Unraveling spatio-temporal variability of arbuscular mycorrhiza fungi in a temperate grassland plot

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Originality-Significance Statement

Our study investigated temporal and spatial variability in arbuscular mycorrhizal fungi (AMF) α- and β-diversity at the plot scale (10 m x 10 m), linking these to shifts in the composition of their symbiotic plant partners within a vegetation period. This is amongst the first studies to characterize such short-term variations in both diversity measures at this small scale.

Summary

Soils provide a heterogeneous environment varying in space and time; consequently, the biodiversity of soil microorganisms also differs spatially and temporally. For soil microbes tightly associated with plant roots, such as arbuscular mycorrhiza fungi (AMF), the diversity of plant partners and seasonal variability in trophic exchanges between the symbionts introduce additional heterogeneity. To clarify the impact of such heterogeneity, we investigated spatio-temporal variation in AMF diversity on a plot-scale (10 x 10 m) in a grassland managed at low intensity in southwest Germany. AMF diversity was determined using 18S rDNA pyrosequencing analysis of 360 soil samples taken at six time points within a year. We observed high AMF alpha- and beta-diversity across the plot and at all investigated time points. Relationships were detected between spatio-temporal variation in AMF OTU richness and plant species richness, root biomass, minimal changes in soil texture, and pH. The plot was characterized by high AMF turnover rates with a positive spatio-temporal relationship for AMF beta-diversity. However, environmental variables explained only ~20% of the variation in AMF communities. This indicates that the observed spatio-temporal richness and community variability of AMF was largely independent of the abiotic environment, but related to plant properties and the co-occurring microbiome.
Introduction

Understanding spatial and temporal patterns in species diversity is one of the fundamental goals of biodiversity research (Gaston and Spicer, 2013). Soil microbial communities exhibit spatial patterns at scales from sub-millimeter to hundreds of meters, determined by heterogeneous environmental conditions at respective scale-dependencies (Grundmann et al., 2001; Ettema and Wardle, 2002; Nunan et al., 2003; Bahram et al., 2015). Simultaneously, dynamic variations in abiotic soil conditions lead to fluctuating soil microbial abundances and functions over time, documented in agricultural (Kandeler and Böhm, 1996; Kandeler et al., 1999), tundra (Björk et al., 2008), and forest ecosystems (Görres et al., 1998; Nacke et al., 2016). Moreover, plant growth and development or changes in vegetation within a year are able to shift soil microbial communities (Chaparro et al., 2014; Nacke et al., 2016). This is especially relevant for obligate biotrophic plant mutualists such as arbuscular mycorrhizal fungi (AMF; Smith and Read, 2010).

Recent studies have aimed to identify general patterns of and major influences on AMF community composition. Some findings have included, for instance, high impacts of land-use intensity (Bouffaud et al., 2017), soil properties (Kivlin et al., 2011; Lekberg et al., 2012), plant community composition (van der Heijden et al., 1998; König et al., 2010; Neuenkamp et al., 2018), and/or host plant identity (Sanders, 2003). Since AMF are obligate root mutualists, most studies have focused on fungus-plant-relationships. Conflicting results have been observed, however, regarding the interactions between plant community composition and AMF communities, ranging from enhanced (Wu et al., 2007, Híiesalu et al., 2014) to reduced plant diversity in the presence of AMF (Antoninka et al., 2011) to no relationship between plant and AMF diversity (Ópik et al., 2008). These contradictory findings may be related in part to study scales (Hempel, 2018), since different environmental forces work at different scales (Chase, 2014); this also applies to AMF (Vályi et al., 2016).

According to ecological theory, niche-related (environmental/deterministic; MacArthur and Wilson, 1967) and neutral (stochastic; Hubbell, 2001) processes in particular shape community composition and habitat colonization. But these processes appear to have different strengths at various scales: niche-related effects are more common at larger (e.g. regional or global) scales, while neutral
processes operate mainly at small spatial scales (Chase, 2014). Many studies have been conducted at
broad geographical scales (Öpik et al., 2006; Hazard et al., 2013; Davison et al., 2015; Bouffaud et al.,
2016; Bouffaud et al., 2017) but to date little is known about richness and occurrence patterns of AMF
at or within plot scales (≤ 50 m × 50 m) in grassland ecosystems (Lekberg et al., 2012; Horn et al.,
2014). One advantage of such small-scale study designs is the focus on environmental conditions and
variations in plant communities within a specific habitat, thereby excluding overriding effects of large-
scale heterogeneity at the landscape level (Berner et al., 2011; Regan et al., 2017). Thus, fundamental
influences on AMF communities can be studied at such plot or subplot scales, ranging from centimeter
to meter. Repeating such sampling scales at one plot adds information on temporal autocorrelations
(Tobler, 1970), providing an opportunity to investigate spatial hot spots and temporal hot moments
simultaneously.

To understand temporal influences and to identify hot moments (Kuzyakov and Blagodatskaya, 2015)
in changing AMF communities (Dumbrell et al., 2011), a study would need to cover the entire
vegetation period by sampling soils at several time points. To date, few of the studies focused on
temporal variation have sampled AMF communities more than twice during the growing season
(Bainard et al., 2014; Liu et al., 2014). Repeated sampling is necessary, however, to account for
seasonal variations in plant cover, which is likely coupled with changes in soil moisture, temperature,
and nutrient fluxes (e.g. phosphate and nitrate), and thus reflected in dynamic soil microbial
communities (Bardgett et al., 2005). Even though a direct connection between AMF diversity, its
abundance, and changes in plant diversity is not always apparent: Dumbrell et al. (2011) showed that
during spring and summer, when plant growth is strong, environmental conditions and AMF
distribution patterns are not constant. However, even fewer studies have investigated both spatial and
temporal variations in AMF communities (Davison et al., 2012; Koorem et al., 2014; Barnes et al.,
2016), and these studies have mainly been done on forest sites. Davison et al. (2012) found seasonal
differences in AMF richness as well as distance decay in community similarity at three 10 m × 10 m
forest plots sampled four times within one year, while Koorem et al. (2014) confirmed the seasonal
variability in AMF by fatty acid analyses at small spatial scales (1.05 m × 1.05 m) sampled twice
during one summer. Combining spatial and temporal sampling also makes it possible to quantify the
beta-diversity of AMF communities, which describes how species composition changes over spatial scales and over time. However, analyses of beta-diversity have only rarely included microorganisms (e.g. Gossner et al., 2016).

Within the research platform “German Biodiversity Exploratories” (Fischer et al., 2010) the project SCALEMIC Experiment established a spatio-temporal sampling design in a low land-use intensity grassland at the plot scale (10 m × 10 m) and assigned six sampling dates from spring to autumn in one vegetation season. Through an interdisciplinary approach, it was previously clarified that plant growth changes plot-scale spatial heterogeneity of soil microorganisms during the vegetation period, and elucidated which driving forces behind this observed microbial heterogeneity (Regan et al., 2014).

We linked existing measures of seasonal and spatial changes in plant diversity, abiotic soil properties, and general microbial community composition (Regan et al., 2014; Regan et al., 2015; Klaus et al., 2016; Regan et al., 2017) to AMF diversity and community patterns. Using high-throughput sequencing technology, this study aimed to answer the following questions: (a) how much variability in AMF alpha- and beta-diversity exists on a spatial scale of 10 m × 10 m and a temporal scale of one season?; (b) are spatial and temporal AMF patterns coupled?; and (c) which environmental drivers are responsible for the observed patterns? We expected a strong relationship between the AMF community and its changing environment, primarily vegetation and phosphate availability.

Results

Taxonomical distribution of AMF

We recovered 1,088,162 AMF SSU rDNA gene reads from all 360 soil samples. After a quality filtering step that included removal of 22,042 potential chimera and non-AMF reads, we had a total of 562,320 AMF reads representing 1,562 reads per sample, and which were clustered into 155 abundant operational taxonomical units (OTUs). As described in detail in the “experimental procedures” section, the removal of rare OTUs (OTUs represented by ≤ 3 reads) had no significant effect on AMF beta-diversity. Thus, the AMF matrix including only abundant OTUs was used for further analyses.
The 155 abundant AMF OTUs were assigned to seven genera: *Acaulospora* (2 OTUs), *Ambispora* (2), *Archaeospora* (3), *Claroideoglomus* (20), *Diversispora* (9), *Glomus* (117), and *Paraglomus* (2). Taxonomical distribution based on the number of observed AMF OTUs differed slightly between sampling dates. The genus *Glomus* was most abundant throughout the entire growing season, ranging from 69% in April to 77% in November (Figure 1, Table S1) with the highest diversity (a total of 106 OTUs) detected in October. Besides *Glomus*, other AMF genera displayed temporal peaks; e.g., *Claroideoglomus* in June (16.4%) and *Diversispora* in April (8%).

**Spatio-temporal variation in AMF richness**

The OTU richness of total AMF was spatially modeled and checked for autocorrelation. Kriged maps were generated for all sampling dates except October, at which date the empirical variogram model was a pure nugget, indicating no spatial autocorrelation at the measured scale (Figures 2a-e). The observed patterns occurred and were distributed throughout the entire AMF community over the entire sampling season. In April and May (Figures 2a and b), AMF diversity was homogeneous with low OTU richness across the plot. An increase in AMF OTU richness was detected in June (yellow areas in Figure 2c). Moreover, first patches developed in June, and became more pronounced in August and November (Figures 2d and e). In general, total AMF OTU richness decreased at the end of the growing season (increase of dark green in the kriged maps), but discrete hot spots and cold spots with high or low AMF OTU richness appeared.

For the two most abundant AMF genera, *Glomus* and *Claroideoglomus*, OTU richness was also spatially modeled and could be visualized through kriged maps (Figures S1 and S2). Spatial distribution of *Glomus* could be modelled in May, June, August and November, while the spatial distribution of *Claroideoglomus* could only be modelled in August and November. As was the case for all AMF OTUs, *Glomus* OTU richness was low to medium in May and June (Figures S1a, b), tending toward spatial patches of low or high richness. Heterogeneity of distribution became more pronounced in August with two spots of high OTU richness (Figure S1c). However, a shift in OTU richness occurred in November (Figure S1d) with areas of low and high OTU richness of *Glomus*. *Claroideoglomus* OTUs exhibited similar spatial patterns in August with areas of low and intermediate
richness (Figure S2a) and in November generally lower richness but more heterogeneous distribution across the plot (Figure S2b).

The effect of sampling date on AMF richness, assessed by linear mixed effect models (LMEM) models, was plotted for all OTUs and additionally for the OTUs of the two most abundant genera, *Glomus* and *Claroideoglomus* (Figure S3). Sampling date significantly influenced richness of both total AMF and *Glomus* OTUs ($p < 0.0001$), but not *Claroideoglomus* OTUs. OTU richness significantly increased in all AMF OTUs from April to June, dropping in August. Total AMF OTU richness peaked in October and dropped significantly in November. The richness of *Glomus* OTUs was similar with significantly lower richness in April and a peak in October. The OTU richness of *Claroideoglomus* did not change over the sampling season.

**Environmental impacts on AMF richness**

Linear mixed effect models, taking into account the impact of all available environmental factors ($n = 34$) on total AMF OTU richness, revealed significant effects of soil- and plant-related parameters in 21 cases, as well as 23 significant effects of environmental variables on *Glomus* OTU richness. *Claroideoglomus* OTU richness was significantly affected by soil carbon content, but this explained only 3% of its variance. To detect those environmental variables most closely associated with the observed temporal effect on total AMF and *Glomus* OTU richness, we combined environmental variables and sampling date as fixed effects in LMEMs. This combination indicated that the measured environmental variables explained less unique variance in total AMF and *Glomus* OTU richness than did sampling date. The final LMEM (Table 1) for total AMF OTU richness pointed to plant species richness, percent silt content and sampling date as the three main drivers at the investigated site, which, taken together, explained 36% of the variance. Silt content had a positive effect, while plant species richness was slightly negatively associated with total AMF OTU richness. Temporal variation was reflected by the significant effects of sampling time and indicated by the different intercepts of the single months (Table 1). *Glomus* richness was driven by plant species richness, root biomass, percent silt content, pH, and sampling date, which together explained 38% of the variance in *Glomus*. Here, we found a slightly negative impact of plant species richness, while root biomass, silt content and pH...
positively affected the OTU richness of *Glomus*. For both total AMF and *Glomus* OTU richness, sampling date explained large proportions of the variance; 27% and 28%, respectively. As plant species richness was only assessed at three sampling dates, we additionally fitted models without this variable for total AMF and *Glomus* OTU richness to determine the best predictor variables across the whole season. When all six sampling dates were analyzed, total AMF OTU richness was not influenced by plant variables; instead there was a slight negative association with soil NH$_4^+$ content. *Glomus* OTU richness was best predicted by legume and root biomass, NH$_4^+$, silt content, pH, the fungal to bacterial ratio, and sampling date over the entire season (see Table 1).

**Spatio-temporal variation in AMF community composition**

AMF beta-diversity (β$_{SOR}$) was lower within time points and within subplots than between these groups (ANOSIM $p$-value = 0.001). When the data set was stratified by sampling date, silt content, pH, microbial biomass, soil C content, and K$_2$SO$_4$-extractable organic N significantly explained variation in β$_{SOR}$. However, these variables together explained less than 10% of the variation in β$_{SOR}$. No relationship between AMF β$_{SOR}$ and any plant variable could be detected. Additionally, a spatial gradient explaining 4% of the variability was observed (Tables S2 and S3).

The AMF β$_{SOR}$ of the subplots between one time point and the one immediately following varied slightly (Figure S4). However, there was no stronger correlation between subplots near each other in comparison to those subplots further distant (Figure S5; for data on *Glomus* and *Claroideoglomus* see Figures S6 and S7, respectively). No significant correlations between the temporal development of β$_{SOR}$ and environmental variables were observed. Turnover (β$_{SIM}$), meaning OTU replacement between time points, and nestedness (β$_{SNE}$), meaning OTU gain and loss from one time point to the next, are summarized in Figure 3 (for data on *Glomus* and *Claroideoglomus* see Figure S8). The turnover in AMF community composition appeared to be constant between 0.3 and 0.4 during the sampling season. The highest AMF β$_{SIM}$ was detected between June and August, which is likely linked to the fact that this difference represented a duration of two months. β$_{SNE}$ peaked later in the season, particularly between August and October but also between October and November. However, kriged
maps revealed hot spots of turnover from April to May as well as from October to November (Figure S9).

Analysis of spatial AMF $\beta_{SOR}$ demonstrated some continuity within subplots early in the growing season (see supplemental material for more details; Figure S10 and Figure S11). The relationship between temporal $\beta_{SOR}$ (the average $\beta_{SOR}$ over time) and spatial $\beta_{SOR}$ (the average AMF $\beta_{SOR}$ with the neighboring subplots) of each subplot displayed a positive trend (Figure 4), indicating that subplots with AMF communities that differed strongly from neighboring subplots also changed more over time. The positive relationship was significantly stronger than the relationship observed in null-models of $\beta_{SOR}$, which were based on random community permutations that maintained each sample’s richness and the overall or sampling date point-specific probability of OTU occurrence (Figure S12). Concurrently, no significant relationships between OTU richness or changes in alpha-diversity with spatial or temporal $\beta_{SOR}$ were observed. The same pattern was observed for the $\beta_{SOR}$ of Glomus OTUs, while Claroideoglomus $\beta_{SOR}$ did not differ significantly from the null-models (Figure S12). No explanatory power was gained by adding environmental variables to the linear model explaining temporal $\beta_{SOR}$ with spatial $\beta_{SOR}$. Among the environmental variables, the mean grass biomass best explained spatial $\beta_{SOR}$ of all OTUs ($p$-value 0.01, adjusted $R^2 = 0.43$).

Discussion

General characterization of AMF

AMF form a multispecies mutualism with over 80% of terrestrial plants, i.e. with more than one fungus per host plant (Smith and Read, 2010). With a total of 155 abundant AMF OTUs on a 10 m × 10 m plot over a vegetation period that extended from April to November, our study found relatively high AMF richness compared to recent studies (Dumbrell et al., 2010; Davison et al., 2012; Horn et al., 2014).

All four orders of the phylum Glomeromycota and seven genera were represented in the observed OTUs. Dominant at all time points was the AMF genus Glomus. This dominance is in agreement with previously published studies in grasslands, forests, and agricultural ecosystems (Daniell et al., 2001; Gai et al., 2009; Öpik et al., 2009). Although AMF OTU richness was high, genera such as
Rhizophagus or Funneliformis, which have often been observed in a comparable German grassland of low land-use intensity (Horn et al., 2014), were not detected in our dataset.

Spatio-temporal variation in AMF alpha-diversity

Our current study determined whether AMF richness exhibits spatio-temporal variation at a small spatial scale in a grassland soil, and which environmental variables shape differences in AMF alpha-diversity. Studies in which other results of the SCALEMIC Experiment have been published observed an increase in biomass of grasses and forbs until June and additionally a gain in biomass of legumes in October as well as significant shifts in mineral nitrogen content of soils over time (Regan et al., 2014). Moreover, temporal shifts in plant biomass and nutrient availability were detected (Klaus et al., 2016). Results of our spatial analyses illustrate clearly how AMF OTU richness varied across the sampled plot and also over the season. The appearance of hot or cold spots of AMF richness showed a dynamic process that developed during the vegetation period. The detected spatial autocorrelation of AMF OTUs with ranges below 10 m across our plot for five of six time points is in accordance with previous studies, e.g., by Bahram et al. (2015), who reported autocorrelation ranges around 9 m. Richness of AMF OTUs therefore shows distance-decay relationships at the investigated plot scale.

Interestingly, although more than 100 OTUs were detected on the entire 10 m × 10 m plot at each sampling date, many of these AMF appeared in patches of 20-30 OTUs per sampling point. This discrepancy between total observed OTU richness across the plot and OTU richness per individual sampling point may be related to carrying capacity (Allen, 1989). We define carrying capacity as the maximum number of AMF OTUs the studied SCALEMIC grassland plot can sustainably support. Accordingly, the local AMF carrying capacity appears to have been patchy but potentially dynamic over time. The observed temporal dynamic could be connected to changes in resource availability. This could be related to asynchronous growth of plants across the site (Yachi and Loreau, 1999). For instance, a changing supply of photo-assimilates could be accompanied by dense AMF population sizes at one sampling point (hot spot), but reduced AMF richness at another point (cold spot) on the plot within or across sample time points. This is in line with findings of the linear mixed effect models, which found a connection between AMF richness and changing environmental variables such as...
as plant species richness, root biomass, pH and NH$_4^+$, To our knowledge, this has not been shown previously and underscores the need for more temporal investigations.

**Restricted impact of environmental variables on AMF richness**

Linear mixed effect model analyses revealed a significant effect of soil texture on the total richness of both AMF and the genus *Glomus* across the plot. The heterogeneous distribution of silt in our soil modified important habitat conditions. Hot spots of high silt content are characterized by larger volumes of medium sized pore space and improved aeration in the surrounding micro-environment (Horn et al., 2010), resulting in favorable habitat conditions for AMF, which lead to increased AMF OTU richness. Although the dependence of AMF on soil texture in grassland soils was shown in a large scale study (Oehl et al., 2017), no studies have yet demonstrated that this effect occurs with small textural changes (changes in silt content < 10%) at the plot scale. Soil texture significantly affected temporal variations in AMF OTU richness even though it was spatially and temporally stable. We suggest that this is because soil texture influences a number of habitat conditions such as nutrient availability, pore space distribution and thereby also the hydrological budget and oxygen supply (Horn et al., 2010), which themselves vary over time. That soil texture was a better measure than single effects, e.g., soil water content, indicates its value as a measurement that captures a range of temporal variations in texture-dependent habitat conditions. This result emphasizes the importance of microhabitat conditions for AMF.

In addition to soil texture, plant species richness was a significant driver of OTU richness for both total AMF and *Glomus* in the months of May, June and October. Interestingly, a reduction in plant species richness led to an increase in total AMF and *Glomus* OTU richness. Argüello et al. (2016) described a positive feedback mechanism for AMF-plant mutualism, leading to stronger cooperation between AMF and plants under the condition of high AMF diversity per plant. In addition, newly emerging plant species over the season may have had different root architectures such as less root biomass, leading to a reduction in AMF OTU richness, as higher root biomass significantly increased the OTU richness of the genus *Glomus*. It has also been suggested that both AMF and plants actively control their associated symbiotic partners (van der Heijden et al., 2015), in which case changes that
resulted in a more cooperative plant community could influence AMF OTU richness. Similarly, the newly emerging plant species may have had more acidic root exudates, which would have reduced the OTU richness of *Glomus*, as *Glomus* OTU richness decreased with decreasing soil pH. A similar effect of pH on AMF OTU richness in grassland ecosystems was also observed by Heyburn et al. (2017). Even when they were evaluated over all six sampling dates throughout the season, the effects of soil texture, pH, and sampling date remained significant. Over the longer time, the impact of plants on total AMF OTU richness was not significant; instead, a negative effect of NH$_4^+$ was detected. The same was true for OTU richness of *Glomus*. As NH$_4^+$ reduces soil pH, this was likely a combined effect of the two soil properties. In addition, an increase in legume biomass led to an increase in *Glomus* OTU richness, which may have been due to increased coverage of the mycorrhizal plant partners such as *Trifolium pratense* (L.) (van der Heijden et al., 1998) and *Vicia sepium* (L.) (Closa and Goicoechea, 2011) over time. The negative relationship between the fungal:bacterial ratio of phospholipid fatty acids and *Glomus* richness was related to an overall increase in saprotrophic fungi at the site (Regan et al., 2014), which was accompanied by a reduction in *Glomus* richness, indicating both competition for resources (Hodge et al., 2001) and interactions due to fungal community composition (Tiunov and Scheu, 2005) at the study site. However, not all AMF genera reacted in the same way. *Claroideoglomus* was affected neither by soil texture nor by any of the above mentioned environmental properties; instead, a small but significant effect of soil carbon content on this genus was detected.

Even though environmental soil properties explained a portion of the variance in AMF alpha-diversity, sampling date was the most important driver of total AMF and *Glomus* OTU richness. Previous studies have reported an increase in AMF OTU richness during the growing season with a decrease in autumn, which could be explained by changing weather conditions within a sampling year (i.e. temperature and precipitation; Kabir et al., 1998; Staddon et al., 2003). In our study, there were significantly fewer AMF OTUs in November than in June or October. This phenomenon may have been related to cold temperatures and less precipitation at this sampling date (see supplemental Fig. A1 of Regan et al. (2014)). Also, the observed decline in AMF OTU richness in August may have been associated with a suppression of plant growth and reduced carbon supply from plant to fungus...
after the mowing event (Gehring and Whitham, 2002). This mowing event and the subsequent regrowth of plants could have led to the high number of AMF OTUs detected in October. It was shown recently that more AMF propagules are present in mown than in unmown soils (Binet et al., 2013), benefiting new AMF infections after mowing. In addition, this is likely connected to increasing root exudation following aboveground plant biomass removal (Waters and Borowicz, 1994). More diverse exudates are likely to recruit a greater AMF diversity (Hugoni et al., 2018). Consequently, mowing leads to emerging micro-niches, which favor a higher variability in AMF. Thereby, AMF can be considered as stress tolerant since they can cope with partition and destruction of their hyphae (Buscot, 2015). Since up to only 10% of the explained variance was directly attributable to measured environmental effects (see Table 1), our results could indicate that neutral processes, stochasticity, or randomness due to natural variability may play a role in the formation of unpredictable AMF patchiness in addition to the contribution from deterministic processes. This applied to both total AMF OTUs and OTUs of the genera *Glomus* and *Claroideoglomus*.

**Pronounced spatio-temporal relationships in AMF beta-diversity**

In addition to alpha-diversity, this study sought to understand whether or not beta-diversity in AMF exhibited similar spatio-temporal patterns. With respect to OTU richness, AMF beta-diversity expressed as Sørenson index indicated spatio-temporal relationships. Theoretically, local AMF communities should be of a common and predictable composition since the species pool at the plot scale is limited; thus, beta-diversity in both spatial and temporal senses should be low according to Powell and Bennett (2016). However, in our study AMF beta-diversity appeared high, with a particularly high turnover rate from one observed time point to the next. Although the turnover rates were high (consistently ≈ 40% of the AMF community changed from one time point to the subsequent one), a certain spatio-temporal stability of AMF communities was observed. Our results suggest that AMF community composition at the first three time points (April-June) was determined by prior communities. It is possible that DNA measures either detected defective and dead cells (Carini et al., 2016), or dormant AMF stages, such as spores from the previous year, which impacted the observed AMF community at the beginning of the vegetation period. This effect was lost during the summer,
which suggests that either legacy effects due to the cyclic character of seasons in temperate regions (Bahram et al., 2015) or the appearance of priority effects (Viana et al., 2016) shaped AMF community composition, as has been shown for soil bacteria (Francioli et al., 2016; Francioli et al., 2018). The plant-AMF interaction may have been set back to zero during winter, resulting in a random start of plant growth and fungal infections during spring. These priority effects could have resulted in high heterogeneity within subplots, which decreases over the season. Coupled with this is the fact that competition amongst AMF emerges only over time (Maherali & Klironomos, 2012). Alternatively, AMF detected from spores in our analyses transformed from spores while colonizing growing plant roots during the second half of the vegetation period. In the second half of the year these AMF may have dropped below the molecular detection limit. Also, the mowing event before sampling in August shuffled AMF community composition, since mowing is known to multiply AMF propagule numbers (Binet et al., 2013). However, we could not identify a direct link between either the plant community taken together or between single plant species and the AMF community. This missing link between these two communities (Hart et al., 2001) supports the “independence hypothesis” which suggests that neither plants nor AMF express any co-variation at all in this mutualism (Zobel and Öpik, 2014). Recent studies mention dispersal limitation of AMF (Davison et al., 2015) as one reason for patchiness in community composition at small scales. This results in “unpredictable assembly” (Powell and Bennett, 2016) of AMF, which corresponds well with our findings that approximately 80% of variation in \( \beta_{\text{SOR}} \) could not be explained by environmental variables. This could indicate that stochastic rather than niche related processes shape AMF \( \beta_{\text{SOR}} \). However, one general pattern could be identified: over time, AMF community composition differed less within than between subplots. More similar environmental conditions found within a subplot appeared to result in significantly lower beta-diversity over time. This indicates that even small environmental differences between two subplots affected AMF community composition. Indeed, it has previously been shown that pH, C, N, P, and soil water content shape AMF grassland communities (Horn et al., 2014). In our study, around 20% of the observed variation in AMF beta-diversity could be explained by measured environmental factors. Nevertheless, it is possible that the low variance explained by environmental variables in our study indicates the influence of important but unmeasured variables, and not stochastic processes as such. To
confirm that neutral processes shape spatio-temporal AMF beta-diversity, future studies should consider microscale effects such as root exudates and pore space to identify currently unidentifiable drivers.

**Conclusions**

Our study of AMF alpha- and beta-diversity found spatio-temporal distribution patterns at the observed plot scale of 10 m × 10 m. We were able to illustrate well both the dynamism of AMF OTU richness, and community development across one vegetation season. Thereby, we demonstrated, albeit indirectly, that stochastic recruitment processes largely shaped our observed patterns of AMF OTU richness and community composition. If seasonal variations in carrying capacity are considered, then shifts in plant growth, diversity, and dominance are likely to favor AMF species already engaged in the symbioses. However, our results revealed high AMF turnover over time, suggesting ongoing recruitment of AMF from formerly dormant propagules. We acknowledge that the detection of niche-based processes could have been limited by the choice of our measured environmental parameters, which were either unable to detect them or wrongly selected for this purpose. Nonetheless, both the scale and spatio-temporal approach of the SCALEMIC Experiment have expanded our understanding of biotic and abiotic interactions at scales that had heretofore not been examined in such detail. Further research, ideally on more than one site, is needed for a deeper and more comprehensive understanding of the spatio-temporal assembly of soil microbes at small scales by assessing and linking functions of bacteria and fungi with plant traits. Likewise, and within the frame of an emerging discussion as to whether AM fungal communities are more structured by the abiotic or biotic environment (Hempel, 2018), future studies should incorporate balanced consideration of environmental variables.

**Experimental procedures**

*Study site and soil sampling*

The studied grassland plot (48°27’31.37”N, 9°27’36.26”E) is one of 300 experimental plots in the large and long-term interdisciplinary research project “Biodiversity Exploratories”, which aims to understand relationships between land-use, multi-trophic biodiversity and ecosystem functioning.
across Germany (Fischer et al., 2010). The grassland plot is located in the Schwäbische Alb in southwest Germany. The plot has never received mineral fertilizers and has never been plowed. Characterized by a rather nutrient-poor substrate, this plot’s soil type is a Rendzic Leptosol (FAO classification). The plot is dominated by \textit{Plantago lanceolata} L., \textit{Festuca rubra} L and \textit{Helictotrichon pubescens} (Huds.) Pilg. and belongs to the phytosociological class of Festuco-Brometea (Oberdorfer, 2001; Klaus et al., 2016). Furthermore, the grassland is usually mown once per year, and grazed briefly by sheep for 1-2 weeks in late summer or early autumn. In 2011, the year of investigation, mowing took place on July 30\textsuperscript{th} and sheep herds grazed on this site in May for five days, in September for seven days and in October for one day.

The \textit{SCALEMIC Experiment} (Regan et al., 2014) encompasses a 10 m × 10 m plot divided into 30 subplots, each 2 m × 1.67 m (see Figure S12). Within each subplot six pairs of sampling locations (each 20 cm × 20 cm) were randomly assigned, with one pair sampled at each of six dates over one growing season. This provided a randomized complete block design for temporal data analysis with sampling date as ‘treatment’ factor, subplots as complete blocks, and pair of sampling locations as randomization unit. Sample pairs were separated by 50 cm to provide appropriate lag distances for geostatistical analyses. Sampling dates were chosen along a seasonal gradient with the following characterization: 1) beginning of vegetation (April 5\textsuperscript{th}), 2) stage of main plant growth (May 17\textsuperscript{th}), 3) peak of plant biomass (June 27\textsuperscript{th}), 4) two weeks after mowing (August 16\textsuperscript{th}), 5) nine weeks after mowing (October 5\textsuperscript{th}) and 6) after the first frost (November 21\textsuperscript{st}). Accordingly, a total of 360 soil samples were collected (60 per date × 6 dates) in the year 2011. Soil samples were collected with core augers (diameter 58 mm). The upper 10 cm layer was taken at each sampling point (see Figure S11) after vegetation was removed and the top one cm, consisting of litter, was discarded from the sample. Subsequently, the soil was immediately stored at 4 \textdegree C and sieved (<5 mm) within 24 h after sampling to remove stones, roots and macrofauna. An aliquot for molecular analyses was stored at -20 \textdegree C before processing in the laboratory. A detailed description of the sampling design and procedure can also be found in Regan et al. (2014).

\textit{DNA extraction and pyrosequencing of AMF amplicons}
DNA was extracted from two replicates of each homogenized soil subsample (300 mg each) according to the manufacturer's protocol using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) as described in Stempfhuber et al. (2016). Independent measurements of DNA concentration from both sample replicates were made on a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The replicates were subsequently pooled and re-measured, confirming the final DNA concentration of each sample, which was diluted to a PCR template concentration of 5 ng DNA ml\(^{-1}\) with ultra-pure water. A semi-nested PCR protocol was used to amplify the ~630 bp-long small subunit (SSU) region of the AMF 18S rDNA via pyrosequencing analysis (454 GS FLX, Roche). In the first PCR run (PCR I) a Glomeromycota-specific region was amplified with the primer set GLOMERWT0/ GLOMER1536 (Wubet et al., 2006), followed by the semi-nested second PCR reaction with the forward general fungal primer NS31 (Simon et al., 1992) including the A adaptor and a 10 bp multiplex identifier (1 of 60 different MIDs), and the B adaptor including the reverse modified AMF primer AM1a and AM1b (Morris et al., 2013). The first PCR was carried out at a 25 µl reaction volume with 0.5 µl of diluted DNA template (5-20 ng µl\(^{-1}\)), 12.5 µl GoTaq Green Mastermix 2x (Promega, Mannheim, Germany), 1 µl of each primer (25 µM) on an Eppendorf Mastercycler DNA Engine Thermal Cycler PCR (Eppendorf, Hamburg, Germany) with the following PCR conditions: 98 °C for 30 s, 5 cycles of 94 °C for 30 s, 60 °C (-1 °C/cycle, 4 cycles) for 30 s, 72 °C for 1 min, and 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and for extension 72 °C for 5 min. For the semi-nested PCR, two separate amplifications were performed using 1 µl of the diluted amplified product of PCR I (1:10), 25 µl GoTaq Green Mastermix 2x, and 1 µl of each primer (25 µM); these 50 µl-reactions were run under the following conditions: 98 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 63 °C for 30 s, 72 °C for 1 min, and 72 °C for 5 min.

Each sample in both PCR amplification steps was amplified in triplicate and accompanied by a negative control. The semi-nested PCR amplified products were pooled per sample, taking into account the amplicon concentration (checked by a 1.5% agarose gel). Pooled samples (30 µl each) were purified with the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer’s recommended protocol. The purified products were quantified by fluorometry using Quant-iT™ PicoGreen ® dsDNA Assay Kit (Life Technologies GmbH, Darmstadt, Germany) as
suggested by Roche Diagnostics GmbH (Mannheim, Germany) for amplicon library preparation. Equimolar concentrations of 60 MID tagged amplicons were loaded into individual lanes on a GS-FLX LUMITRAC 600 plate (Titanium Series) separated with a four-lane gasket and sequenced at the Department of Soil Ecology, UFZ – Helmholtz-Centre for Environmental Research (Halle/Saale, Germany).

**Bioinformatic analysis of sequence data**

Sequence read quality filtering and splitting of the dataset into individual samples was performed using mainly MOTHUR (Schloss et al., 2009). Sequences were trimmed using the “keepfirst” comment in order to discard sequences with less than 300 bp and chopping at least 50 bp from potential noisy sequence ends. Simultaneously, all sequences with average quality scores of below 20 as well as MID- and primer sequences were removed. Sequences were then downsampled to the smallest read number per sample (1,562 sequences per sample) and potential chimeric sequences were identified and removed by UCHIME (Edgar et al., 2011) as implemented in MOTHUR. These quality-filtered sequences were clustered into OTUs based on the algorithm implemented in CD-HIT-EST (Huang et al., 2010) with a sequence similarity threshold of 97%. The representative sequence for each resulting OTU was compared to a GAST (Global Alignment for Sequence Taxonomy)-based taxonomic assignment of an NCBI based fungal reference data set (Huse et al., 2008) at the 97% similarity level. All non-Glomeromycota OTUs were removed from the dataset (~11% of sequences). Representative sequences (most abundant sequence per OTU) of the Glomeromycota OTUs were further taxonomically assigned by using the MaarjAM virtual taxa reference database (web-based database for studies of the diversity of arbuscular mycorrhizal fungi, version 0.8.1 beta; Öpik et al., 2010).

The raw SSU DNA sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under study accession number SRP137677. In addition to all measured values, the analyzed and processed data used in this study can be found in the BExIS database (https://www.bexis.uni-jena.de/).

**Environmental properties**
The interdisciplinary approach of the SCALEMIC Experiment permits data from previous works on various environmental properties of the site to be used in the present study. We collected information on plant diversity (richness, Shannon index) on three sampling dates (May, June, and October) and plant biomass (aboveground biomass of grasses, herbs and legumes; root and litter biomass; Regan et al. (2014), Regan et al. (2015), Klaus et al. (2016)). We also measured soil texture, bulk density, water content, mineral nitrogen \( N_{\text{min}} = \text{NH}_4^+ + \text{NO}_3^- \), total carbon and nitrogen, extractable organic carbon and extractable nitrogen (EOC, EN), bioavailable phosphate (PO\(_4^{3-}\)), pH, bacterial and fungal biomass (phospholipid fatty acid (PLFA) content; Regan et al. (2014)); as well as bacterial and archaeal abundances (qPCR on 16S rRNA; Regan et al. (2017)). These variables were used in statistical analyses to determine their explanatory power with respect to AMF OTU richness and community composition (see detailed list of environmental properties in Table S4).

**Statistical analyses**

Statistical analyses were performed using the software R (version 3.4.0; R Development Core Team, 2017) unless stated otherwise. First, to test whether rare AMF taxa (OTUs represented by \( \leq 3 \) sequence reads per sample) affected estimates of beta-diversity, we performed Procrustes correlation analysis based on Bray Curtis dissimilarity using the protest function (Peres-Neto and Jackson, 2001) of the “vegan” R-package (Oksanen et al., 2018). This approach provides information about congruence between two non-metric multidimensional scaling (NMDS) ordinations; in our case AMF data matrices comprising all OTUs and only abundant OTUs (OTUs represented by \( > 3 \) sequence reads per sample) with 999 permutations. Results indicated nearly identical ordinations in the presence or absence of rare AM fungal OTUs on AMF beta-diversity (Procrustes correlation coefficient = 0.9915, \( p=0.001 \)). Hence, all subsequent analyses were performed using the relative abundance AMF community matrix excluding singletons, doubletons and tripletons.

To assess the spatial distribution of the richness of all AMF OTUs, and of the OTUs belonging to the genera *Glomus* and *Claroideoglomus*, semivariogram analyses were performed using the R package “gstat” (Pebesma, 2004). Data were checked for normality of distribution and were log or square root transformed if necessary according to McBratney and Webster (1986). As environmental data did not
show general distribution trends across the study site in preliminary analyses, isotropy was assumed for semivariogram analysis. Subsequently, empirical semivariograms for the three AMF groups at each sampling date were computed separately. In cases where empirical semivariograms indicated spatial autocorrelation, semivariogram models were fitted using the “fit.variogram” function. Bin sizes were restricted to minimum 35 points per bin; spherical, exponential and linear models were fitted using the default method of the “fit.variogram” function. The model with the lowest sum of squared error (SSErr) was selected. To estimate the amount of variance that was spatially correlated, the percent spatial structure was calculated by subtracting the nugget effect from the sill, and dividing the remaining, or partial sill, variance by the sill variance. Kriged maps for semivariogram models were generated with ArcGIS (ESRI, 2010, Environmental Research Institute, Redlands, CA, USA).

The effects of sampling date and environmental variables on the OTU richness of all AMF and of the genera *Glomus* and *Claroideoglomus* were assessed using LME models accounting for the spatial sampling design. First, different model structures were tested with SAS 9.4 (SAS Institute Inc., Cary, USA) using subplot number as random block effect, and models were evaluated and chosen based on Akaike’s information criterion (AIC) (see Table S5). The addition of spatial autocorrelation structures as well as addition of a random effect for pairs of sampling locations did not substantially improve model fit, and this was also the case for an addition of temporal autocorrelation structure. As the model was to be used repeatedly for selection of important covariates, we chose a model that included a subplot effect and residual error as random effects. The resulting model structure was as follows:

\[ y_{ijk} = \mu + b_j + \beta_1 x_{ijk(1)} + \cdots + \beta_n x_{ijk(n)} + e_{ijk} \]

where \( y_{ijk} \) is the value of the response variable for the \( i \)-th sampling date on the \( j \)-th subplot at the \( k \)-th sampling location, \( b_j \) is the random effect for the \( j \)-th subplot, \( \beta_{1-n} \) are the slopes of the regression on the predictor variables (= fixed effects) \( x_{ijk(1-n)} \), and \( e_{ijk} \) is the independently normally distributed error term with constant variance. This model structure was subsequently used for all LMEMs, which were computed in R using the package “nlme” (Pinheiro et al., 2017). We separately assessed the effects of sampling date and environmental variables in univariate models for each independent variable for total AMF OTUs, *Glomus*, and *Claroideoglomus*. To detect those environmental variables which were most strongly related to the observed temporal effects, additional LMEMs were computed on the
combination of individual environmental variables together with sampling date. The best predictors among plants as well as abiotic and biotic soil properties were then included in the final LMEMs. These contained all significant drivers, and were set up separately for total AMF OTUs, *Glomus*, and *Claroideoglomus*. Models with best predictor variables were selected based on lowest AIC (based on full maximum likelihood) using the “stepAIC” function with forward and backward selection, and checked for homoscedasticity and normal distribution of residuals. Spearman correlations of environmental variables in the final models revealed no considerable multicollinearity. Explained unique variance of dependent variables by independent variables and random effects was assessed following the approach of Nakagawa and Schielzeth (2013) using the function “sem.model.fits” of the “piecewiseSEM” package (Lefcheck, 2016). Separate models were calculated for models that contained plant diversity data because these were only available at three sampling dates.

To assess AMF beta-diversity, Sørensen distances ($\beta_{\text{SOR}}$), as well as their turnover ($\beta_{\text{SIM}}$) and nestedness ($\beta_{\text{SNE}}$) components, were calculated using a function generalizing the “beta.sample” algorithm from the R package “betapart” (Baselga and Orme, 2012); see supplement material for further details). In accordance with the nested study design, the means of the distances between all combinations of within-subplot repetitions were calculated. Significance of groupings of community composition by sampling date and subplot were assessed by analysis of similarities (ANOSIM), as implemented in the R package “vegan” (Oksanen et al., 2018). To compare community structures at different sampling dates within and between subplots, the complete Sørensen distances between all samples were calculated and the median values of all pairwise distances matching each comparison were extracted and visualized using the R package “beanplot” (Kampstra, 2008). Significance of comparisons was established using the non-parametric Mann-Whitney test.

To detect which environmental variables were sources of variation in $\beta_{\text{SOR}}$, permutational multivariate analysis of variance based on the Sørensen distance matrix was performed using the function “adonis” from the R package “vegan” (Oksanen et al., 2018). First, all z-transformed environmental variables were applied in separate univariate models, with stratification by sampling date. In a second model, all significant variables were combined in descending order of their significance in the first run, again
with stratification by sampling date. Spearman correlations of the significant variables revealed no considerable multicollinearity.

Spearman correlations between the temporal developments of $\beta_{\text{SOR}}$ between consecutive sampling dates (delta) at each subplot were calculated and visualized by hierarchical clustering of the inverse correlation by Ward’s criterion using the R packages “dendextend” (Galili, 2015) and “vegan” (Oksanen et al., 2018). Correlation between patterns in temporal development of mean turnover to environmental parameters at each subplot was assessed using the “vegan” (Oksanen et al., 2018) implementation of Mantel’s test.

In order to relate spatial and temporal patterns in $\beta_{\text{SOR}}$, the approach of Mellin et al. (2014) was adapted to the present dataset. Briefly, for spatial $\beta_{\text{SOR}}$, the average of the $\beta_{\text{SOR}}$ values for each sampling date of AMF communities between each subplot and its neighbors was calculated. For temporal $\beta_{\text{SOR}}$, the mean delta at each sampling plot was calculated. Linear models between both $\beta_{\text{SOR}}$ terms were fitted for 1000 different rarefactions and compared to null-models based on 1000 draws of species identities (based on their relative probability of occurrence among samples), while holding constant the total number of species in each sample. To detect further variables contributing to the spatio-temporal patterns in $\beta_{\text{SOR}}$, OTU richness at each subplot and environmental variables and their changes over time were included in the models.

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Authors declare no conflict of interests.

**Author Contributions**

KG, RSB and SK contributed equally to this work. EK, SM and HPP designed the SCALEMIC Experiment. KG, RSB, SK, KMR, EK, FB and TW developed the concept of the current study and KG, RSB and SK drafted the manuscript. KMR conducted DNA extractions and SK conducted AMF DNA amplification and preparation of sequencing libraries. SK and TW performed bioinformatic processing of sequences. RSB, SK, KMR, VK, DP, DB and SM provided data. RSB performed geostatistics and together with HPP fitted linear mixed effect models. KG and AHB performed multivariate statistics. KMR edited and corrected the manuscript’s English. All authors read and revised the manuscript.

**References**


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Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and R Development Core Team (2017) nlme: Linear and nonlinear mixed effects models.


## Tables and Figures

### Tables

**Table 1** LMEM results for richness of entire AMF, *Glomus*, and *Claroideoglomus* for three and six sampling dates. Given are significant environmental variables with their coefficients (data z-transformed for comparison between coefficients), number of samples in the model as well as explained variances. Subplot number was used as random effect (intercepts not displayed). n = number of samples.

<table>
<thead>
<tr>
<th>Sampling dates</th>
<th>Target</th>
<th>Model coefficients of fixed effects</th>
<th>n</th>
<th>Percentage explained variance</th>
<th>Random effects</th>
<th>Environmental variables</th>
<th>Sampling time</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>all AMF OTU</td>
<td>(-0.57 \times \text{plant species no.} + 1.7 \times \text{silt content} + 23.39 \text{ (for sampling time May)} + 25.47 \text{ (for sampling time June)} + 30.95 \text{ (for sampling time October)})</td>
<td>180</td>
<td>48</td>
<td>9</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>all AMF OTU</td>
<td>(-0.7 \times \text{NH}_4^+ + 1.3 \times \text{silt content} + 22.25 \text{ (for sampling time April)} + 22.81 \text{ (for sampling time May)} + 25.3 \text{ (for sampling time June)} + 22.82 \text{ (for sampling time August)} + 30.5 \text{ (for sampling time October)} + 22.96 \text{ (for sampling time November)})</td>
<td>360</td>
<td>45</td>
<td>6</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Glomus</em> OTU</td>
<td>(-0.41 \times \text{plant species no.} + 0.5 \times \text{root biomass} + 1.5 \times \text{silt content} + 0.75 \times \text{pH} + 15.78 \text{ (for sampling time May)} + 16.73 \text{ (for sampling time June)} + 22.9 \text{ (for sampling time October)})</td>
<td>180</td>
<td>60</td>
<td>10</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>Glomus</em> OTU</td>
<td>(0.37 \times \text{legume biomass} + 0.34 \times \text{root biomass} – 0.65 \times \text{NH}_4^+ ) + (1.25 \times \text{silt content} + 0.35 \times \text{pH} – 12.73 \times \text{fungi:bacteria ratio} + 15.46 \text{ (for sampling time April)} + 16.73 \text{ (for sampling time May)} + 18.06 \text{ (for sampling time June)} + 17.41 \text{ (for sampling time August)} + 23.82 \text{ (for sampling time October)} + 17.96 \text{ (for sampling time November)})</td>
<td>360</td>
<td>54</td>
<td>7</td>
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<tr>
<td>6</td>
<td><em>Claroideoglomus</em> OTU</td>
<td>(0.57 \times C_{\text{total}})</td>
<td>360</td>
<td>11</td>
<td>3</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1 Bar graphs representing the temporal distribution of AMF OTUs of Glomeromycota genera detected across the entire plot.

Figure 2 Geostatistical data analysis of AMF OTU richness with all AMF OTUs grouped together per sampling date: a) April, b) May, c) June, d) August and e) November. Spatial patterns within the data were analyzed and calculated as semivariogram models (lower panels in figure) and visualized as kriged maps using these models (corresponding upper panels in figure). Dimensions of all maps are 10 m x 10 m.

Figure 3 Patterns of variability within AMF assemblages across the studied plot from one time point to the next. Stacked bars represent overall beta-diversity ($\beta_{SOR}$) observed in the partial data sets, computed using the R-package betapart (Baselga, 2010); dark grey sections of the bars represent the contribution of the turnover of AMF ($\beta_{SIM}$), light grey sections account for the nestedness of AMF ($\beta_{SNE}$); error bars represent variability between SCALEMIC subplots.

Figure 4 Relationship between spatial and temporal $\beta_{SOR}$ of total AMF, Glomus or Claroideoglomus, respectively. Spatial indices of AMF turnover (x-axis) represent the AMF turnover between each subplot and its neighbors averaged over all sampling dates. Temporal indices (y-axis) represent the mean delta (turnover from one time point to the subsequent time point). Regression lines (black) are based on linear models and 95% confidence intervals (grey dotted lines).
The bar chart shows the change in β-diversity over different months.

- **Apr-May**: Dark brown area representing β_SIM is approximately 0.3, and the gray area representing β_SNE is about 0.1.
- **May-Jun**: The dark brown area (β_SIM) is slightly less than the previous month, and the gray area (β_SNE) is larger than the previous month.
- **Jun-Aug**: The dark brown area (β_SIM) decreases further, and the gray area (β_SNE) increases.
- **Aug-Oct**: The dark brown area (β_SIM) continues to decrease, and the gray area (β_SNE) remains relatively stable.
- **Oct-Nov**: The dark brown area (β_SIM) decreases, and the gray area (β_SNE) increases slightly.

The chart indicates a trend where the dark brown area (β_SIM) generally decreases over time, while the gray area (β_SNE) increases, especially noticeable in the periods from Jun-Aug to Oct-Nov.