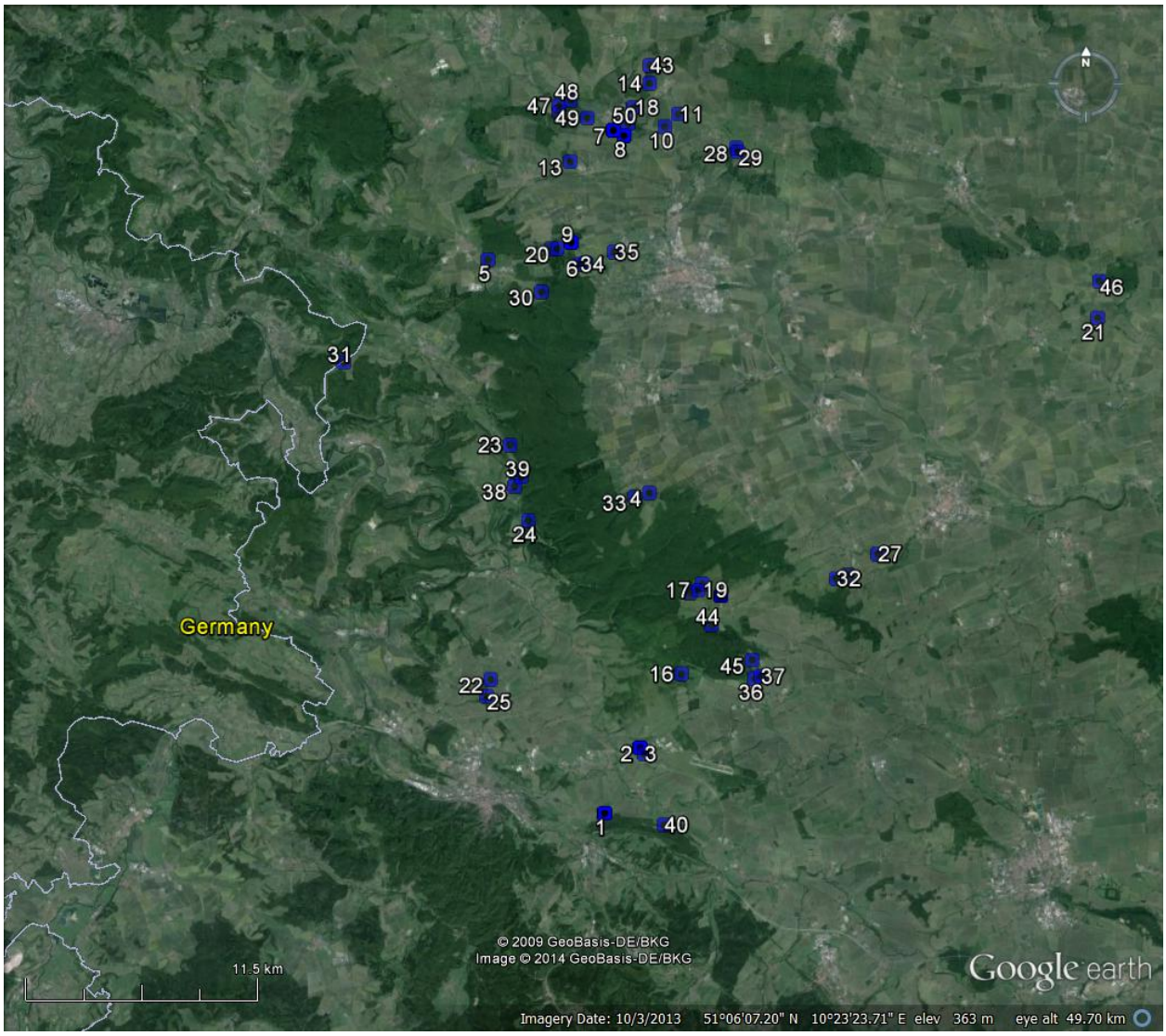

Fig. S1b.: Location of the sampling plots in the sampling area (Hainich-Dün) in Germany, Europe. Created from the plot coordinate database by Andreas Ostrowski, from the Biodiversity Exploratories Information System (BEXIS): http://exploratories.bgc-jena.

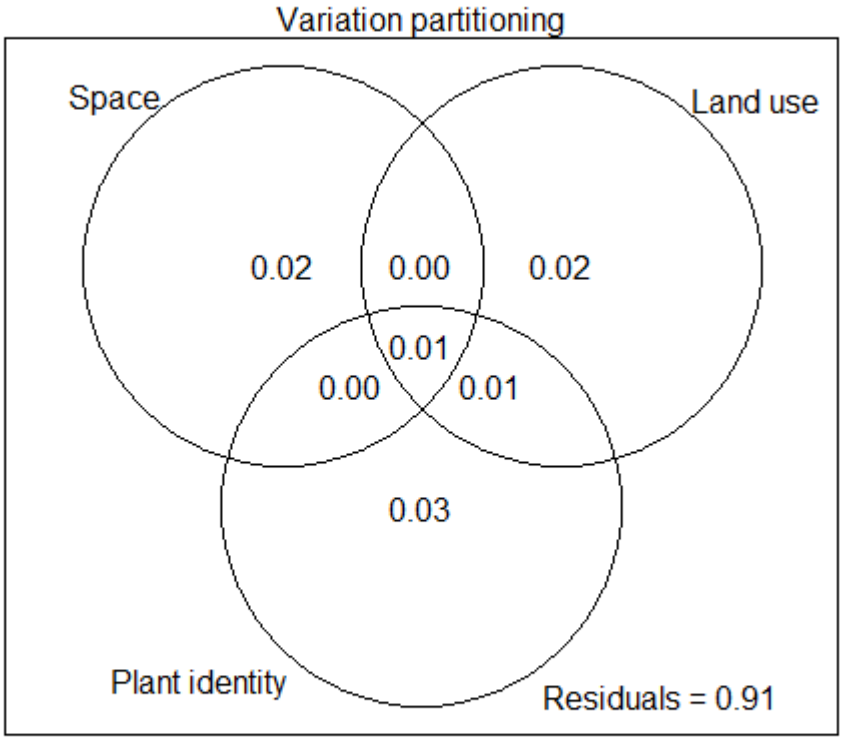

Fig. S2 *Variance partitioning of AMF community composition variance between land use intensity, plant identity and spatial data matrices using all 190 samples. Using rda and anova we have found that the components explained by all three matrices are significant*

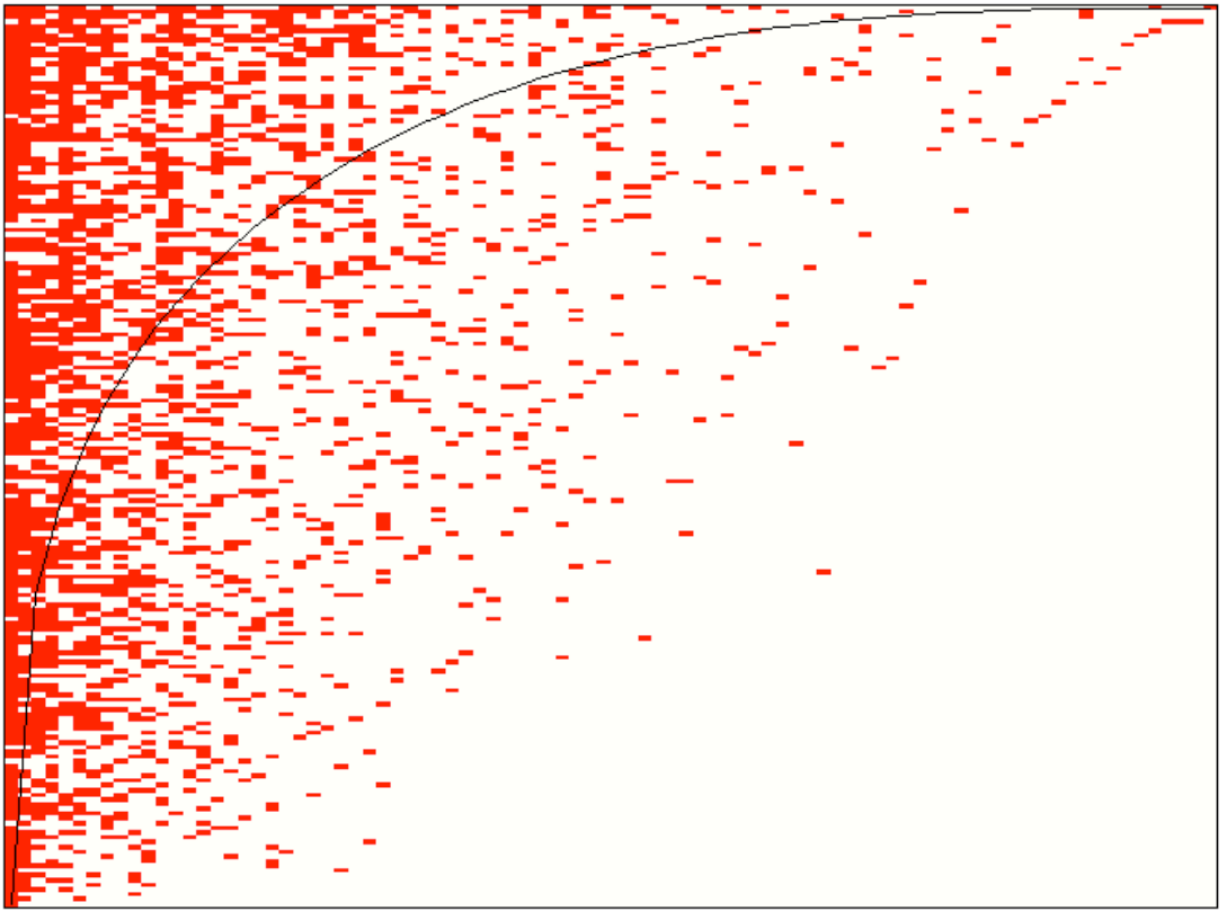

Fig. S3: Maximally stacked matrix of arbuscular mycorrhizal fungal presence in the sampling plots. Columns are virtual taxa, rows are samples (root pieces).

Table S1 Taxonomic information on the virtual taxa found in the resampled dataset and morphologically described species included where available. Source: MaarjAM database (status 31/03/2013), http://maarjam.botany.ut.ee/, VT files, family and genus names are taken from MaarjAM, morphological species nomenclature follows Redecker et al. (2013).

VT	Family	Genus	Morphological species [1]
VTX00030	Acaulosporaceae	Acaulospora	*A. scrobiculata*
VTX00231	Acaulosporaceae	Acaulospora	none
VTX00379	Acaulosporaceae	Acaulospora	none
VTX00008	Archaeosporaceae	Archaeospora	none
VTX00009	Archaeosporaceae	Archaeospora	none
VTX00245	Archaeosporaceae	Archaeospora	*Arch. trappei, Arch. schenckii*
VTX00056	Claroideoglomeraceae	Claroideoglomus	none
VTX00057	Claroideoglomeraceae	Claroideoglomus	none
VTX00193	Claroideoglomeraceae	Claroideoglomus	*C. claroideum, C. lamellosum, C. luteum, C. etunicatum, C. viscosum, Entrophospora infrequens*
VTX00225	Claroideoglomeraceae	Claroideoglomus	none
VTX00276	Claroideoglomeraceae	Claroideoglomus	none
VTX00340	Claroideoglomeraceae	Claroideoglomus	none
VTX00358	Claroideoglomeraceae	Claroideoglomus	none
VTX00054	Diversisporaceae	Diversispora	*D. celata, D. auratia, Otospora bareae, Entrophospora nevadensis*
VTX00061	Diversisporaceae	Diversispora	*D. epigaea, D. spurca*
VTX00263	Diversisporaceae	Diversispora	*D. spurca*

VTX00306	Diversisporaceae	Diversispora	none
VTX00347	Diversisporaceae	Diversispora	*D. trimurales*
VTX00380	Diversisporaceae	Diversispora	none
VTX00064	Glomeraceae	Glomus	*Septoglomus constrictum, Funneliformis africanum*
VTX00065	Glomeraceae	Glomus	*Funneliformis caledonium, F. geosporum, F. fragilistratum*
VTX00067	Glomeraceae	Glomus	*Funneliformis mosseae*
VTX00069	Glomeraceae	Glomus	*Sclerocystis sinuosa*
VTX00072	Glomeraceae	Glomus	none
VTX00074	Glomeraceae	Glomus	none
VTX00083	Glomeraceae	Glomus	none
VTX00105	Glomeraceae	Glomus	*Rhizophagus intraradices*
VTX00108	Glomeraceae	Glomus	none
VTX00113	Glomeraceae	Glomus	*Rhizophagus intraradices, Rh. fasciculatus*
VTX00114	Glomeraceae	Glomus	*Rh. intraradices, Rh. irregulare*
VTX00115	Glomeraceae	Glomus	*Rh. intraradices, Rh. irregulare, Rh. vesiculiferus*
VTX00125	Glomeraceae	Glomus	none
VTX00129	Glomeraceae	Glomus	none
VTX00130	Glomeraceae	Glomus	none
VTX00135	Glomeraceae	Glomus	none
VTX00140	Glomeraceae	Glomus	none
VTX00143	Glomeraceae	Glomus	none

VTX00148	Glomeraceae	Glomus	none
VTX00151	Glomeraceae	Glomus	none
VTX00153	Glomeraceae	Glomus	none
VTX00155	Glomeraceae	Glomus	*G. iranicum*
VTX00163	Glomeraceae	Glomus	none
VTX00165	Glomeraceae	Glomus	none
VTX00166	Glomeraceae	Glomus	none
VTX00167	Glomeraceae	Glomus	none
VTX00177	Glomeraceae	Glomus	none
VTX00185	Glomeraceae	Glomus	none
VTX00186	Glomeraceae	Glomus	none
VTX00187	Glomeraceae	Glomus	none
VTX00188	Glomeraceae	Glomus	none
VTX00195	Glomeraceae	Glomus	none
VTX00199	Glomeraceae	Glomus	*G. macrocarpum, G. hoi*
VTX00202	Glomeraceae	Glomus	none
VTX00212	Glomeraceae	Glomus	none
VTX00214	Glomeraceae	Glomus	none
VTX00219	Glomeraceae	Glomus	none
VTX00222	Glomeraceae	Glomus	*G. indicum*
VTX00234	Glomeraceae	Glomus	none
VTX00244	Glomeraceae	Glomus	none
VTX00295	Glomeraceae	Glomus	none
VTX00307	Glomeraceae	Glomus	none

VTX00324	Glomeraceae	Glomus	none
VTX00326	Glomeraceae	Glomus	none
VTX00333	Glomeraceae	Glomus	none
VTX00342	Glomeraceae	Glomus	none
VTX00344	Glomeraceae	Glomus	none
VTX00369	Glomeraceae	Glomus	none
VTX00387	Glomeraceae	Glomus	none
VTX00390	Glomeraceae	Glomus	none
VTX00395	Glomeraceae	Glomus	none
VTX00413	Glomeraceae	Glomus	none
VTX00416	Glomeraceae	Glomus	none
VTX00417	Glomeraceae	Glomus	none
VTX00281	Paraglomeraceae	Paraglomus	*P. laccatum*

[1] The apparent discrepancy between VT nomenclature and morphological species nomenclature is due to the mismatch of morphologically defined species to VT, which varies throughout Glomeromycota (Öpik et al., 2014), due to some morphological species with yet unresolved phylogenetic position and due to the fact that not all nomenclatural changes recently suggested for Glomeromycota (Redecker et al. 2013) have been integrated into MaarjAM.

Appendix

Methods S1: PCR conditions.

For PCR I. and II. a/b KAPA HiFi PCR Kit was used (Kapa Biosystems, Boston, Massachusetts, USA).

PCR I., per well

1 µl DNA extract

Mastermix:

Fidelity Buffer: 5 µl

dNTP: 0.75 µl

GlomerWT0: 0.75 µl of 20 µM primer solution

Glomer1536: 0.75 µl of 20 µM primer solution

H2O 16.25 µl

KapaHifi 0.5 µl

PCR I program (minutes:seconds)

Once:

95°C - 3:30

---

5 times:

98°C - 0:20

60°C* - 0:15

72°C - 0:30

---

25 times:

98°C - 0:20

55°C - 0:15

72°C - 0:30

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Once:

72°C - 03:30

4° C - ∞

*=-1° / cycle

PCR IIa/b per well (two reactions in parallel)

PCR I + 25 microliter H2O: 0,75 microliter

NS31_A_MID: 0,75 microliter of20 µM primer solution (A is Adaptor A for Pyrosequencing, MID is the barcode)

Mastermix:

Fidelity Buffer: 5 µl

dNTP: 0.75 µl

AM1a_B or AM1b_B: 0.75 µl of 20 µM primer solution

H2O: 16.75 µl

KapaHifi 0.5 µl

PCR IIa/b program (minutes:seconds)

Once:

95°C - 3:30

---

30 times:

98°C - 0:30

63°C - 0:30

72°C - 1:00

Once:

72°C - 05:00

4° C - ∞

Afterwards PCR II a+b products of the same sample were mixed.

Host plant identification

Per well/tube

1.5 µl DNA extract

Mastermix:

5 µl FIREPol® 5 x Master Mix "Ready to Load" (Solis BioDyne, Tartu, Estonia)

0.5 µl Trnl-C 20 µM primer solution

0.5 µl Trnl-D 20 µM primer solution

17.5 µl H_2O

Program (minutes:seconds)

Once:

98°C - 0:30

Appendix

35 times:

95°C - 0:30

50°C - 0:30

72°C - 2:00

Once:

72°C – 1:00

4° C - ∞

Appendix B: Supplementary material to Chapter 3

Methods S1: PCR Conditions: See Appendix A.

Table S1: Plant species and their frequencies, based on BLAST search of their trnL-intron sequences in the NCBI and matched with vegetation survey on the field. Root traits data: A=available, NA=not-available

Plant species name (NCBI)	Number of samples	Root traits data
Agrostis capillaris	8	A
Agrostis stolonifera	16	A
Alopecurus geniculatus	2	A
Alopecurus pratensis	40	A
Anthoxanthum odoratum	3	A
Anthriscus sylvestris	1	NA
Arrhenatherum elatius	73	A
Bellis perennis	1	A
Brachypodium pinnatum	12	A
Briza media	2	A
Bromus erectus	13	A
Bromus hordeaceus	11	NA
Bromus inermis	2	A
Carex flacca	1	A
Carex hirta	1	NA

Cerastium fontanum	1	NA
Cerastium holosteoides	1	NA
Dactylis glomerata	47	A
Deschampsia cespitosa	2	A
Elytrigia repens	50	A
Festuca ovina	3	A
Festuca pratensis	22	A
Festuca rubra	25	A
Helictotrichon pratense	3	A
Helictotrichon pubescens	7	A
Holcus lanatus	7	A
Juncus articulatus	1	A
Koeleria pyramidata	1	A
Lolium perenne	27	A
Luzula campestris	2	A
Phalaris arundinacea	3	A
Phleum pratense	4	A
Picris hieracioides	1	NA
Plantago lanceolata	7	A
Plantago media	1	A
Poa angustifolia	39	A
Poa pratensis	33	A
Poa trivialis	42	A
Prunella vulgaris	1	A

Ranunculus acris	1	A
Ranunculus bulbosus	1	A
Ranunculus repens	5	A
Sesleria albicans	1	NA
Stellaria graminea	2	A
Taraxacum sect. Ruderalia	1	A
Thymus pulegioides	1	NA
Trifolium montanum	2	A
Trifolium pratense	7	A
Trifolium repens	15	A
Trisetum flavescens	36	A
Urtica dioica	1	NA
Veronica chamaedrys	2	A