Linking the community structure of arbuscular mycorrhizal fungi and plants : a story of interdependence?

Linking the community structure of arbuscular mycorrhizal fungi and plants: a story of interdependence?

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Abstract

Arbuscular mycorrhizal fungi (AMF) are crucial to plants and *vice versa* but little is known about the factors linking the community structure of the two groups. We investigated the association between AMF and the plant community structure in the nearest neighborhood of *Festuca brevipila* in a semi-arid grassland with steep environmental gradients, using high-throughput sequencing of the Glomeromycotina (former Glomeromycota). We focused on the Passenger, Driver and Habitat hypotheses: i) plant communities drive AMF (passenger); ii) AMF communities drive the plants (driver); iii) environment shapes both communities causing covariation. The null hypothesis is that the two assemblages are independent and this study offers a spatially explicit novel test of it in the field at multiple, small scales. The AMF community consisted of 71 OTUs, the plant community of 47 species. Spatial distance and spatial variation in the environment were the main determinants of the AMF community. Community structure of the plant neighborhood of the focal plant was a poor predictor of AMF communities, also in terms of phylogenetic community structure. Some evidence supports the passenger hypothesis but the relative roles of the factors structuring the two groups clearly differed, leading to an apparent decoupling of the two assemblages at the relatively small scale of this study. Community phylogenetic structure in AMF suggests an important role of within-assemblage interactions.

Introduction

Arbuscular mycorrhizal fungi (AMF) are one of the most important symbiont groups for plants, forming relationships with the majority of land plants and playing a significant role in the acquisition of phosphorus (Smith and Read 2008). Yet, despite some important progress in recent years, especially in relation to interactions with other soil biota or how AMF respond to management (Alguacil *et al.,* 2014, Caravaca and Ruess 2014, Leifheit *et al.,* 2015, Knegt *et al.,* 2016), there are many aspects of the assembly processes regulating the community ecology of these organisms that are poorly understood: a key challenge remains disentangling the relative contribution of dispersal limitation, environmental filtering and biotic interaction on AMF community structure (Vályi et al. 2016). The cryptic nature of the group and the complexity of the three-way interaction between plants, AMF and the environment complicate the study of the factors that regulate AMF community structure. Dispersal limitation remains one of the most complex aspects of AMF ecology (Zobel and Öpik 2014): as for example reviewed in Vályi *et al.* (2016), AMF can disperse via local mycelium spread but also spores, hyphal fragments, and colonized root fragments, and the importance of these mechanisms could be scale dependent, although direct evidence is missing. Still, large AMF spores and fragments are mostly spread via zoochory, which implies limited dispersal capability andseems reflected by small scale patterns in community structure (Mummey and Rillig 2008; Dumbrell *et al.,* 2010a, Horn *et al.,* 2014). Still, the effects of dispersal limitations are entangled with those of environmental gradients, biotic interactions within the AMF assemblage, and between AMF and plants (e.g. Mummey and Rillig 2008; Dumbrell *et al.,* 2010a, Horn *et al.,* 2014, Martinez-Garcia *et al.* 2015, Garcia de Leon *et al.* 2016a, Garcia de Leon *et al.* 2016b).

The study of AMF in grasslands is of particular importance since grassland ecosystems cover a significant proportion of the earth's surface, harbor the majority of herbaceous plant

diversity (Shantz 1954), and it is in grasslands that AMF reach their highest abundance and diversity (Treseder and Cross 2006, Kivlin *et al.,* 2011). Studies on plant biodiversity in grassland ecosystems on small scales have revealed connections between species richness of AMF and plants (Hiiesalu *et al.*, 2014) and host plant effects on AMF community composition (Vályi *et al.*, 2015). Still, effects can be very localized: AMF can form extended hyphal networks but spatial autocorrelation in their distribution is typically found at sub-meter scales (Mummey and Rillig 2008), with a potential role for biotic interactions (Vályi *et al.,* 2016). To date, only a few studies have taken this fact into account and applied a sufficiently fine-grained sampling design for a solid statistical analysis of the patterns generated by local processes (Dumbrell *et al.,* 2010b, Horn *et al.,* 2014).

AMF and plants form two sets of communities associated with each other but assembled through different processes that take place at different spatial and temporal scales (Zobel and Öpik 2014). The plant set can drive the fungal set or vice versa (Fig. 1) but which group is driving might depend on successional stage, which is linked to differences in dispersal processes between plants and AMF. Zobel and Öpik (2014) have used the concept of difference in dispersal between AMF and plants to revisit the Driver and Passenger hypotheses originally proposed by Hart *et al.* (2001). Zobel and Öpik (2014) also formulated the Habitat hypothesis to distinguish a situation where AMF and plant communities co-vary but are not directly causally linked, as opposed to the null hypothesis of no co-variation ("independence"). For example, during primary succession, plants typically arrive before AMF and then act as a potential filter to AMF: AMF are Passengers as they are following plants. However, dispersal limitation in an established AMF assemblage can cause the AMF assemblage to more strongly determine which plants will establish during secondary succession: the AMF assemblage becomes the Driver (Zobel and Öpik 2014). Zobel and Öpik (2014) further predict that the Habitat hypothesis would be most common in regions with a

stable community (e.g. climax vegetation) where environmental variation within regions will cause a non mechanistic covariation between AMF and plant communities. The general null hypothesis is that plants and AMF may vary independently of each other, which could possibly happen at very broad or global scales, where plants are more disperal limited than AMF seem to be (Kivlin *et al.*, 2011, Öpik *et al.*, 2013, Davison *et al.*, 2015). Accordingly, Vályi *et al.* (2016) have recently proposed that the host effect is minimal on regional and global scales, when differences in optima such as optimal C:N:P ratios between AMF and plants (Johnson 2010) might become structuring.

There are studies that have touched upon components of these hypotheses. For example, AMF taxa are generally found to be able to colonize any AM (as opposed to non-AM) plant species (Klironomos 2000), although there may be a bias towards easily cultivable species (Ohsowski *et al.*, 2014) and "specificity" might be quantitative rather than qualitative (Vályi *et al.*, 2015). Prevailing AM fungal communities and plant communities may still be directly causally correlated despite the perceived generalism of the AM symbiosis. A thorough account of the studies supporting the various hypotheses is given in Zobel and Öpik (2014) and we are aware of only two recent, observational studies that have addressed the subject (Martinez-Garcia *et al.* 2015, Garcia de Leon *et al.* 2016). However, a problematic aspect of observational field studies remains to tease apart cause and effect in the correlations observable between the two organism groups in the presence of spatial structure in the environment (Fig. 1). To solve this problem, we applied a spatially explicit design to sample AMF and plant communities along a replicated steep but short (≈15m) soil environmental gradient (Horn et al. 2014). We could therefore control for spatial patterns and environmental effects when testing for the effects of plants on AMF communities and vice versa. We used a standardized focal plant of high abundance to investigate environmental, plant and AMF community variation at sufficiently small scales. We also took into account the phylogenetic

community structure of both plant and AMF assemblages to allow community relationships to occur at levels other than species/OTU between and within the groups.

Our main aim was to collect for the first time multiple scale and high spatial resolution data to test the general null hypothesis that plant community structure, including phylogenetic structure, is independent of AMF community structure and vice versa. If the hypothesis were rejected, given the scales included in the study, we aimed to collect support for one or more of the three alternative hypotheses (Fig. 1), with the overall goal of shedding light on the mutual relationships between plant and AMF communities

Methods

Study area and sample collection

Sampling was conducted in a nature protection area located in north-eastern Germany (Brandenburg, 52°27.778' N, 14°29.349' E), a Natura 2000 biodiversity hotspot which contains over 200 different plant species and combines floral elements of steppes and coastal habitats. Given the high diversity of plants (Ristow *et al.*, 2011) and AMF (Horn *et al.*, 2014), the area is very suitable for this study. We sampled by a hierarchical nesting of plots in April 2011: twelve 3 x 3m plots were sampled at the four corners of three 15 x 15m larger plots (henceforth called "macroplots") located on the slope of a hillside (Fig. S1). The distances between the macroplots ranged from 20 to 500m (Fig. S2), leading to overall inter-sample distances from a few cm to 3m (within a plot) and up to 500m between macroplots. The uphill-downhill axes of the three macroplots were characterized by a steep textural gradient from sandy-loamy (uphill) to highly sandy (downhill) soils (Fig. S3). Soil parameters varied significantly and to a large extent (e.g. almost 3 units of pH) along the texture gradient (Horn *et al.*, 2015).

We assessed the local AM fungal community in the roots and surrounding soil of *Festuca brevipila* plants plus the neighboring plant species around these *Festuca* plants. *Festuca brevipila* is one of the most abundant species in sampled plots (Ristow *et al.,* 2011, Horn *et al.,* 2015). Soil cores (5 cm radius, 15 cm deep) were taken from five *F. brevipila* plants per plot, resulting in 60 (5 plants x 12 plots) sampling locations. Each sample position was random within the plot (minimum distance of 30 cm between any two samples in the same plot, Fig. S1). Plant presence / absence was assessed in the surrounding area in a radius of 15cm around each soil core to target local interactions present in the rhizosphere of our focal plant (neighborhood plant community structure). This scale is consistent with the minimal observed spatial autocorrelation of AM fungi (30-100 cm, Mummey and Rillig 2008).

Soil cores, including roots and plant material, were stored at -20°C prior to analysis. Each soil core was thoroughly homogenized and subsampled for soil chemical analyses (supplementary information part *a*.). We measured water content, pH, carbon, nitrogen and phosphorus content of the soil, which are known to affect AMF community variation (Camenzind *et al.,* 2014, Horn *et al.,* 2014, Horn *et al.,* 2015). Additionally, dehydrogenase activity was assessed as a proxy for microbial activity. Roots were washed in Millipore water before analysis.

DNA extraction, 454-pyrosequencing and OTU delineation

We extracted genomic DNA twice from each core, once from 150 mg of washed, fine-ground *Festuca brevipila* roots and once from 250mg of soil material which was sieved through a 2mm mesh. We used the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc.) following the procedure in the manufacturer's manual. We then created 454-pyrosequencing amplicon pools for the AMF using a nested PCR design. We utilized the AMF-specific primer set SSUmAf and LSUmAr for the first and SSUmCf and LSUmBr for the second, nested PCR

(Krüger *et al.*, 2009). We amplified a region spanning genes for the small ribosomal subunit (SSU), the complete ITS region and a part of the large ribosomal subunit (LSU). Subsequently, amplicons of about 600bp in length were created from the AMF-specific PCR fragments using general fungal primers located in the LSU gene modified with 454 adapters and sample specific barcode sequences (Supplementary Information part *b*). The 454 sequencing was done on a Roche GS FLX+ system with titanium chemistry at the Göttingen Genomics Laboratory at the Georg-August University of Göttingen.

Sequences were denoised using the PyroNoise approach (Quince *et al.*, 2009) implemented in Mothur (Schloss *et al.*, 2009). The denoising approach removes bad quality sequences, creates sequence clusters and removes chimera sequences. After denoising and preclustering, sequences from roots and soil were clustered into operational taxonomic units (OTUs) using CROP (Hao *et al.*, 2011), which utilizes a Bayesian clustering algorithm. This approach addresses species delineation uncertainty better than hierarchical clustering methods due to its flexible cut-off, thereby creating significantly less artifact OTUs than fixed cut-off clustering approaches (Hao *et al.*, 2011). We checked the final OTU sequences against chimeras using the Mothur implementation of the uchime algorithm and the Krüger et al. (2012) SSU-ITS-LSU alignment, as well as the slayer algorithm against the sequences themselves. Default settings were used for both algorithms.

Due to the nature of pyrosequencing, we found differences in read numbers for every sampling location, so we resampled the read numbers to equal amounts of 500 reads per sample using a bootstrap approach with 10,000 iterations per sample (Efron 1979, Wehner *et al.*, 2014). Samples with considerably lower read numbers than the estimated resampling threshold (less than 350 reads, equal to 70% of the resampling threshold) were discarded prior to resampling. Additionally, singletons were removed. All subsequent statistical analyses were done in R 3.1 (R Core Team 2015).

194

195 *Phylogenetic tree calculation*

196 OTUs were annotated according to the results of a BLAST search against the NCBI
197 nucleotide database (nt) prior to phylogenetic tree calculation. We calculated a phylogenetic
198 tree for the AMF OTUs using RAxML (Stamatakis 2006) in order to further refine the OTU
199 definitions following our approach from a previous study (Horn *et al.,* 2014). About 110
200 representative sequences of an SSU-ITS-LSU AMF reference alignment (Krüger *et al.,* 2012)
201 plus an out-group sequence from the Chytridiomycota were added to our own sequences to
202 determine the phylogenetic position of our OTUs. With the help of the phylogenetic tree we
203 removed sequences which clustered outside the Glomeromycotina and are therefore likely to
204 be erroneous or non-AMF sequences.

205

206 *Null model analysis and Phylogenetic community structure*

207 In order to account for non-random species associations potentially linked to biotic influences
208 of AMF and plant on each other, we performed null model analysis on plant and AMF
209 species, respectively. Null models were created in EcoSim (Gotelli and Entsminger 2012;
210 details in Supplementary Information part *c*)

211 We included phylogenetic sorting of the respective communities as a potential driver of
212 community structure (Horn *et al.,* 2014). This approach tests the hypothesis that the
213 relationship between AMF and plant communities is reflected at a phylogenetic level
214 including, but not restricted to species/OTUs. We analyzed phylogenetic diversity (PD)
215 within the AMF and plant communities separately. We chose the Daphne plant tree for our
216 plant phylogenetic analysis (Durka and Michalski 2012), which provides a complete set of
217 phylogenetic distances for our plant dataset. Phylogenetic distances between AMF OTUs
218 were calculated using the Needleman-Wunsch implementation of Esprit (Sun *et al.,* 2009).

The distances between plant species were calculated as pairwise distances from the trimmed Daphne phylogenetic tree using the cophenetic.phylo function of the ape package (Paradis *et al.*, 2004). Using the picante package (Kembel *et al.*, 2010), we obtained two estimates of PD: the standardized effect size of mean pair wise distance (SES-MPD), which calculates the net relatedness index (NRI) from beta-diversity with a null model, and inter-community mean pair wise distance (IC-MPD), i.e. phylogenetic distance between communities (Supplementary Information part *d*). The mean values of the NRIs of all samples of AMF were then used as the alpha-diversity measure to judge the clustering (positive) or segregation (negative) of the overall AMF or plant community. IC-MPDs were calculated as pair-wise phylogenetic distances of the samples, based on pair-wise genetic distances between OTUs and plant species. In order to include the IC-MPD information in a subsequent variance partitioning analysis (Legendre and Legendre 1998, Caruso *et al.*, 2012), the distance matrices of plants and AMF were subjected to a principal coordinate analysis (PCoA).

Models of correlations between plants and AMF

To robustly test the null hypothesis of the study (i.e. independence), we used three main multivariate and multiple regression analysis based on redundancy analysis (Horn *et al.*, 2015 and supplementary information part *e*) to quantify how plant community variation was affected by variation in phylogenetic distance and community structure of AMF, plus the vice-versa analysis using plant phylogenetic community structure and plant community structure as a predictor of AM fungal community structure.

To visualize patterns of community structure, we used Principal Coordinate Analysis (PCoA), a generalization of ordinary PCA (Legendre and Legendre 1998) that is also the basis of distance based RDA. For AMF, PCoA was applied to Hellinger transformed data to prevent inflation in the weights of rare OTUs and work on an ecologically meaningful

Euclidean space (Legendre and Legendre 1998). For plants, PCoA was applied to the Jaccard distance matrix of the presence/absence matrix. We also used the kriging estimator (Ribeiro and Diggle, 2001) to display spatial structures in environmental variables and the PCoA axes. PCoA axes of the two assemblages were also plotted on a scatter plot to visualize correlation between the assemblages. We used Moran eigenvector mapping (MEM) to account for spatial autocorrelation at multiple scales (Dray *et al.,* 2006, Legendre *et al.,* 2009, Supplementary Information part *e*): the analysis produce a number of vectors that describe spatial patterns in species distribution at all the spatial scales resolvable by the sampling design. These vectors are sometimes referred to as "spatial factors" or "spatial effects", which implicitly describe spatial variation that may originate from a multitude of factors such as spatially structured environmental variation but also spatial variation not related to environmental variation, and/or unmeasured but spatially structured factors such as dispersal and biotic interactions. Spatial effects independent of environmental variables are often called "pure space" (e.g. Legendre and Legendre 1998).

We then used redundancy analysis and variance partitioning to test and quantify the effects of the community structure of one group on the other group by controlling for other covarying effects (space, environment, phylogeny).

Finally, to increase the statistical power of multivariate analysis (Warton *et al.,* 2012) and so robustly test the null hypothesis , we also tested the generalized linear response of the relative abundance of AM fungal taxa to the plant community and vice-versa using the manyglm function from the mvabund package (Wang *et al.,* 2012, Warton *et al.,* 2012). The test was performed on residuals after removing the contributions of environmental and spatial covariates.

All multivariate calculations were done in R, using the vegan (Oksanen *et al.,* 2012), the spacemakeR (Dray 2011) and geoR (Ribeiro and Diggle 2001) packages.

Results

454-pyrosequencing and OTU delineation

The clustered and denoised data set consisted of 325 putative AM fungal OTUs. During the resampling, we removed seven root and one soil sample based on minimal read numbers of 500 reads. Species accumulation curves showed a sufficient sampling depth (Fig. S5). After resampling and removal of singletons, 88 OTUs remained of which 17 were removed since they clustered outside the Glomeromycotina subphylum (former Glomeromycota, see Spatafora et al. 2016, after Schüßler et al. 2001) as it is currently described. This resulted in a total of 71 OTUs used in all subsequent analyses. The OTUs found in our tree span all known AMF families, indicating a fairly exhaustive covering of the Glomeromycotina subphylum(Fig. S5). The root data set eventually consisted of 68 OTUs and the soil dataset of 62 OTUs. Overall OTU richness per macroplot was comparable between these datasets, ranging from 30 to 43 in roots and from 28 to 43 in soil (Table 1). The dominant fungal groups in our soils and roots were *Glomus* spp. and *Rhizophagus* spp.

Community structure of AMF excluding plants

The AMF community was significantly segregated at the level of the entire dataset. However, for the AMF communities in root samples the effect was significant only for one of the macroplots and the whole dataset (Table 1). For the soil community two out of three macroplots had significantly segregated assemblages and effect sizes were considerably higher in soil than in root data sets (Table 1).

There were no significant NRI differences overall. Neither the root nor the soil sets of the phylogenetic data showed significantly segregated or aggregated communities on a per-macroplot or per-data-set basis.

All measured environmental variables display a clear spatial gradient along the uphill direction (see four examples in Fig. 2), although sometimes with a component of variaion along the direction orthogonal to the uphill direction. At the macroplot scale, the spatial gradient in the first two axes of the PCoA of AMF (accounting for almost 2/3 of total variance) follow the environmental gradient more than the equivalent PCoA axis of plants do (Fig. 3). When we excluded plants from the analysis and removed spatial effects, the effect of the measured environmental variables (pH, water content, C, N, C/N ratio, phosphorus, dehydrogenase activity) on AMF community structure was overall low. With an exception of the root data set from one macroplot, environmental data explained less than 10%. Pure space was a major predictor of the overall data set and within each macroplot, showing significant and large proportions (up to 31%) of explained variation (Table S2). Phylogeny was the second largest explanatory component in the variance partitioning of the AMF without plants. As described above, up to 30% of variation could be explained by the phylogenetic distance of the AMF in our data set (Table S2). Additionally, we found the spatial-phylogenetic effects accounted for a large fraction of the AMF variance.

AMF-plant correlations

A PCoA ordination of all samples from all plots show that the plant assemblage seemed the most structured spatially: macroplot 3 clustered against macroplot 1 and 2 (see also Fig. 4). The same clustering was not observed in AMF, neither in root nor in soil. Scatter plots (Fig. 5) correlating the first two PCoA of AMF and plants revealed that gradients in the community structure of the two assemblages are correlated but with a confounding effect of spatial patterns at the broad scale separating the three macroplots (see for example Fig 5a and c). Still, after filtering out spatial autocorrelation plant community structure accounted for a statistically significant amount of variation in the root AMF community, while plant

phylogeny was not a significant predictor (Table 2). Instead, when we used the AMF community as a predictor of the plant community, the variation explained by the fungi was very low and not significant (Table S3). Overall, these results reject the null hypothesis of the study although the amount of variation uniquely attributable to the effect of plants on AMF is small (Table 2). GLM results were consistent with these results: plant community structure had significant effects on the AMF community in roots ($P<0.001$) and soil ($P<0.001$) but AMF communities did not show any significant effects when used as a predictor of plant community structure.

Discussion

Is the community structure of AMF independent of that of plants?

AMF and plants may affect each other's community dynamics depending on spatial and temporal scale, the latter especially in relation to succession (Zobel and Öpik 2014). Evaluating which group is driving which other group is challenging because both groups may influence each other to some extent and possibly at different spatial and temporal scales (Martinez-Garcia *et al.* 2015, Garcia de Leon *et al.* 2016). Also, in stable ecosystem (e.g. climax) regional covariation between AMF and plants could arise as the effect of environmental gradients (Habitat hypothesis). Our results reflect this complexity in plants and AMF interactions in a species rich grassland area at a range of small spatial scales but made clear some important points. First, AMF community variance is mostly accounted for by spatial factors and phylogenetic distance patterns in OTU composition. Second, plant communities are also strongly influenced by the soil environment. Overall, AMF and plants showed different spatial structures and the relative roles of the tested factors neatly change between plant and AMF, which rules out the Habitat hypothesis. Also, the strong influence of spatial factors on AMF communities could have offered some support to the Driver

hypothesis if this played some role but we did not find an effect of AMF on plants (Zobel and Öpik 2014). Instead, when plant communities were used as a predictor of AMF, after taking into account all other effects (i.e. environment, space), we found a significant effect of plants on AMF communities. We can thus reject the statistical null hypothesis that the groups are independent. Specifically, there is some support for AMF being Passengers. We have to note that reversing response and predictors (i.e. AMF passenger or driver) in these multivariate statistical models is not trivial. For example, there is additional and not invertible information in the phylogenetic trees of each set of species.

Notwithstanding the aforementioned technicality and the statistical rejection of the null hypothesis, the complex set of correlations linking plants and AMF are weak and barely significant (whatever group plays the role of predictor or response), which implies that the interaction between plants and AMF are weak at the community level: plant community structure remains a modest predictor of AMF community structure compared to the other predictors employed in the analysis.

All these results are overall consistent with theory (Zobel and Öpik 2014): the scale of the study is relatively small, with a steep but short soil environmental gradient replicated a number of times at various distances (within plots and between plots), from tens of meters to a few hundred meters. At these scales, we can expect absence of or not strong dispersal limitation for plants but some dispersal limitation in AMF, and the textural gradient sampled along the hills may mimic a primary succession gradient in the plant assemblage (Horn et al. 2015). Under these conditions, the passenger ”effect” should be at its strongest.

Which mechanisms could underlie the observed patterns? More specifically, if AMF are passengers why is the effect of plants apparently weak? It has been shown that plants may reward the best fungal partners with more carbohydrates (Bever *et al.*, 2009, Kiers *et al.*, 2011, Verbruggen *et al.*, 2012) and that particular plant communities may cause the

development of specific AMF communities (Hausmann and Hawkes 2009). This is consistent with our observation that the neighborhood plant community of a dominant focal plant is a significant although not very strong predictor of the AMF community in its roots. Interestingly, we observed this effect only for the root assemblage and not for the soil assemblage and plant community phylogenetic structure seems to play no role in these effects.

The weakness of the observed effects of plant communities on AMF communities may be particular to the study system. For instance, the dominance of *Glomus* spp., *Rhizophagus irregularis* and other generalist taxa may cause effects to be less strong than in systems with higher evenness and/or specialist taxa. Another potential explanation is that other ecological interactions overwhelm the effect, as evidenced from the non-random phylogenetic community pattern of the AMF assemblage. Also, the grassland is dominated by several C3 grasses, which are not very dependent on mycorrhiza (Reinhart *et al.,* 2012), and there is increasing evidence that these plants associate with generalist AMF taxa (Helgason *et al.,* 2007, Öpik *et al.,* 2009, Vályi *et al.,* 2015).

Are AMF communities assembled through interspecific interactions?

As recently reviewed by Vályi et al. (2016), AMF communities are structured by a range of different processes, including environmental filtering, dispersal and biotic interactions (Lekberg *et al.,* 2007, Peng *et al.,* 2009, Dumbrell *et al.,* 2010a, Dumbrell *et al.,* 2010b, Silva and Batalha 2011). Biotic interaction at the interspecific level could play a major role in some cases. For example, negative interactions between AMF species competing for the same root space may result in the superior competitor persisting on the root (Hart *et al.,* 2001, Thonar *et al.,* 2014). In addition, greenhouse studies as well as field observational work has shown that net phylogenetic distance patterns are connected to co-occurrence (Maherali and Klironomos

2007, Horn *et al.,* 2014) and AMF traits are phylogenetically conserved (Powell *et al.,* 2009) For example, mechanisms such as facilitation or feedbacks between plants and AMF could be signaled by net phylogenetic distance patterns in community structure if closely related species received similar facilitation (Anacker *et al.,* 2014). Here, the AMF assemblage resulted strongly segregated while phylogenetic aggregation or segregation patterns were not significant but with mean pairwise distances between communities overall quite low. This slightly contrasts with a previous analysis of AMF communities in the same sampling area as well as findings from other authors, which found local species pools to be phylogenetically aggregated (Kivlin *et al.,* 2011, Saks *et al.,* 2014, Horn *et al.,* 2014, Grilli *et al.,* 2015). However, the overall segregation in a non-phylogenetic null model analysis was found in both studies and may point to processes of a competitive nature, which seem independent of phylogenetic distribution and dispersal limitation. Integrating all the available evidence, including previous work (Horn *et al.,* 2014), AMF communities seem phylogenetically structured and very much determined by spatial structure in the environment and plants. Given the amount of variation accounted for by these effects and the fact that for plants environmental variation was the main structuring factor, we conclude that AMF communities in our sampling area assembled mostly independently of the plant community with a possibly important role of interaction within the AMF community. However, there is shared variation between environment, space and phylogenetically structured variation in AM fungal communities.

The processes behind shared variation (e.g., spatially structured covariation between environmental and phylogenetic variation) cannot be explained solely on the basis of observational evidence. Experimental work will in the future be necessary to understand how this shared variation is generated. As already suggested by Zobel and Öpik (2014), in an ideal experiment either the plant or AMF community should be kept constant while varying the

other community, also in relation to changing environmental conditions (e.g. soil properties such as pH) and different degrees of dispersal limitation. These experiments are challenging under field conditions but we suggest that surveying AMF communities in plant assemblages under a range of primary and secondary succession stages (e.g. Garcia de Leon *et al.* 2016) and manipulating vegetation to control the succession process will offer a valid starting point to move from patterns to the mechanisms. In that perspective, our study suggests to experimentally test for a potentially important role of biotic interactions within the AMF assemblage.

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Conflict of Interest

The authors declare no conflict of interest

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682 **Figure Captions**

683

684 **Figure 1.** Autocorrelation (Semivariogram) and trends in environmental variables create
685 (arrow a) spatial structure and environmental gradients. Variation in the environment
686 generates variation in plants and AMF (arrows b). AMF and plants can thus be structured by
687 changes in habitat conditions, which can then simply lead to covariation between the two
688 assemblages (Habitat hypothesis). Alternatively, AMF could either drive the plant
689 assemblage (Driver hypothesis, arrow c) or be driven by the plant assemblage (Passenger
690 hypothesis, arrow d). In all cases, the driving factors/assemblage (b, c, and d) have a spatial
691 structure that will be, at least partially, reflected by spatial structure in the driven assemblage.
692 This spatial dependence calls for a spatially explicit approach to the testing of the three
693 hypotheses. Spatial scale and successional stage have also been hypothesized to be the major
694 factors in determining which among the Habitat, Driver and Passenger hypotheses apply to
695 real systems. In addition to all these factors, AMF can also be structured by interactions
696 within the assemblage, independently of plants, which has been hypothesized to happen at
697 local scale and that could create very patchy distribution. All data are simulated.

698

699 **Figure 2.** Kriging interpolation of four of the measured environmental variables as measured
700 in one of the three macroplots (macroplot 1, see Supporting Information). Plots were by
701 construction aligned along a soil textural gradient on the slopes of a hillside (Fig. S1), with
702 the gradient running along the uphill-downhill axis (y-axis; Fig. S2 and 3). As we expected,
703 the main gradient in major soil variables followed the uphill-downhill axis, although in the
704 case of macroplot 1, water showed a patchy distribution.

705

706 **Figure 3**. Kriging interpolation of the first two PCoA (see also Fig. 4) axes of AMF and
707 plants. Data are shown for macroplot 1, and are so directly comparable to those shown for
708 environmental variables in Fig. 2. Spatial patterns in the structure of the two assemblages
709 appear to be only poorly correlated. Similar patterns were observed in the other macroplots
710 (not shown).

711

712 **Figure 4.** PCoA ordination plots of Plants and AMF. Individual samples are colour labeled
713 by macroplot (M1, blues; M2, red; M3, black) and symbol label in terms of uphill (up,
714 triangle) or downhill (down, square) position of individual samples within the macroplot (see
715 also Fig. S1). The plant assemblage appears to be more spatially structured in terms of the

716 separation between M3 and M2 + M1, with the latter two being geographically much closer
717 to each other (Fig. S2). This clustering pattern is not observed in AMF.
718
719 **Figure 5**. Bivariate covariation of PCoA 1 and 2 of both AMF (roots) and plants (see Fig. 4)
720 in all four possible combinations: a) PCoA1 AMF vs. PCoA1 plants; b) PCoA1 AMF vs.
721 PCoA2 plants; c) PCoA2 AMF vs. PCoA1 plants; d) PCoA2 AMF vs. PCoA2 plants. Pearson
722 correlation coefficient (r) and relative p-value (p) is reported for each set of correlations.
723 Individual samples are colour labeled by macroplot (M1, blues; M2, red; M3, black). Some
724 significant correlation is observed but seems driven by spatial structure between macroplots.
725 For example, in panel b and c, M3 samples are clustered on the right-hand side while in panel
726 d) the observed positive correlation between the PCoA2 axes of plants and AMF is driven by
727 variation internal to macroplot 1. These results suggest spatial dependence in the covariation
728 between AMF and plants.
729
730 **Tables**
731
732 **Table 1:** AMF phylogeny and null model results from community abundance data. Column
733 names are: sample size, numbers of OTUs; MPD, the mean pair wise phylogenetic distance
734 between individual communities (i.e. samples). Positive effect sizes (C-score) and mean pair
735 wise distances indicate segregated communities (species repel each other), while negative
736 values represent an aggregated community (species attract each other). MP = macroplot. The
737 rows "all MPs" show result across macroplots while the other rows within each macroplot.
738
739

			phylogeny	null model	
	sample size	OTUs	MPD	effect size	P
all MPs root	53	68	0.01	11.75	<0.001
MP1 root	16	43	-0.02	4.08	0.002
MP2 root	18	30	-0.07	1.13	0.137
MP3 root	19	43	0.00	-0.73	0.250
all MPs soil	59	62	0.01	19.42	<0.001

MP1 soil	20	41	0.08	10.96	<0.001
MP2 soil	19	28	-0.14	10.66	<0.001
MP3 soil	20	43	0.08	1.61	0.068

740

741

Table 2: Variance partitioning of the AMF community matrix with the plant community also included as a predictor of the AMF community. The table is divided in two main blocks: phylogeny and presence/absence of plants. These blocks refer to how the effect of plants on AMF was evaluated. In the first two columns of results (phylogeny, root and soil) the effects of plants (row wise) is assessed by using plant phylogeny as a predictor of AMF. In the second two columns (presence/absence, root and soil) we used plant community structure as predictor of AMF. The other predictors were environment or env (soil properties) and space (geographic position). The plus sign in the Source of variance column stands for shared variation (it is not the sum of the variances explained by each predictor, e.g. env + space is the spatially structured effect of the environment). Figures are percentage values of total variance. Significance: *** = P<0.001; ** = P<0.01; NS = not significant, NT = not testable.

Source of variance	phylogeny		presence/absence	
	root	soil	root	soil
environment	0 ^{NS}	0 ^{NS}	3 ***	0 ^{NS}
space	30 ***	29 ***	19 ***	24 ***
plants	0 ^{NS}	0 ^{NS}	4 **	0 ^{NS}
env + space	4 ^{NT}	3 ^{NT}	11 ^{NT}	5 ^{NT}
space + plants	0 ^{NT}	6 ^{NT}	11 ^{NT}	10 ^{NT}
env + plants	0 ^{NT}	0 ^{NT}	0 ^{NT}	0 ^{NT}
env + space + plants	3 ^{NT}	3 ^{NT}	0 ^{NT}	2 ^{NT}
unexplained	63	59	52	54