Abiotic rather than biotic filtering shapes the arbuscular mycorrhizal fungal communities of European seminatural grasslands

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Summary

- Although it is well known that arbuscular mycorrhizal fungi (AMF) play a key role in the functioning of natural ecosystems, the underlying drivers determining the composition of AMF communities remain unclear.
- In this study, we established 138 sampling plots at 46 grassland sites, consisting of 26 acidic grasslands and 20 calcareous grasslands spread across 8 European countries, to assess the relative importance of abiotic and biotic filtering in driving AMF community composition and structure in both the grassland soils and in the roots of 13 grassland plant species.
- Soil AMF communities differed significantly between acidic and calcareous grasslands. In root AMF communities, most variance was attributable to soil variables while very little variation was explained by host plant identity. Root AMF communities in host plant species occurring in only one grassland type closely resembled the soil AMF communities of that grassland type and the root AMF communities of other host plant species occurring in the same grassland type. The observed AMF-host plants networks were not modular but nested.
- Our results indicate that abiotic conditions, rather than biotic filtering through host plant specificity, are the most important drivers in shaping AMF communities in European semi-natural grasslands.

Keywords: abiotic filtering, modularity, nestedness, specificity, semi-natural grassland, calcareous grassland, *Nardus* grassland

Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous fungal symbionts that play a key role in the functioning of ecosystems worldwide (Smith & Read, 2008). They constitute root symbionts of up to 80% of all terrestrial plant species and supply nutrients to the host plant, protect them against soil pathogens and alleviate drought stress in exchange for plant-assimilated carbon compounds (Augé, 2001; Veresoglou & Rillig, 2012). Furthermore, AMF improve soil formation and soil aggregation through their large network of hyphae, which provides protection against soil erosion (Wilson *et al.*, 2009). By mediating competitive interactions among plant species, AMF communities also shape local plant species diversity and community composition (van der Heijden *et al.*, 1998; Dostalek *et al.*, 2013).

According to modern coexistence theory, two main hypotheses explain the structuring of AMF communities (HilleRisLambers *et al.*, 2012; Nemergut *et al.*, 2013). Firstly, the 'abiotic filtering' hypothesis states that local habitat conditions act as a main filter, selecting only the AMF taxa that are adapted to the abiotic conditions in that habitat. Strong supporting evidence for abiotic filtering comes both from studies comparing the AMF communities between distinct habitats (e.g. Moora *et al.*, 2014; Rodriguez-Echeverria *et al.*, 2017) and from studies that have evaluated the response of AMF communities to anthropogenic land use changes and natural succession (e.g. De Beenhouwer *et al.*, 2015; Valyi *et al.*, 2015). For instance, a study investigating AMF communities in the Gorongosa National Park in Mozambique revealed a strong differentiation of AMF communities between forested areas, flooded savannas and grasslands (Rodriguez-Echeverria *et al.*, 2017). Alternatively, the 'biotic filtering' hypothesis states that host plant species act as the major filter, selecting only specific AMF taxa that are the most compatible with the host (HilleRisLambers *et al.*, 2012). It has been suggested that plant host specificity may drive AMF community composition, although this hypothesis has been subject of debate for decades (Zobel & Öpik, 2014). Early research has concluded that AMF-host plant specificity must be very low because there are much more potential host species than AMF taxa (Stahl, 1949; Gerdemann, 1955). Although the number of known AMF taxa has strongly increased due to the use of metagenomic approaches (341 AMF virtual taxa; Öpik *et al.*, 2013), it remains very low compared to the *c.* 200.000 possible plant species they may associate with (Brundrett, 2009). Yet, evidence is increasing that biotic filtering in AMF is more important than previously thought. For instance, herbaceous plant species that co-occur in the same grasslands have been shown to harbor distinct AMF communities (Vandenkoornhuyse et al. 2003, Valyi et al. 2015, but see Honnay *et al.*, 2017). A meta-analysis of 435 crop inoculation experiments also showed that specific combinations of

AMF taxa and host plants were more beneficial for growth promotion as compared to others, suggesting a functional AMF-host plant specificity (Van Geel *et al.*, 2016).

If biotic filtering is important for structuring AMF communities, host plant species should be able to discriminate between specific AMF partners. Consequently, it can be expected that AMF-plant interaction networks are comprised of densely connected, non-overlapping subsets of taxa, i.e. that a modular network will be formed (Olesen *et al.*, 2007). Modularity has already been shown in mutualistic networks between plants and their pollinators (Olesen *et al.*, 2007), and between orchid species and orchid mycorrhizal fungi (Jacquemyn *et al.*, 2015). Montesinos-Navarro *et al.* (2012) showed that AMF-plant networks originating from xeric shrubland in Mexico were strongly internally connected, i.e. modular. Yet, the architecture of the AMF-host plant network has received relatively little attention (Torrecillas *et al.*, 2012), and large scale studies analyzing AMF-host plant networks are still lacking.

Despite the significance of AMF, insights into the relative importance of abiotic and biotic filtering on AMF abundance and distribution is still fragmentary (Zobel & Öpik, 2014; Valyi *et al.*, 2015), and hampered by a shortage of systematic data from natural ecosystems (Ohsowski *et al.*, 2014). Nevertheless, the recent availability of metagenomic approaches gradually increases our understanding by providing tools to detect the distribution and composition of AMF communities at a larger scale. To assess the relative importance of abiotic and biotic filtering on AMF community composition, we established 138 sampling plots in a total of 46 grassland sites spread across eight European countries comprising 26 acidic grasslands and 20 calcareous grasslands. AMF communities were determined in both the grassland soils and in the roots of 13 grassland plant species. We sampled plant species occurring in both grassland types, plant species exclusive to acidic grasslands and plant species exclusive to calcareous grasslands. More specifically, we tested whether: (i) soil and root AMF communities were characterized by distinct AMF communities between both grassland types, which would provide support for the hypothesis that AMF communities are shaped by abiotic filtering; or (ii) root AMF communities associating with different host plant species were distinct and modular in nature, independent of grassland type, which would provide support for the hypothesis that AMF communities are shaped by biotic filtering. To explicitly test for these hypotheses, we determined whether either soil variables, representing abiotic filtering, or host plant data, representing biotic filtering, explained most of the variation in soil and root AMF communities. Next, we also performed formal network analyses on root AMF communities. Finally, we investigated whether specific AMF taxa could be identified that are indicative for a particular grassland type or host plant species.

Materials and Methods

Study sites and sampling

For this study, we established 138 sampling plots in a total of 46 grassland sites between May and June 2013. Sampling plots were spread across eight European countries (Estonia, Sweden, United Kingdom, The Netherlands, Belgium, France and Switzerland; Fig. 1). The grassland sites consisted of 26 acidic grassland sites (*Nardus* grasslands) and 20 calcareous grassland sites, two common European semi-natural grassland types. The grassland sites were selected out of a dataset of 501 previously surveyed semi-natural grasslands in Europe (Ceulemans *et al.*, 2014).

At each grassland site, we randomly positioned three sampling plots of 1 × 1 m, ensuring that soil surface and vegetation composition in these plots was homogenous. For instance, no ditches, major soil disturbances or clumped dominance of single plant species were allowed within the plots. Per plot, we recorded the presence and abundance by visually estimating the relative surface area of all vascular plant species. Per plot, we also collected three pooled top soil samples (0-10 cm) with an auger of 2 cm diameter, yielding a total of 414 soil samples (138 sampling plots × 3 replicates). Finally, we took root samples of the three most dominant plant species and from 27 species that were included in a pre-determined list containing characteristic plant species that are commonly found in both grassland types, or are unique to one of both grasslands types (Table S1). Root samples were collected by digging up three separate individuals per species per plot with a small clump of soil. Both soil samples and root samples were stored in water tight zip lock bags at 4 ºC prior to laboratory analyses. Ultimately, only roots of the 13 plant species that were present in at least five records were used for further analyses of AMF communities (*Achillea millefolium* subsp. *millefolium*, *Agrostis capillaris, Antennaria dioica*, *Briza media*, *Bromus erectus*, *Chamaespartium sagittale*, *Danthonia decumbens*, *Festuca rubra* subsp. *rubra, Nardus stricta, Pilosella officinarium*, *Potentilla erecta*, *Sanguisorba minor* and *Succisa pratensis*).

Soil chemical analyses

For each soil sample, soil pH was quantified using a glass electrode in a 1:10 soil/water mixture. As a measure of the plant-available N content of the soil, ammonium and nitrate were quantified by shaking 10 g of soil in 200 mL of 1 M potassium chloride solution for one hour. Extracts were analyzed colorimetrically using a segmented flow auto analyzer (Skalar, Breda, the Netherlands). As a measure of the plant-available P content of the soil, Olsen P values were quantified by shaking 2 g dry soil for 30 minutes with 0.5 M sodium bicarbonate at pH 8.5 and subsequent colorimetric analysis of the

extracts using the molybdenum blue method (Robertson *et al.*, 1999). Moisture content was quantified by the weight loss of 10 g of fresh soil after evaporation of water content at 105 °C. Organic carbon content was quantified by weight loss of 5 g of dry soil after combustion of organic matter at 700 °C.

DNA extraction, PCR amplification and pyrosequencing

We performed molecular analyses on both root and soil samples. For the root samples, we first carefully brushed off the soil clump around the roots and then rinsed the roots twice with sterile distilled water. Next, roots with a diameter of 3 mm or less of the three replicate plants per plots were mixed and then cut in pieces of less than 5 mm. Then, 100 mg of this root material was used to extract DNA, using the UltraClean Plant DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer's instructions. For the soil samples, we first removed any stones or roots and then thoroughly homogenized the sample. Subsequently, DNA was extracted from 250 mg soil using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer's instructions. PCR amplification of all root and soil DNA extracts was performed using the primer pair AMV4.5NF-AMDGR (Sato et al., 2005), as this primer pair is AMF specific and able to consistently describe AMF communities using 454 pyrosequencing based on the most variable part of the small subunit (SSU) rRNA gene region (Van Geel et al., 2014). 'Fusion' primers were designed according to the guidelines for 454 GS-FLX Titanium Lib-L sequencing containing the Roche 454 pyrosequencing adapters and a sample-specific MID barcode in between the adapter and the forward primer. In total, 60 MID barcodes (recommended by Roche, Mannheim, Germany) were used for sample-specific amplicon tracking. PCR reactions were performed on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) in a reaction volume of 20 µl, containing 0.15 mM of each dNTP, 0.5 µM of each primer, 1x Titanium Taq PCR buffer, 1U Titanium Taq DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), and 1 µl genomic DNA. Before amplification, DNA samples were denatured at 94°C for 2 min. Next, 35 cycles were run, consisting of 45 s at 94°C, 45 s at 65°C and 45 s at 72°C, followed by a final elongation of 10 min at 72°C. After separation of amplicons by agarose gel electrophoresis, amplicons within the appropriate size range were cut from the gel and purified using the Qiaquick gel extraction kit (Qiagen, Hamburg, Germany). Purified dsDNA amplicons were quantified using the Quant-iT PicoGreen® dsDNA Assay Kit and the Qubit fluorometer (both from Invitrogen, Ghent, Belgium), and pooled in equimolar quantities over ten amplicon libraries, each representing 60 samples tagged with a unique MID barcode. The quality of the amplicon libraries was assessed using the Agilent Bioanalyzer 2100 (Agilent

Technologies, Waldbronn, Germany). The amplicon libraries were each loaded on a 1/4th of a 454 Pico Titer Plate and pyrosequencing was performed by Macrogen (Korea) using the Roche GS-FLX instrument and Titanium chemistry according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

Bioinformatics

Sequences obtained from the 454 pyrosequencing run were clustered into operational taxonomic units (OTUs) using the UPARSE algorithm, following the recommended pipeline (Edgar, 2013). First, quality filtering of the reads was performed with the 'fastq_filter' command, allowing a maximum expected error of 0.5 for the individual sequences. In order to optimize the number and length of retained sequences, truncation length was set to 222 bp. Next, the sequences were dereplicated and sorted by abundance. Sequences occurring only once in the entire dataset were removed prior to clustering as this has been shown to improve the accuracy of diversity estimates (Brown et al., 2015). Afterwards, sequences were clustered into OTUs defined at 97% sequence similarity, which is commonly used to define SSU-based OTUs in AMF, with the 'cluster_otus' command. In this step, chimeric OTUs predicted by the *de novo* method built from more abundant reads were discarded as well. However, as advised by Edgar (2013) all obtained OTUs were double-checked for chimeric sequences against the MaarjAM database (Öpik et al., 2010) using the 'uchime_ref' command. OTUs were assigned to a taxonomic identity by querying representative sequences (as determined by the 'cluster_otus' command) against GenBank using the BLAST algorithm (Altschul et al., 1990). Taxonomic assignments were considered reliable when a ≥200 BLAST score value was obtained (Lumini et al., 2010). OTUs not belonging to the Glomeromycota or having a BLAST score lower than 200 were discarded. To accurately identify the obtained AMF OTUs, representative sequences for each OTU were also queried against the MaarjAM database (Öpik et al., 2010; accessed November 13, 2016), a database that aims to provide a quality-controlled repository for published sequence data from Glomeromycota. Representative AMF OTUs were submitted to GenBank (Genbank accession numbers MF153547-MF153908).

Data analysis and statistics

Multitag pyrosequencing might introduce bias to AMF community composition as certain barcodes may amplify better than others (Berry *et al.*, 2011). Therefore, all samples were resampled to 200 AMF sequences per sample. Samples with less than 200 AMF sequences were omitted from further analyses (see Supporting information Fig. S1 for further details on the rationale to resample to 200 AMF

sequences per sample). To assess the adequacy of the sampling effort, rarefaction curves using a resampling without replacement approach were made in MOTHUR (Schloss et al., 2009) for both grassland types and for all host plant species.

To explicitly test whether plant communities differed between grassland types, we used PerMANOVA (1000 permutations) using the adonis function of the *vegan* package in R on the vegetation data matrix (Anderson, 2001; Oksanen *et al.*, 2016). To determine which soil variables significantly explained variation in plant community data, we performed forward selection using the 'forward.sel' function of the R-package Packfor (Dray *et al.*, 2009).

To assess differences in AMF communities between grassland types and host plants species, we first performed non-metric multidimensional scaling (NMDS) using Bray-Curtis distances (metaNMDS function, Vegan package, Oksanen *et al.*, 2016). Next, we tested whether soil AMF communities differed between grassland types with multivariate permutational analysis of variance (PerMANOVA) (1000 permutations) using the *adonis* function of the *vegan* package in R. Subsequently, we performed variance partitioning on both the soil and root AMF communities using the 'varpart' function (Legendre, 2008) of the R-package Vegan. Variance partitioning allows to efficiently investigate the contribution of two or more explanatory matrices to explain the total variation in the dataset. In our case, three data matrices were used, namely geography (to account for spatial positions), soil data (to represent abiotic filtering) and host plant data (to represent biotic filtering). For the geography explanatory matrix, we calculated a set of spatial predictors from the geographical coordinates of the grassland sites by principle coordinates of neighbor matrices (PCNM) using the *pcnm* function of the *vegan* package in R (Borcard & Legendre, 2002; Borcard *et al.*, 2004). Only the significant explanatory variables, as determined by forward selection ('forward.sel' function of the R-package Packfor, Dray *et al.*, 2009), in each of the three matrices were included in the variance partitioning. For the root AMF communities, the host plant data is represented by the plant identity, i.e. the host plant species. For the soil AMF communities, the host plant data is represented by the two NMDS coordinates (NMDS1 and NMDS2) of the plant community data recorded per plot (vegetation composition). The venneuler package in R was used to create Venn diagrams to present the variation partitioning results. Finally, we used indicator species analysis in PC-ORD (McCune & Mefford, 2006) to investigate whether some AMF taxa were significantly indicative for a particular grassland type or host plant species. To prevent false positive indicator OTUs, indicator OTUs with an indicator value lower than 30 were omitted from the results.

Next, two community-level structural properties were calculated to study the architecture of the AMF-host plant interaction networks in each grassland site, i.e. modularity and nestedness. First, to test whether the network of interactions between host plant species and associating AMF taxa was modular, we used the simulated annealing algorithm developed by Guimera and Amaral (2005). The algorithm identifies modules whose nodes have the majority of their links inside their own module and provides an index of modularity that measures the extent to which taxa have more links than expected if linkage is random. To test the significance of modularity, 1000 random networks with the same species distribution as the original network were constructed and the observed modularity was compared with the modularity of the random networks. Second, to test whether the network of interactions between host plant species and associating AMF taxa was significantly nested, we calculated the NODF metric (based on overlap and decreasing fill) using the software package ANINHADO (Guimarães & Guimarães, 2006). Almeida-Neto *et al.* (2008) demonstrated that the NODF metric is less sensitive to both matrix size and shape compared to matrix temperature. To test the significance of nestedness, two different randomization models were used. In the first model (ER), presences are randomly assigned to any cell within the matrix. In the second model (CE), the probability of each cell being occupied depends on the number of presences in the row and column (Almeida-Neto et al., 2008). To prevent bias due to sample size, we only calculated modularity and nestedness of AMF-host plant networks with five or more host plant species per grassland site.

Results

Pyrosequencing

After removal of non-Glomeromycota sequences, pyrosequencing yielded a total of 502 004 AMF sequences with a minimal length of 222 bp containing the correct barcode and primer sequence. To prevent bias due to different sequencing depth, all samples were rarefied to 200 AMF sequences per sample, leaving 83 000 sequences and 415 samples (239 soil and 176 root samples) for further analysis (Table S2).

After rarefying, 362 AMF OTUs were detected across all plants species and soils (Table S3). The majority of OTUs belonged to the Glomeraceae (82.9%, 300 OTUs), whereas the others belonged to the Acaulosporaceae (5.8%, 21 OTUs), Claroideoglomeraceae (5.3%, 19 OTUs), Diversisporaceae (2.2%, 8 OTUs), Gigasporaceae (1.6%, 6 OTUs), Archaeosporaceae (1.1%, 4 OTUs), Ambisporaceae (0.8%, 3 OTUs) and Paraglomeraceae (0.3%, 1 OTU). The rarefaction curves of all host plant species

saturated, except for *Chamaespartium sagittale* which is probably due to the low number of samples in the dataset (5 samples, Fig. S2). The rarefaction curves of soil and root communities in both grassland types also saturated. The soil communities showed higher AMF richness than the root AMF communities in both grassland types (Fig. S3).

AMF community composition

PerMANOVA analysis showed strong significant differences in plant communities between acidic and calcareous grassland types ($F = 7.88$, $R^2 = 0.069$, $P < 0.001$). During stepwise selection of the soil variables, pH ($F = 3.50$, $R^2 = 0.032$, $P < 0.001$), nitrogen content ($F = 2.94$, $R^2 = 0.027$, $P = 0.002$) and Olsen P ($F = 2.58$, $R^2 = 0.024$, $P = 0.002$) were all selected as the explanatory variables significantly explained variation in plant community composition.

Both root and soil AMF communities plotted on the NMDS ordination clearly separated by grassland type (Fig. 2). Visual inspection of the NMDS ordinations obtained for host plant species occurring in both grassland types suggests that differences between grassland types were consistent within all host plant species (Fig. 3a). The NMDS ordination obtained for host plant species occurring only in one grassland type showed that AMF communities in plant species from calcareous grasslands (*Bromus erectus* and *Sanguisorba minor*) differed from AMF communities from plant species from acidic grasslands (*Agrostis capillaris*, *Danthonia decumbens*, *Nardus stricta*, and *Succisa pratensis*) (Fig. 3b). In agreement with the NMDS ordination, PerMANOVA analysis showed highly significant differences in soil AMF communities between acidic and calcareous grassland types ($F = 39.69$, $R^2 = 0.078$, $P < 0.001$) (Fig. 2).

Among the geographical variables, the forward selection procedure selected only the spatial predictor pcnm5 as significantly related to the soil AMF communities ($P < 0.001$). During stepwise selection of the soil variables, two variables were selected as the most important predictors of soil AMF communities, i.e. pH and Olsen P (both $P < 0.001$). Nitrogen, moisture and organic carbon content of the soil were not significantly related to the soil AMF communities. Both NMDS1 and NMDS2 variables, reflecting the vegetation composition (Table S4), were significantly related to the soil AMF communities ($P = 0.001$ and 0.004 respectively). All three separate explanatory matrices were significantly related to the soil AMF communities: geography $F = 3.51$, $P < 0.001$; soil $F = 6.55$, $P < 0.001$; and vegetation $F = 6.95$, $P < 0.001$. Comparison of the three different explanatory matrices using variance partitioning revealed that geography explained the smallest part of the total variation in

soil AMF communities (Table S5, Fig. 4a). Both vegetation composition and soil variables explained a unique part of the variation in soil AMF communities, but the largest part of the variation (R^2 adjusted = 0.053) explained by the soil variables was shared with the vegetation composition, indicating that soil variables and vegetation composition were strongly intercorrelated (Table S5, Fig. 4a).

In the root AMF communities, the forward selection procedure selected only spatial predictor pcnm4 as a significant predictor (P = 0.002). In agreement with the variance portioning of the soil AMF communities, stepwise selection of the soil variables revealed pH and Olsen P (both $P < 0.001$) as significantly related to the root AMF communities. Host plant identity was also significantly related to the root AMF communities (P = 0.027). All three separate explanatory matrices were significantly related to the root AMF communities: geography F = 3.32, $P < 0.001$; soil F = 7.84, $P < 0.001$; and host plant identity F = 2.22, P = 0.027. The variance partitioning revealed that, compared to site geography and host plant identity, the soil variables showed the strongest relationship with the root AMF communities. In contrast to the soil AMF communities, the effect of soil variables on root AMF communities was more important, with the amount of explained variation due to soil variables (R^2 adjusted = 0.061) being more than 3 times larger than the site geography (R^2 adjusted = 0.020). Host plant identity explained the least variation in root AMF communities (R^2 adjusted = 0.002) (Table S6, Fig. 4b).

Indicator species analysis

In agreement with the strong difference in AMF communities between grassland types, the indicator species analysis detected 11 OTUs significantly indicative of acidic grasslands and 10 OTUs significantly indicative of calcareous grassland (Table 1). Although variance partitioning revealed that the soil variables contributed the most to the composition of root AMF communities as opposed to host plant identity, we still found two indicator OTUs that were significantly associated with *Sanguisorba minor* (Table 2).

Network architecture

The modularity analysis showed that none of the tested AMF-host plant networks were significantly modular. M_{obs} ranged from 0.301 to 0.367 and was never significantly higher than the mean modularity index of the random networks (M_{rand}) (Table 3). Therefore, no modules in which taxa have more links than expected if linkage is random could be identified. In contrast, the nestedness analysis showed that all tested AMF-host plant networks were highly nested. Both the matrix $NODF_{ER}$ and $NODF_{CE}$ metric

indicated that all AMF-host plant networks were significantly more nested than expected by chance (Table 3).

Discussion

Our study showed that abiotic, rather than biotic filtering, shapes AMF communities in semi-natural grasslands. This was supported by (i) the clear differences in both soil and root AMF communities between acidic and calcareous grasslands, (ii) the explained variability in root AMF communities that was mostly attributed to soil variables, whereas very little variation was explained by host plant identity and (iii) the observation that AMF-host plants networks were not modular but nested.

Abiotic filtering as a dominant driver of AMF communities

We tested the hypothesis of abiotic filtering by comparing the soil AMF communities and root AMF communities between two different grassland types. If abiotic filtering was responsible for structuring AMF communities, we expected that both soil and root AMF communities would be determined by grassland type, not host identity. Indeed, we found clear differences between acidic and calcareous grasslands in soil AMF communities. The same differences were also found in root AMF communities of the seven host plant species occurring in both grassland types. Furthermore, the root AMF communities in host plant species occurring in only one grassland type closely resembled the soil AMF communities of that grassland type and the root AMF communities of other host plant species occurring in the same grassland type. The variance partitioning of root AMF communities also revealed that the amount of explained variability due to soil variables was much larger compared to the host plant identity. These results suggest that abiotic filtering constrains the AMF taxa to species adapted to the specific habitat conditions. The results are in agreement with Moora *et al.* (2014) who found differences in AMF communities between northern boreal forests and grasslands in Estonia, and Rodriguez-Echeverria *et al.* (2017) who revealed a strong differentiation of AMF communities between forested areas, flooded savannas and grasslands in the Gorongosa National Park in Mozambique, and thus also provided evidence that abiotic filtering by edaphic properties shapes AMF communities.

Indicator species analyses further identified 11 OTUs that were characteristic for acidic grasslands, and 10 OTUs characteristic for calcareous grassland. OTU_28 and OTU_13 (both *Glomus sp.*), indicative of calcareous grasslands, were identified as VTX00153 and VTX00114 in the MaarjAM database (Öpik *et al.*, 2010). In agreement with our results, both taxa also occurred in soils with alkaline pH levels and

not with acid pH levels (pH 7.7 and 7.8, respectively) (Kovacs *et al.*, 2007; Alguacil *et al.*, 2008), suggesting that they prefer calcareous habitats over acidic habitats. Similarly, OTU_16, indicative of acidic grasslands in our study, was identified as VTX00074 in the MaarjAM database and was also found in previous studies in slightly acidic soils (pH 5-7; Santos *et al.*, 2006; Öpik *et al.*, 2008; Davison *et al.*, 2011). The results of the indicator species analysis thus provide further evidence for abiotic filtering as it would seem that only those AMF taxa 'tolerant' to the pH levels of the habitat are present.

Host plant identity explained very little variation in root AMF communities

If biotic filtering was responsible for structuring AMF communities, we expected that plant host identity explained differences in root AMF communities, irrespective of soil AMF communities. We also expected that host plant species occurring in both grassland types showed similar root AMF communities irrespective of grassland type. Host plant identity, however, explained very little variation in root AMF communities in comparison to the soil variables. Furthermore, root AMF communities of host plant species occurring in both grassland types were similar to the soil AMF communities of the grassland type they were growing in. Our results suggest that biotic filtering is largely unimportant for the 13 host plant species sampled in our dataset. However, one has to be cautious when generalizing that biotic filtering plays little role in shaping AMF communities, given the relative limited number of host plant species in our dataset (13) compared to the total number of recorded plant species (303). Furthermore, we have only surveyed relatively dominant plant species, which may have similar ecological requirements. Further research must confirm whether our results hold across rare plant species and across functional groups. Our findings suggest that the 13 host plant species sampled in our dataset form opportunistic associations with the extant AMF taxa that are determined by abiotic properties instead of selectively forming associations with a host plant specific set of AMF taxa. Similarly, Öpik *et al.* (2003) failed to identify AMF taxa specifically associated with *Pulsatilla patens* and *Pulsatilla pratensis* from Estonian boreal forest and grassland habitats. Öpik *et al.* (2009) also showed that specificity of AMF communities in 10 boreonemoral forest plants occurred at the level of ecological groups rather than at the species level. Nevertheless, some studies did report plant host specific differences in AMF communities in the roots of plant species co-occurring in the same grassland (Vandenkoornhuyse *et al.*, 2003; Torrecillas *et al.*, 2012; Valyi *et al.*, 2015). Therefore, compared to our study which considered relatively few host plant species per site across a large geographical scale, stronger effects of biotic filtering might be expected when surveying more host plant species per site across a small geographical scale and less contrasting environments.

In agreement with our results, van der Heijden *et al.* (2015) predicted a strong specialization between mycorrhizal fungi and orchids and a low specialization between AMF and host plants. A strong specialization in orchid mycorrhiza may be explained by the critical dependency of orchid germination and seedling growth on mycorrhizal fungi colonizing the endosperm-lacking orchid seeds (Rasmussen & Rasmussen, 2009; Jacquemyn *et al.*, 2015). In contrast, AMF are known to colonize a broad range of plant hosts and may not have any physiological or physical constrains that may be reflected in specialization (Mosse, 1975; McGonigle & Fitter, 1990; Smith & Read, 2008).

Although host identity explained relatively little variation in root AMF communities, we detected two OTUs significantly associated with *Sanguisorba minor*. As this species only occurred in calcareous grasslands, it is likely that these OTUs were not selectively associated with this host plant species, but were rather characteristic for the grassland type. Indeed, OTU_1726 and OTU_3963 (both *Glomus sp.*) also occurred in 40 and 58 other samples originating from calcareous grasslands, while they only occurred in 11 and 12 samples originating from acidic grasslands, respectively.

Both plant and AMF communities follow changes in abiotic conditions

Variance partitioning revealed that both vegetation composition and soil variables explained unique variation in soil AMF communities. The unique variation explained by the soil variables suggests abiotic filtering of soil AMF communities by abiotic conditions, while the unique variation explained by the vegetation composition indicates that AMF and plant communities co-vary. The co-variation of plant and AMF communities has been explained by the driver (AMF drive plant communities) and passenger hypothesis (AMF follow plant communities) (Zobel & Öpik, 2014). The observational nature of this study does not allow to separate between both hypotheses. The largest part of the variation explained by the soil variables, however, was shared with the vegetation composition, suggesting that the vegetation composition also co-varies with the abiotic conditions. Indeed, we found that plant communities significantly differed between acidic and calcareous grasslands, and pH, nitrogen and phosphorus content of the soil significantly explained variation of the plant communities. This suggests that both plant and AMF communities are shaped by abiotic conditions, in accordance with the habitat hypothesis, which postulates that both plant and AMF communities follow changes in abiotic conditions (Zobel & Öpik, 2014).

Architecture of the network of arbuscular mycorrhizal associations

The observed AMF-host plant networks were not significantly modular, i.e. no subset of species that interacted more with a group of partners than with other groups could be found. Olesen *et al.* (2007) showed that larger networks (>150 species) are more likely to be modular than smaller networks (<50 species). The seven observed AMF-host plant networks in this study ranged from 7 to 5 plant species and from 74 to 100 total species (host plant species and AMF OTUs). The absence of modularity in the observed AMF networks may possibly be explained by the relative small size of the observed networks. Yet, the observed host plant networks were all significantly nested, indicating that the networks are organized around a central core of AMF interacting with almost all host plant species (i.e. generalist AMF taxa) and more specific AMF interacting only with a subset of plant species that also interact with the generalist AMF taxa (i.e. specialist AMF taxa). This pattern was consistent in all seven spatially separated AMF-host plant networks with five to seven host plant species, indicating the robustness of the AMF-host plant network architecture. Although the observed AMF-host plant networks were relative small in size, our observations are in agreement with the proposed relationship between host specificity, modularity and nestedness in associations between mycorrhizal fungi and host plants of van der Heijden *et al.* (2015). On the one hand, mycorrhizal fungi-orchid networks are predicted to show high modularity and low nestedness, reflecting the high specificity between mycorrhizal fungi and orchid partners. Indeed, Jacquemyn *et al.* (2015) showed that the network between mycorrhizal fungi and 20 orchid species co-occurring in a species-rich Mediterranean grassland was significantly modular but not nested, reflecting the strong specialization between mycorrhizal fungi and orchids resulting from physiological, physical or spatial constraints. On the other hand, AMF-host plant networks are predicted to show low modularity and high nestedness, reflecting the low specificity between AMF and host plants. Indeed, our results suggest that host plant specificity had no effect on AMF communities and the observed AMF-host plant networks were not modular but nested.

Implications for AMF community ecology

This study indicates that abiotic filtering plays a dominant role in the assembly of AMF communities. This leads to a better understanding of how AMF communities in natural grassland ecosystems are likely to respond to increasing human impact, such as nutrient pollution and land use changes. Nutrient pollution through atmospheric nitrogen deposition and overuse of fertilizers can strongly impact the abiotic conditions of natural ecosystems (Peñuelas *et al.*, 2012), and thus our findings suggest that AMF

taxa adapted to the abiotic conditions will be selected. To increase our understanding of AMF ecology in an era of ever increasing nutrient pollution, we suggest that future studies investigate AMF in natural ecosystems across a strong gradient of nutrient pollution.

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Author contributions

MVG and TC designed the research. MVG, TC, KVA, JP, LS and LK collected the root and soil samples. GP, TC and KVA performed the lab analysis. MVG and TC analyzed the data with helpful insights from HJ. MVG, OH and TC wrote the first draft of the manuscript. All authors reviewed and edited the final manuscript. OH and TC supervised the research.

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Figure legends

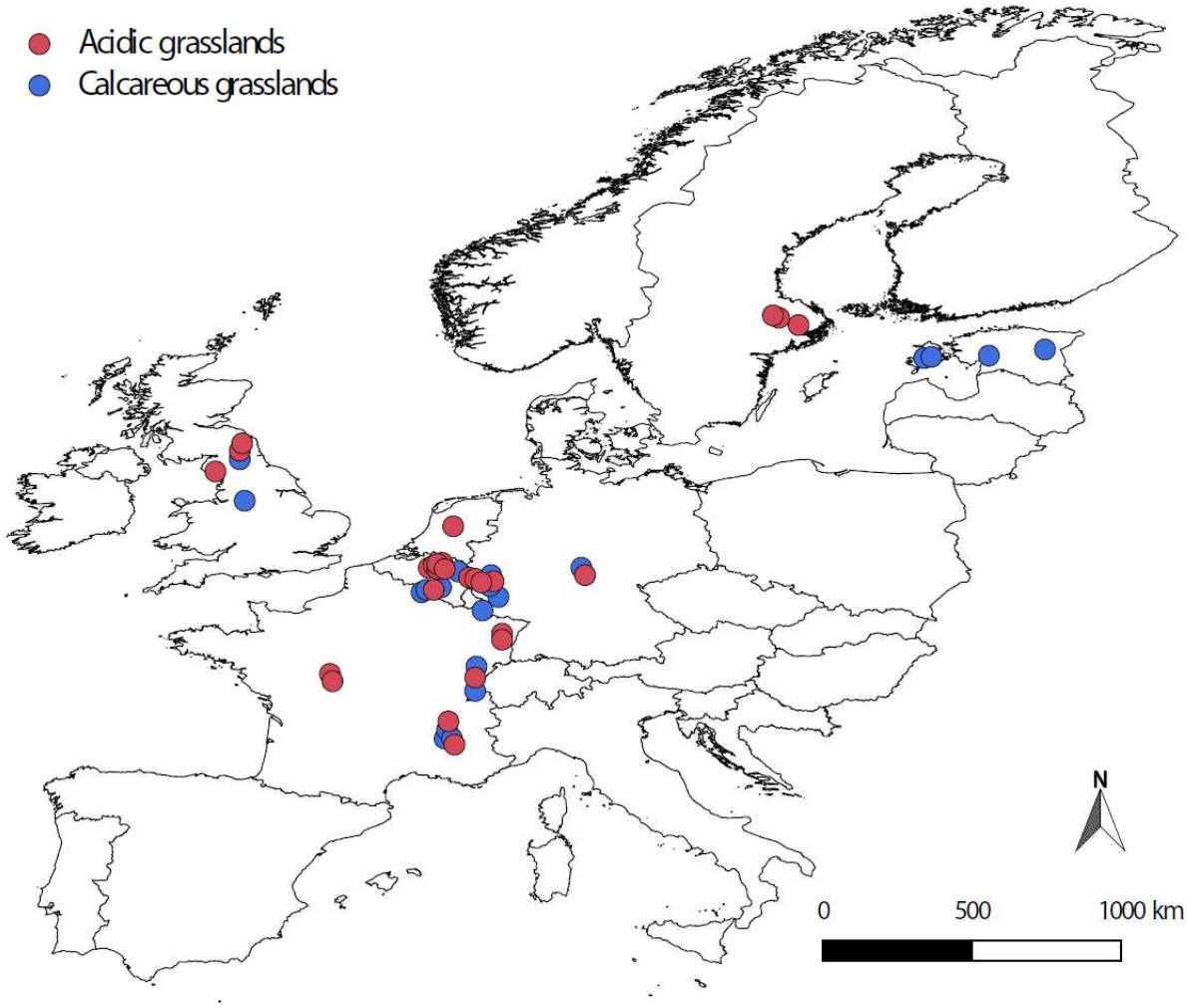

Figure 1 Map of Europe showing the surveyed grassland sites (N = 46). Acidic grasslands are shown in red (N = 26) and calcareous grasslands in blue (N = 20).

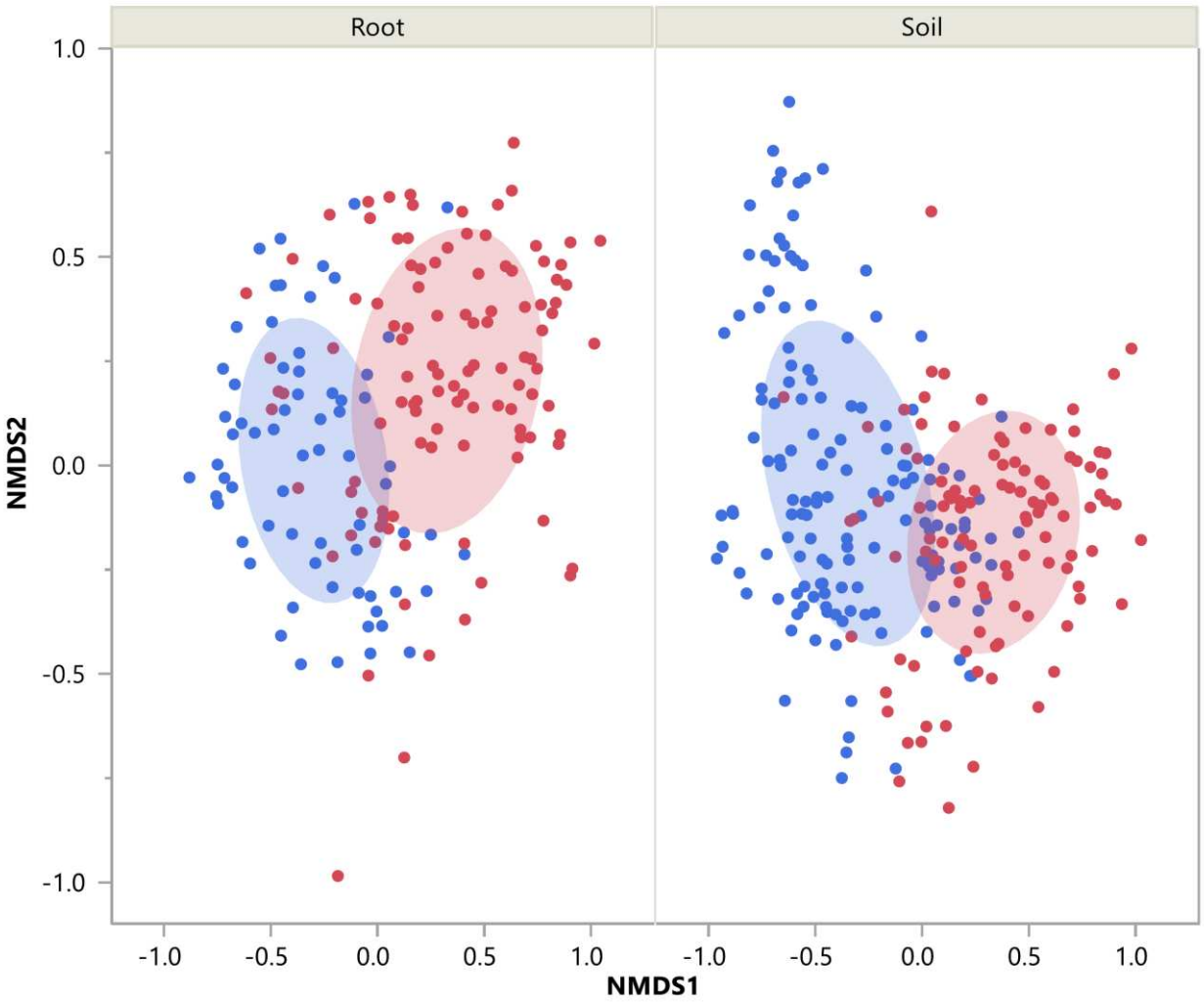

Figure 2 NMDS ordination plot of root and soil AMF communities from acidic (red) and calcareous (blue) grasslands. PerMANOVA analysis showed significant differences in soil AMF communities between acidic and calcareous grasslands (Table 1). Ellipses are dispersion ellipses using the standard deviation of the mean.

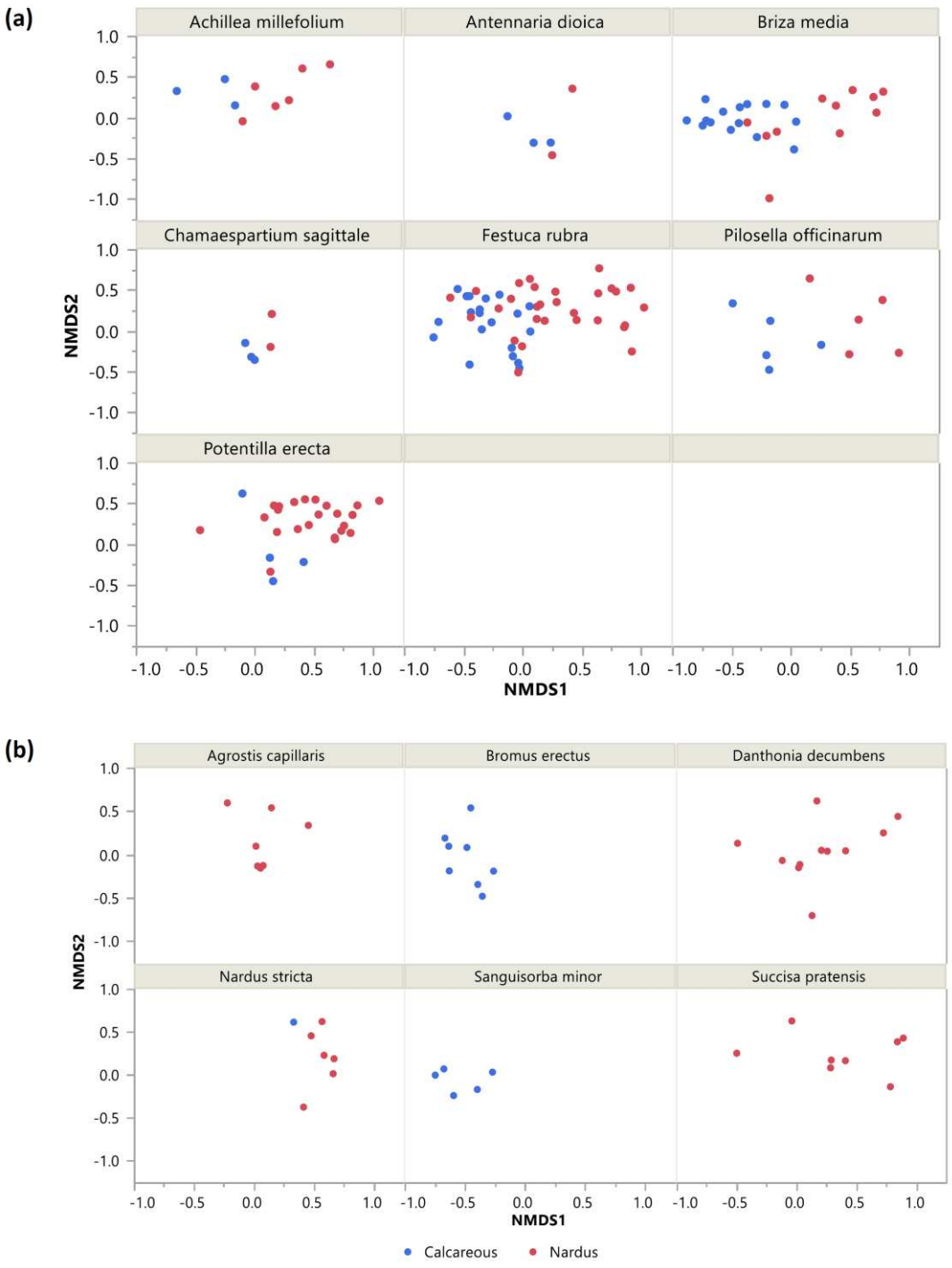

Figure 3 NMDS ordination plot of AMF communities in the roots of host plant species occurring in both acidic (red) and calcareous (blue) grasslands (a), and of specialist host plants species only occurring in one grassland type (b).

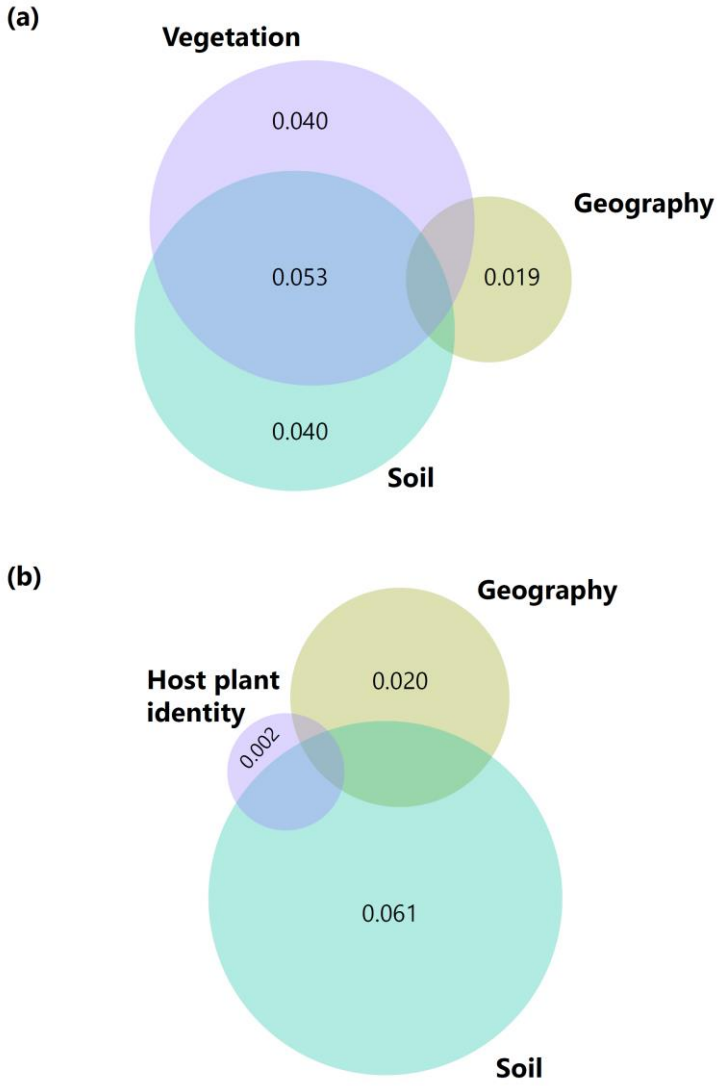

Figure 4 Venn diagrams representing variance partitioning of soil (a) and root (b) AMF communities among three explanatory matrices, i.e. geographical variables, soil chemical variables and host plant data. For the soil AMF communities (a), the host plant data is represented by the two NMDS coordinates (NMDS1 and NMDS2) of the vegetation composition recorded per plot. For the root AMF communities (b), the host plant data is represented by plant identity, i.e. the host plant species. The size of the circles is proportional to the variability in AMF communities as explained by a particular explanatory matrix, while overlap of the circles represents the shared variation among explanatory matrices. Numbers indicate adjusted R^2 values.

Tables

Table 1 Indicator OTUs detected for acidic and calcareous grasslands. Significance levels are obtained by Monte Carlo permutation tests.

	OTU_ID	Family	Genus	No. of sequences*	Indicator value	*P*
Acidic grassland	OTU_16	Glomeraceae	Glomus	4760	55.0	<0.001
	OTU_49	Glomeraceae	Glomus	1345	54.0	<0.001
	OTU_5	Claroideoglomeraceae	Claroideoglomus	4666	48.3	<0.001
	OTU_239	Glomeraceae	Glomus	1023	47.9	<0.001
	OTU_18	Glomeraceae	Glomus	2672	47.1	<0.001
	OTU_37	Glomeraceae	Glomus	1966	46.8	<0.001
	OTU_4148	Glomeraceae	Glomus	1820	43.2	<0.001
	OTU_4035	Glomeraceae	Glomus	1067	41.7	<0.001
	OTU_2850	Glomeraceae	Glomus	1443	36.9	<0.001
	OTU_58	Glomeraceae	Glomus	688	34.2	<0.001
	OTU_116	Acaulosporaceae	Acaulospora	544	33.9	<0.001
Calcareous grassland	OTU_28	Glomeraceae	Glomus	3829	70.1	<0.001
	OTU_745	Glomeraceae	Glomus	969	58.0	<0.001
	OTU_13	Glomeraceae	Glomus	4556	55.7	<0.001
	OTU_35	Glomeraceae	Glomus	3034	49.9	<0.001
	OTU_53	Glomeraceae	Glomus	1933	48.8	<0.001
	OTU_698	Glomeraceae	Glomus	789	45.1	<0.001
	OTU_187	Glomeraceae	Glomus	1409	44.9	<0.001
	OTU_236	Glomeraceae	Glomus	550	36.2	<0.001
	OTU_120	Glomeraceae	Glomus	370	32.9	<0.001
	OTU_353	Glomeraceae	Glomus	503	30.4	<0.001

*In rarefied dataset

Table 2 Indicator OTUs detected for different host plant species. Significance levels are obtained by Monte Carlo permutation tests.

	OTU_ID	Family	Genus	No. of sequences*	Indicator value	*P*
Sanguisorba minor	OTU_1726	Glomeraceae	Glomus	119	40.7	<0.001
	OTU_3963	Glomeraceae	Glomus	302	40.3	<0.001

*In rarefied dataset

Table 3 Modularity and nestedness analysis of all observed AMF-host plant networks with five or more plant species. Significance of modularity was determined by comparing the observed modularity (M_{obs}) with the mean modularity of 1000 random networks (M_{rand}). Significance of the NODF nestedness metric was based on two different randomization models (ER and CE). All AMF-host plant networks were not modular but were significantly nested.

Grassland site	No. of plant species in the network	M_{obs}	M_{rand}	*P*	$NODF_{ER}$	*P*	$NODF_{CE}$	*P*
Germany9	7	0.301	0.323	0.998	18.5	<0.001	24.77	0.010
Belgium8	6	0.310	0.327	0.989	19.4	<0.001	26.74	<0.001
UK4	6	0.329	0.328	0.444	19.3	<0.001	26.75	<0.001
Germany8	6	0.326	0.332	0.813	18.9	<0.001	24.57	<0.001
Germany4	6	0.353	0.373	0.998	17.6	<0.001	24.57	0.010
Sweden2	5	0.367	0.387	0.993	18.5	<0.001	24.55	0.010
UK5	5	0.310	0.344	0.999	20.4	<0.001	26.77	0.010

Supporting information

Table S1 List of sampled host plant species.

Table S2 The rarefied sample*OTU matrix and all accompanying environmental data.

Table S3 List of the 362 operational taxonomic units (OTUs) identified at a 3% sequence dissimilarity cut-off.

Table S4 Vegetation composition per plot.

Table S5 Variance partitioning results of soil AMF communities

Table S6 Variance partitioning results of root AMF communities

Figure S1 The relation between the number of reads per sample after rarefying and the percentage of samples and total reads retaining in the dataset.

Figure S2 Rarefaction curves of AMF richness for all host plant species.

Figure S3 Rarefaction curves of AMF richness for root and host plant communities in both grassland types.