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Steeper spatial scaling patterns of subsoil microbiota are shaped by deterministic assembly process

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Abstract :

Although many studies have investigated the spatial scaling of microbial communities living in surface soils, very little is known about the patterns within deeper strata, nor is the mechanism behind them. Here, we systematically assessed spatial scaling of prokaryotic biodiversity within three different strata (Upper: 0-20 cm, Middle: 20-40 cm, and Substratum: 40-100 cm) in a typical grassland by examining both distance-decay (DDRs) and species-area relationships (SARs), taxonomically and phylogenetically, as well as community assembly processes. Each layer exhibited significant biogeographic patterns in both DDR and SAR ($P < 0.05$), with taxonomic turnover rates higher than phylogenetic ones. Specifically, the spatial turnover rates, β and z values respectively, ranged from 0.016±0.005 to 0.023±0.005 and 0.065±0.002 to 0.077±0.004 across soil strata, and both increased with depth. Moreover, the prokaryotic community in grassland soils assembled mainly according to deterministic rather than stochastic mechanisms. By using normalized stochasticity ratio (NST) based on null model, the relative importance of deterministic ratios increased from 48.0 to 63.3% from Upper to Substratum, meanwhile a phylogenetic based method revealed average βNTI also increased with depth, from -5.29 to 19.5. Using variation partitioning and distance approaches, both geographic distance and soil properties were found to strongly affect biodiversity structure, the proportions increasing with depth, but spatial distance was always the main underlying factor. These indicated increasingly deterministic proportions in accelerating turnover rates for spatial assembly of prokaryotic biodiversity. Our study provided new insight on biogeography in different strata, revealing importance of assembly patterns and mechanisms of prokaryote communities in below-surface soils.

Keywords : assembly mechanism, biodiversity, biogeography, grassland, prokaryote, spatial scaling

1. Introduction

The microbiome is one of several biological communities in terrestrial soil ecosystems (Fierer, 2017), and plays several important ecological roles, such as decomposition and geochemical cycling, while soils themselves provide unique habits for a variety of microorganisms (Serna-Chavez, Fierer & Van Bodegom, 2013). Soil profiles are often meters in depth, and changes in soil structure across depth are associated with shifts in microbial communities across strata (Fierer, Schimel & Holden, 2003; Hartmann, Lee, Hallam & Mohn, 2009), representing distinct compositional divergences between microbial communities from deep soil strata and those from the surface (Eilers, Debenport, Anderson & Fierer, 2012; Stone, DeForest & Plante, 2014). For example,

previous studies in soil microbial communities have shown that decreases in their abundance and richness (Fierer et al., 2003; Eilers et al., 2012; Jiao et al., 2018), and increasing differentiation in structure (Kramer, Marhan, Haslwimmer, Ruess & Kandeler, 2013; Chu et al., 2016) occurred with soil depth. According to these observations, one may expect a greater spatial dissimilarity among deep-soil microorganisms compared to that of shallower strata along soil profiles, and consequently different scaling patterns should be observed in different soil strata.

One of the central goals of ecology is to characterize and understand fundamental patterns in the structure of the biosphere (Shade et al., 2018). The spatial scaling of biodiversity (i.e., taxonomic and phylogenetic diversity) is one such pattern, corresponding to how observables at a given scale change (Green & Bohannan, 2006). Understanding spatial scaling can give important information about the structure of a community and provide insights for setting conservation priorities. Over the past few decades, robust spatial scaling patterns have been observed in various system, providing evidence for the biogeography of organisms, from the smallest to the largest (Green & Bohannan, 2006; Ranjard et al., 2013; Paul, 2014; O'Brien et al., 2016; Meyer et al., 2018; Shade et al., 2018). The distance-decay (DDR) and species-area relationship (SAR) are regarded as the two best-documented fundamental laws of community ecology (Nekola & White, 1999; Horner-Devine, Lage, Hughes & Bohannan, 2004). DDR typically describes the fact that the similarity in the composition of biological assemblages decreases with increasing geographic or environmental distance (Nekola & White, 1999; Nekola & McGill, 2014). The rate at which similarity decreases is generally summarized by the slope of the DDR, β (Nekola & McGill, 2014). SAR reflects how the observed species richness increases with the size of the sampled area (Horner-Devine et al., 2004), and is generally summarized by $S = cA^z$, where the z parameter represents the rate at which species accumulate with increasing area (He & Hubbell, 2011). Both β and z values represent the rates at which spatial replacement occur across community diversity. Although microbial turnover rates have been well documented in many soil ecosystems, the majority of these studies have focused on surface soils (0-20 cm), paying limited attention to rates along vertical soil profiles. Therefore, a general understanding of whether spatial scaling of microorganisms exists in deeper soil strata, and what relationships they present among different strata, remains lacking.

Behind the simple description of microbial spatial distribution patterns, an increasing number of studies have attempted to disentangle the mechanisms and potential processes responsible for the observed patterns

(Ofiţeru et al., 2010; Prach & Walker, 2011; Zhou et al., 2013). Two fundamental types of ecological mechanisms, determinism and stochasticity, have been proposed as underlying factors influencing the assembly distribution of microbial communities. Deterministic theory is based on the concept of ecological niche and stresses the key role of environmental selection (or environmental filtering) imposed by abiotic and biotic factors in the assembly of microbial communities (Stegen, Lin, Konopka & Fredrickson, 2012; Wang et al., 2013). Meanwhile, stochasticity is related to neutral theory, which considers all organisms as equal in their ecological characteristics, and asserts that community structure is largely governed by the history of stochastic events such as dispersal, birth, death, extinction, or speciation (Chase & Myers, 2011). It is now generally accepted that both deterministic and stochastic processes occur simultaneously during the assembly of local communities (Chase, 2010; Dumbrell, Nelson, Helgason, Dytham & Fitter, 2010; Langenheder & Székely, 2011; Zhou et al., 2014). Recently, a number of studies have defined the ecological context and factors that can explain the relative importance of the different assembly mechanisms, and determine their dynamics in space, with several distinct approaches (Langenheder & Lindström, 2019). In practice, however, separating the deterministic and stochastic processes controlling ecological succession across different scales is challenging and remains poorly understood (Nemergut et al., 2013). Knowledge on how the fundamental ecological processes that shape the assembly of microbial communities vary along soil profiles is essential to better understand microbial ecology (Powell et al., 2015; Chu et al., 2016; Evans, Martiny & Allison, 2016). To our present knowledge, it is still unclear to us how the relative importance of stochasticity versus determinism will govern the spatial patterns of microbial communities, and what is the main underlying process affecting communities along soil profiles.

Grasslands play significant ecological roles in the Earth's biosphere, and soil prokaryotic communities are a key factor in maintaining their function and stability (Blair, Nippert & Briggs, 2014; Schloter, Nannipieri, Sørensen & van Elsas, 2018). Recent technological advances promise a greater ability to detect microbial diversity and describe the spatial scaling of microbial communities. Hence, we characterized the prokaryotic taxonomic and phylogenetic biodiversity using high-throughput 16S ribosomal RNA (rRNA) gene sequencing, and quantified their spatial patterns (DDR and SAR) and assembly mechanisms in three strata of grassland soils (Upper: 0-20 cm, Middle: 20-40 cm, and Substratum: 40-100 cm, respectively). We looked to test the following hypotheses: (i) taxonomic and phylogenetic turnover rates respond differently to spatial scale within a given strata; (ii) spatial patterns also exists in deeper soil strata, and turnover rates for both DDR (β) and SAR (z) will

differ between strata; and (iii) prokaryotic turnover rates at deeper strata are under greater influence of determinism in community assembly as compared with shallower strata.

2. Materials and Methods

2.1 Study area

For this study, a typical grassland ecosystem was selected in Duolun County (116°17′E, 42°02′N), Inner Mongolia Autonomous Region of China, which belongs to a temperate zone habitat, characterized by a semiarid continental monsoon climate (Zhang, Johnston, Li, Konstantinidis & Han, 2016). The experiment site was free from anthropogenic disturbances (i.e., grazing) and the ecosystem was dominated by perennial herbs species, including *Stipa klemenzii Roshev.*, *Artimesia frigida*, *Potentilla acaulis*, *Agropyron cristattum, Allium bidentatum*, and *Cleistogenes squarrosa* (Zhang et al., 2016). Long-term mean annual precipitation was approximately 383 mm, with ninety percent of the total precipitation distributed across six months, from May to October (Ru, Zhou, Hui, Zheng & Wan, 2018). Monthly average temperature varied from -17.5°C in January to 18.91°C in July (Ru et al., 2018). According to the FAO, the soil type in this area was classified as Haplic Calcisols, which is a loam composed of 62.7% sand, 20.3% silt, and 17% clay (Liu, Zhang & Wan, 2009). The soil pH was neutral (6.84 ± 0.07) and an average bulk density of approximately 1.31 g· cm^{-3} (Liu et al., 2009).

2.2 Sampling procedures

Soil sampling was carried out in August 2017 according to a nested design, in which smaller sample areas are nested within larger ones (FIGURE 1). In this study, the center plot (red point, area of 8 m^2) was used as the reference point, 12 plots of 1m^2 were set in a cross manner with four plots abutting a central plot and four plots placed at 10, 100 and 1000 m from the central plot in each cardinal direction (FIGURE 1). Sample soils were taken using a soil sampler tube (4 cm diameter × 100 cm deep) over an area of 2 km^2. The center plot consisted of 17 individual soil cores, while each 1 m^2 plot was composed of 5 cores. Each soil core was divided into three different subsamples based on obvious changes in soil appearance (i.e., color and structure): Upper (0-20 cm), Middle (20-40 cm) and Substratum (40-100 cm), which are close to the depths of A, B and C horizons in situ. In total, 231 soil samples (77 sample cores ×3 soil strata) were obtained. After sieving to remove coarse roots and stones, soils were immediately transported to the laboratory. A part of each sample was stored at 4 °C for determination of physicochemical parameters, while the other part was stored at -80 °C for DNA extraction.

2.3 Soil physicochemical analyses

For each sample, soil moisture content (%) was estimated as the mass difference between soil before (15 g) and after drying for 48h in an oven at 55 °C. Soil pH was measured after creating a soil suspension, which consisted of a soil: water ratio of 1: 2.5 (weight/g: volume/ml), with a pH meter according to the standard protocol (Sartorius). Other physicochemical parameters used in this study were measured according to the protocols in Shi et al.'s descriptions (Shi et al., 2018). In short, the air-dried soil samples were titrated with potassium dichromate standard solution and concentrated sulfuric acid to determine total organic carbon (SOC). To determine the total nitrogen (TN) content, the soil samples were dissolved with potassium persulfate solution in a pressure cooker at 121 °C for 40 min. After cooling, the solution was taken out and the concentration of the digestion solution was determined by ultraviolet spectrophotometer. The contents of ammonia-nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) in soil were determined by centrifugation filtration after the soil sample was added to 2 mol/L potassium chloride solution after shaking for 1 h. The absorbance of the supernatant was measured at 420 and 210 nm, respectively, from which the contents of NH_4^+-N and NO_3^--N were calculated. Detailed information on soil characteristics is given in TABLE S1.

2.4 Soil DNA extraction, amplification of 16s rDNA and sequencing

Soil total DNA was extracted from 0.5 g of thoroughly mixed soil using MP FastDNA™ Spin Kit for Soil (MP Biomedicals, LLC, USA) according to the manufacturer's instructions.

Extracted DNA was amplified using the 16S rRNA universal primer set 515 forward (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 reverse (5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V4 hypervariable regions of the prokaryotic 16S rRNA genes and supplemented with sample-specific barcodes (Caporaso et al., 2012; Yarza et al., 2014). The polymerase chain reaction (PCR) amplification was conducted in a 50 μl reaction containing 0.5 μl Taq DNA Enzyme (TaKaRa), 5 μl 10× PCR buffer, 1.5 μl dNTP mixture, 1.5 μl of both 10 μM forward and reverse primers, 1 μl of template DNA within 20–30 ng μl^{-1} and 39 μl ddH_2O. The thermal cycle conditions were as follows: denaturation at 94°C for 1 min, 30 cycles of 94°C for 20 s, 57°C for 25 s and 68°C for 45 s, thereafter extension at 68°C for 10 min and then held at 4°C. PCR products were tested by 1% agarose gels and purified with a E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, Inc., USA), then concentrations calculated with Nanodrop 2000 and stored.

Samples were sent for sequencing on the Illumina Hiseq platform at Magigene Biotechnology Co., Ltd. (Guangzhou, China).

2.5 Sequencing data analysis

The raw reads of the 16S rRNA gene were submitted to a publicly available sequence analysis pipeline (http://mem.rcees.ac.cn:8080) integrated with various bioinformatics tools (Feng et al., 2017). The reads were assigned to different samples according to their barcodes, allowing for a single mismatch, after which the barcode and primer sequences were trimmed. Forward and reverse reads of the same sequence were merged using FLASH (Salzberg & Magoč, 2011). Reads were filtered using Btrim with an average Quality Score >20 and minimum length of 140 bp (Kong, 2011). Any sequences with ambiguous bases were removed and only reads with length between 245 and 260bp were retained for further analysis. The Greengenes reference data set (DeSantis et al., 2006) was used as reference for chimera checking, while clustering of sequences into operational taxonomic units (OTUs) was performed using UPARSE (Edgar, 2013) with a 97% sequence similarity threshold. Singletons were retained as rare species account for significant shifts in β diversity (Jousset et al., 2017). Due to the large difference in the number of sequences for each sample, all the samples were randomly resampled to the same total number of reads (20,291). The resampled OTU table was used for subsequent community analysis. The Ribosomal Database Project (RDP) 16S classifier was used to assign 16S rRNA representative sequences to taxonomic annotations with 80% confidence estimates (Wang, Garrity, Tiedje & Cole, 2007). Representative sequences were aligned utilizing PyNAST (Caporaso et al., 2009) and phylogenetic tree was generated by FastTree (Price, Dehal & Arkin, 2009; Price, Dehal & Arkin, 2010).

2.6 Statistical analyses

After ensuring the values of each group are normally distributed, the differences in soil physicochemical variables among the three strata were tested using ANOVA and Tukey post-hoc tests. Dissimilarity between prokaryotic communities was estimated using Bray-Curtis and Sørensen indexes for taxonomic diversity and (un)weighted UniFrac for phylogenetic diversity, using the vegan (Dixon, 2003) and phyloseq (McMurdie & Holmes, 2013) R packages, respectively. These dissimilarity matrices were used to visualize differences in community composition using Principal Coordinate Analysis (PCoA). Differences in community composition and multivariate dispersion among the different soil strata were tested using Permutational Analyses of Variance (PERMANOVA) (Anderson, 2001) and Permutational Analyses of Multivariate Dispersions (PERMDISP) (Anderson, Ellingsen & McArdle, 2006), respectively. A geographic distance matrix was estimated using Euclidean distance based on sampling sites coordinates (Figure 1, the red point was the origin).

As different facets of diversity could scale differently across space (Webb, Ackerly, McPeek & Donoghue, 2002), we simultaneously examined the DDRs based on taxonomic and phylogenetic dissimilarity matrices. The rates (β) of distance decay were estimated through regression of log-transformed dissimilarity against log-transformed geographic distance. DDRs were represented by the following equation:

$$\log(Dissim) = \beta \times \log(D) + c \quad (1)$$

where *Dissim* is the community dissimilarity, c is the intercept parameter, D is the geographic distance and β is the slope of DDR, or spatial turnover rate.

Generally, SAR used to be described as a function of power law or its logarithmic form, but many other functions have been considered (Dengler, 2009). To examine relationships between area and diversity (species richness or phylogenetic diversity, PD), we performed several nonlinear multimodel SARs, and this was implemented by fitting nonlinear relationships based on possible SAR models using package mmSAR (Guilhaumon, Mouillot & Gimenez, 2010) in R. We selected the reasonable model by ranking them based on coefficients of determination (R^2) and the Akaike Information Criterion (AIC), as well as documented z values in the range. Within each 1m^2 sample plot, five soil cores were composited for SAR analysis. The distances of each sampling location to the plot center were $\sqrt{2}/2$, 1, $\sqrt{2}$, 2, 10, 100 and 1000 m, yielding sampling areas of 1, 2, 4, 8, 200, 2×10^4 and 2×10^6 m^2, respectively. Permutation tests were then performed to determine whether the turnover rates (β and z values) were significantly different between strata. Estimated turnover coefficients were compared with the observed ones by one-tailed t test after 10,000 bootstraps between dissimilarity versus distance and richness versus area (Zhou, Kang, Schadt & Garten, 2008). All log-transformations were performed using log10 transformations.

Null-modeling-based approaches were preferentially performed in this study to infer community assembly mechanisms. The first approach referred to the method proposed by Chase et al. (Chase, Kraft, Smith, Vellend & Inouye, 2011), with a null model algorithm maintaining constant species frequency and richness, to qualify the significance of the observed difference of the prokaryotic communities in the three strata from random expectation for all communities. If the observed ecological community variations are statistically different from null expectation, the community dynamics are regarded as largely shaped by deterministic processes. Otherwise, they are considered to be dominated by stochastic processes. We then applied the modified method of Ning et al. (Ning, Deng, Tiedje & Zhou, 2019), which was tested with simulated communities by considering abiotic

filtering, competition, environmental noise, and spatial scales, to calculate the stochastic ratio (Normalized Stochasticity ratio, NST) for estimating the relative importance of stochasticity in shaping community structure. Furthermore, a two-step statistical framework that quantifies the relative contributions of various ecological processes to microbial assembly was applied (Stegen et al., 2012; Stegen et al., 2013). We first calculated β-mean-nearest taxon index (βNTI) for pairwise phylogenetic turnover between communities to estimate the proportion of determinism. A value of $|\beta NTI| > 2$ indicates that observed turnover between a pair of communities is governed primarily by determinism, which could be divided into homogeneous selection ($\beta NTI < -2$) and heterogeneous selection ($\beta NTI > +2$). On the contrary, a value of $|\beta NTI| < 2$ indicates that observed differences in phylogenetic composition between a pair of communities is governed primarily by stochasticity. Then the relative contributions of stochastic processes were estimated with Bray-Curtis-based Raup-Crick matrix (RC_{bray}). If the value of $|RC_{bray}| > 0.95$, community assembly is considered significantly dominated by dispersal, either by homogenizing dispersal ($RC_{bray} < -0.95$) or by dispersal limitation ($RC_{bray} > +0.95$). However, if $|RC_{bray}| < 0.95$, then community is undominated (including weak selection, weak dispersal, diversification and drift processes).

Additionally, variation partitioning and distance approaches, including Mantel tests (Horner-Devine, Carney & Bohannan, 2004) and Multiple regression on distance matrices (MRM) (Lichstein, 2007), were then used to identify the potential contributions of geographic distance versus edaphic variables to prokaryotic community dissimilarity. They are able to partition variation in community composition between environmental and spatial distances, while accounting for spatial autocorrelation of environmental variables or distances (Tuomisto & Ruokolainen, 2006). While MRM was advanced to investigate linear, nonlinear or nonparametric relationships between a multivariate response distance matrix and any number of explanatory distance matrices. Partial regression coefficients of an MRM model provided a measure of the rate of change in microbial community similarity for variables of interest when other variables were held constant. Generally, if an edaphic parameter is significant, that indicates selection processes are important, inversely, if spatial distance is significant and it indicates dispersal limitation. Before applying calculations, values of edaphic variables were standardized to give an equal weight to each variable.

All the statistical analyses were carried out using R software (https://www.r-project.org/) with related R packages, “vegan” (Dixon, 2003), “stats” (Field, Miles & Field, 2012), “ggplot2” (Wickham, 2016), “phyloseq”

(McMurdie & Holmes, 2013), "mmSAR" (Guilhaumon et al., 2010), "picante" (Kembel et al., 2010), "ape" (Paradis, Claude & Strimmer, 2004) and "NST" (Ning et al., 2019).

3. Results

3.1 Edaphic properties of the samples

The edaphic physicochemical parameters exhibited a high range of variation across soil strata (TABLE S1). All tested soil properties (moisture, pH, TN, NH_4^+-N, NO_3^--N, SOC), were significantly influenced by depth. TN and SOC were the most responsive variables as every pair of strata differed significantly (ANOVA, $P < 0.05$). Other properties (moisture, pH, NH_4^+-N) significantly differed between the Upper and two lower strata, while NO_3^--N showed significant differences between the top two strata and the Substratum (TABLE S1). These results indicated that soils exhibited a strong vertical variation in environmental conditions, specifically in nutrient content.

3.2 Prokaryotic community diversity, composition and structure across different strata

In this study, a total of 89,744 OTUs were obtained across the 231 soil samples. For each sample site, the alpha diversity of Upper community taxa was higher than Middle and Substratum displaying a decreasing trend with depth (ANOVA, $P < 0.01$), suggesting that Upper prokaryotic assemblages varied more between samples than Middle and Substratum communities (FIGURE S1a). The overlap in the OTU composition among different strata was assessed using a Venn diagram (FIGURE S1b). We observed that 37% (n = 18,315) of the OTUs were shared by all three strata. The Upper layer exhibited the twice as many unique OTUs (n = 10,204) as the two other strata (n = 5,257 and 5,813 for the Middle and Substratum, respectively).

The relative abundances of taxa in the soil samples were investigated at the phylum level (FIGURE 2a). Except for the unclassified, among the nine most abundant phyla across all samples, eight were classified as bacteria, one phylum belonged to archaea. Furthermore, Actinobacteria (15.0%-21.0%), Thaumarchaeota (16.9%-19.4%), Proteobacteria (12.3%-16.8%), Acidobacteria (11.0%-12.2%), Verrucomicrobia (5.85%-11.56%), Firmicutes (4.12%-6.98), Bacteroidetes (2.62%-4.60%), Planctomycetes (1.37%-2.15%) and Gemmatimonadetes (0.50%-0.92%) phyla accounted for more than 70% of abundance across all soil strata. Contrary to the changes observed in OTUs, taxonomic composition of the communities appeared to be affected by depth in only a limited manner, but with their ranking changed between the three soil strata. For example, there were significant differences in Unclassified and Bacteroidetes ($P < 0.05$), whereas Thaumarchaeota and

Acidobacteria displayed no obvious differences across the three layers ($P > 0.05$). The remaining phyla showed significant differences between the top layer and deeper strata (TABLE S2).

The dissimilarity in community structure among strata was visualized using principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity. The samples from the three strata were clearly separated on the first two PCoA axes, which explained ~35% of total variance (FIGURE 2b). Communities generally clustered by soil depth with significantly different prokaryotic communities occurring overall between sampling strata (PERMANOVA, $P < 0.001$, TABLE S3), suggesting community composition was affected by depth. PERMDISP confirmed differences in community dispersion among soil strata ($P < 0.001$, TABLE S3) and specifically between Upper versus Substratum and between Middle versus Substratum (TABLE S3). Within individual layers, the prokaryotic communities of the Upper layer were tightly clustered in FIGURE 2b, whereas the strongest compositional variabilities were found within Substratum samples (FIGURE S2), indicating that there was more homogeneous dispersion of Upper communities, vice versa in Middle and Substratum. These results suggested that each soil stratum harbored divergent microbial diversity, composition and structure.

3.3 Spatial turnovers of prokaryotic community structure in different soil strata

In order to measure and compare the spatial turnover rates of microbial biodiversity in the three soil strata, we estimated DDRs and SARs for each stratum, separately. First we analyzed DDRs and observed a significant correlation between taxonomic composition dissimilarity (Sørensen distance) and geographic distance (Globally, $\beta = 0.01398$, $R^2 = 0.43$, $P < 0.001$). In addition, the slopes (β) of the DDRs differed between strata (FIGURE 3a), showing a significant increasing trend from the Upper stratum ($\beta = 0.00860$, $R^2 = 0.40$, $P < 0.001$), to the Middle ($\beta = 0.01378$, $R^2 = 0.51$, $P < 0.001$), and to the Substratum ($\beta = 0.02011$, $R^2 = 0.59$, $P < 0.001$). As different aspects of microbial biodiversity might scale differently across space, we estimated DDRs for community structure and phylogenetic diversity using Bray-Curtis and (un)weighted UniFrac, respectively. Using unweighted phylogenetic dissimilarity matrix to calculate DDRs, the global scaling parameter (β) of phylogenetic diversity was significantly ($P < 0.001$) lower than for taxonomic diversity (Globally, $\beta = 0.009582$, $R^2 = 0.44$, $P < 0.001$). Again, we observed an increase of the scaling parameter with increasing depth (FIGURE 3b), with an almost flat DDR in the Upper layer ($\beta = 0.00551$, $R^2 = 0.32$, $P < 0.001$), an increase in the Middle layer ($\beta = 0.00948$, $R^2 = 0.46$, $P < 0.001$), and the largest β value observed in the Substratum ($\beta = 0.01423$, $R^2 = 0.58$, $P < 0.001$). Similarly, the differences in abundance-weighted taxonomic (Bray-Curtis, FIGURE 3c) and

phylogenetic (weighted UniFrac, FIGURE 3d,) structure of the prokaryotic communities exhibited a significant increase with geographic distance ($P < 0.001$). Spatial turnover parameters of both taxonomic and phylogenetic biodiversity were very similar across the three strata, displaying a linear increase of dissimilarity with geographic distance. Moreover, the scaling exponent, β, was the flattest in the Upper and increased towards the subsurface strata.

Next we analyzed SARs, which constitute another distribution pattern to characterize the spatial scaling of taxonomic and phylogenetic diversity turnover of the microbial community over geographic distance. After comparing nonlinear SAR models, model fitting results indicated that the power law model always produced the best fits (TABLE S4), showing an increasing trend along with DDRs, while others had no such phenomenon. In its logarithmic form, species richness was strongly and positively related with area (Globally, $z = 0.07668$, $R^2 = 0.89$, $P < 0.001$). Similarly, the scaling parameters of taxonomic SARs (z) exhibited an increasing trend with depth (FIGURE 3e), with the Upper layer showing the smallest value ($z = 0.07065$, $R^2 = 0.93$, $P = 0.003$), followed by the Middle ($z = 0.07505$, $R^2 = 0.93$, $P = 0.002$), and the Substratum ($z = 0.08435$, $R^2 = 0.92$, $P = 0.003$). Importantly, the global scaling parameter (z) of phylogenetic diversity increased with area at a slower pace than species richness ($z = 0.06494$, $R^2 = 0.90$, $P < 0.001$). In keeping with taxonomic SARs, a similar trend was observed for phylogenetic SARs (FIGURE 3f), with z values of 0.06320 ($R^2 = 0.94$, $P = 0.002$), 0.06325 ($R^2 = 0.94$, $P = 0.002$) and 0.06831 ($R^2 = 0.93$, $P = 0.003$) for Upper, Middle, and Substratum, respectively. Together, these results showed that both taxonomic and phylogenetic prokaryotic biodiversity scale differently and significantly across soil strata, with an increasingly stronger turnover with depth.

3.4 Ecological assembly processes of prokaryotic communities

Null model approaches were used to determine the relative importance of deterministic and stochastic mechanisms in the assembly of prokaryotic diversity within the different soil strata. A null model approach based on taxonomic beta-diversity showed that dissimilarities of microbial communities from the three strata were significantly higher than expected under null expectations (ANOVA, $P < 0.001$, TABLE 1), indicating that communities in this grassland ecosystem assemble mainly in a deterministic manner. To further quantify the relative importance of deterministic processes in shaping soil prokaryotic community succession, stochastic (NST) and deterministic ratios were calculated by proportioning the observed dissimilarity for each pairwise comparison and the null expected dissimilarity. Though determinism was the predominant mechanism overall,

its quantitative ratios varied in a depth-dependent manner. Along the vertical profile, importance of determinism contributed to community variations at 48.0%, 53.2%, and 63.3% for the Upper, Middle, and Substratum, respectively (TABLE 1). Another null model approach, constituted of taxonomic and phylogenetic matrices, was used to further disentangle different assembly processes and to quantify their relative importance. Across the three soil strata, average phylogenetic dissimilarity between a pair of communities showed an increasing βNTI of -5.29, 2.75 and 19.59 with depth, and a concomitant increase in the deterministic percentage of specific ecological processes (specially, heterogeneous selection) (FIGURE S3), demonstrating that the relative contribution of deterministic processes played a more important role than stochastic processes. Overall, they indicate that deterministic mechanisms applied increasing influence to community differences with depth.

Geographic distance and various edaphic parameters are two detectable deterministic factors shaping prokaryotic communities. Variation partitioning and distance methods allow determination of whether environmental or spatial factors can explain variation or differences in community composition across horizonal spaces. First we performed Mantel tests on community taxonomic composition (i.e., Sørensen dissimilarity) (TABLE S5). Prokaryotic composition dissimilarity increased consistently with geographic distance ($P = 0.001$) and edaphic variations ($P = 0.001$), indicating compositional variation was spatially and environmentally structured across all strata. Meanwhile, Mantel and partial Mantel tests showed that the importance (regression coefficient, R^2, TABLE S5) of distance and edaphic variables in structuring prokaryotic community turnover dramatically increased along the soil profile, while geographic distance always showed a stronger effect on community dissimilarity. The full MRM model composed of all variables (geographic distance, moisture, pH, TN, NH_4^+-N, NO_3^--N, SOC) explained 19%, 24% and 35% of the variability in community similarity ($P = 0.001$, TABLE 2) for Upper, Middle, and Substratum, respectively. Geographic distance was the only variable that significantly influenced variation in community dissimilarity across the three strata ($P = 0.001$). Moisture and TN explained a significant proportion of variation in community dissimilarity in both Middle (Moisture, $P = 0.004$; TN, $P = 0.007$) and Substratum (Moisture, $P = 0.006$ for; TN, $P = 0.025$). SOC explained a significantly proportion of prokaryotic community variation in the Upper stratum ($P = 0.030$), while NO_3^--N was significant in the Substratum ($P = 0.043$). It is worth noting that neither pH nor NH_4^+-N contributed significantly in any stratum (TABLE 2). Importantly, community turnover continued to increase in relation to spatial distance, which was consistent with the Mantel and partial Mantel tests, indicating a potential deterministic position of dispersal

limitation shaping community turnover variation with depth.

4. Discussion

An important topic of microbial ecology is to determine the patterns and assembly mechanisms amidst complex and uncertain microbial relationships (Thompson et al., 2017). Previous reports on spatial scaling of soil microbial communities have largely suggested that spatial patterns were variable across different habitats (Martiny, Eisen, Penn, Allison & Horner-Devine, 2011; Wang et al., 2017). While soils profiles provide ideal conditions for studying microbial spatial scaling (Fierer, 2017), our understanding of the fundamental patterns and mechanisms that underlie microbial biogeographic patterns across vertical soil structures remain limited. Here, our objective was to describe, compare, and understand the spatial scaling of prokaryotic communities across multiple soil strata in a typical grassland ecosystem. Our study will be helpful for understanding the spatial scaling of microorganisms, and provide insights into the complexity and maintenance of natural biodiversity.

This study demonstrated that the total alpha diversity of prokaryotic microorganisms in the soil of a typical grassland displays a regular decrease along vertical soil depth. Within one-meter soil profiles, the species richness (OTU number) decreased significantly with increasing soil depth (FIGURE S1). This trend of decreasing microbial alpha diversity is consistent with many other previous studies (Deng et al., 2015; Tang et al., 2018; Feng, Guo, Wang, Song & Yu, 2019), which have shown that the richness, abundance, and composition of the soil microbiome displayed layer specificity and distinct differences across strata. The community distance showed clearly that community variability increased within sites from Upper layer to Substratum (FIGURE S2), suggesting community heterogenization was occurring. These continuous trends also have important reference value for understanding soil stratification. The soil profile in this study has a strong environmental gradient, and many soil factors change with increasing depth (TABLE S1). The correlation between these soil environmental parameters and the microbiome indicates that all factors directly or indirectly affected the structure of the community, in particular the significant decline in microbial OTU richness as depth increased, and the consistent trend between TN and SOC content decreasing with depth. Thus, the vertical differences in these microbiomes can be attributed in a large extent to the decline in the availability of various resources with soil depth (Wu et al., 2020). Together, the above results show that soil depth is an important factor affecting microbial diversity and structure, and the gradient change of soil environment caused by soil

depth may be the most important factor in the vertical distribution characteristics of microbial species.

In microbial ecology, spatial patterns like DDR and SAR have received growing attention over the last decade, however most previous studies have only focused on just one of these relationships (Zhou, Kang, Schadt & Garten, 2008; Liang et al., 2015; Deng et al., 2016; Wang et al., 2017; Deng et al., 2018). Though, biogeographical patterns are acknowledged scale-dependent, in this study, we observed significantly positive DDRs and SARs ($P < 0.05$, FIGURE 3) for both taxonomic and phylogenetic prokaryotic diversities in all three soil strata. Overall, the slopes of taxonomic and phylogenetic DDRs (β) are relatively smaller and remarkably similar to previous reports for bacteria (Horner-Devine et al., 2004; Wang et al., 2017), indicating specifically slower microbial scaling characteristics. Meanwhile, z values for taxonomic and phylogenetic SARs were in accordance with previous observations of relatively low slope range of 0.001-0.1 (Green & Bohannan, 2006; Woodcock, Curtis, Head, Lunn & Sloan, 2006; Dengler, 2009), suggesting mild spatial turnover rates in microbes. Importantly, different facets of diversity scaled differently with space, with phylogenetic diversity exhibiting lower turnover rates (β and z) than taxonomic ones, indicating that the divergence of communities in phylogeny is slower than that in taxonomy. This was consistent with our first hypothesis and suggests that despite the observation of different species turnover with distance, the phylogenetic origin of the microbes was more conserved and might reflect local adaptation of communities.

It is worth noting that the pattern of beta diversity was stratum specific, more importantly, we observed that turnover rates (β and z) became steeper with depth in both DDRs and SARs (FIGURE 3), which demonstrated that the turnover of prokaryotic communities across space is faster within the deeper soil strata than in superficial soil layers. In ecological terms, this suggested that deep soil prokaryotic communities change more with distance (spatial and environmental) than surface ones, likely due to the higher isolation between them as compared to the surface strata. These results confirmed our second hypothesis, that is, the existence of DDRs and SARs in deeper soil strata with significant greater turnover rates. We also observed a decline in richness (FIGURE S1), associated with the larger divergence in community composition with depth (TABLE S3 and FIGURE S2). The above results revealed a remarkable simultaneity in spatial scaling properties of prokaryotic different facts of diversities (alpha and beta diversities) across different strata.

Understanding the mechanisms mediating the underlying spatial variations in biodiversity is essential to advancing fundamental knowledge of microbial biosphere (Langenheder & Lindström, 2019). The majority of

studies based on the null model approach have indicated that succession and assembly of the microbial community are controlled by the interplay between deterministic and stochastic processes (Stegen, Lin, Konopka & Fredrickson, 2012; Stegen et al., 2013; Wang et al., 2013). To detect the relative influences of stochastic and deterministic processes over community succession, we performed an updated null model test calculation based on taxonomic dissimilarity (Ning et al., 2019). Here, we found that deterministic mechanisms strongly structured prokaryotic community assembly in all strata (TABLE 1) and their importance increased substantially with depth, ranging from 48.0 to 63.3%, suggesting that growing higher isolation between strata. Inspiringly, Stegen's framework based on taxonomic and phylogenetic beta diversity also resulted in an increasing deterministic proportion at deep soils (FIGURE S3). Even though these two null model approaches are different at their core, they verified each other, proving that this conclusion is reliable. These findings are in agreement with our third hypothesis and support previous studies demonstrating that determinism is a primary factor underlying microbial spatial scaling (Freedman & Zak, 2015; Powell et al., 2015; Guo et al., 2018).

Furthermore, another typical working framework is that microbial communities are primarily controlled by dispersal limitation acting at the largest spatial scales with environmental filtering of local sites (Mittelbach & Schemske, 2015). We compared the spatial space and environmental factors responsible for variations of prokaryotic communities. Indeed, in any single soil stratum, environmental differences are likely to increase with increasing geographical distance or sampling area, and environmental filtering is expected to alter the microbial community composition and increase divergence across the geographic distance (Cox, Newsham, Bol, Dungait & Robinson, 2016). In addition, spatial distance can limit the dispersal of microorganisms and accelerate speciation (Diniz-Filho & Telles, 2000), which also contributes to the divergence of community compositions. It has been shown that the surface microbial community can be more efficiently dispersed by actions such as water runoff, wind, and animal transportation, which tend to homogenize communities, and thus leading to weaker spatial patterns (Vellend & Agrawal, 2010). According to our results of MRM (TABLE 2) and partial Mantel tests (TABLE S5), we found that both the coefficient and Mantel r values between community structure and all measured edaphic parameters, while excluding geographic distance, became stronger when the upper layer was compared with middle and substratum layers, indicating environmental filtering increased with soil depth. In addition, geographic distance partitioning of edaphic parameters showed a clearly enhanced trend (Coefficient: 0.0101, 0.0150, 0.0306; Mantel r: 0.22, 0.28, 0.39; all $P < 0.01$) with increasing depth. These

results suggested that environmental selection and spatial dispersal limitation were both strengthened in the microbial community with soil depth. Collectively, these results showed that prokaryotic communities were greatly impacted by geographic distance and environmental selection in the three strata, with both having an increasingly stronger effect on community structure with depth. However, dispersal limitation had proportionally larger effect on species establishment in successful colonization than environmental filtering. They both further validated the observation of stronger diversity scaling in deeper strata compared with surface soil. It was believed that microbial turnover in soils was largely determined by environmental selection with various environmental factors (Wang, Gonzalez Perez, Ye & Huang, 2012; Zhang, Wei, Chen & Han, 2014). Indeed, across such a geographic range we surveyed, it is totally impractical to address the effects of important environmental variables, such as the edaphic parameters what we did not measure (Wang et al., 2017). As a result, the soil parameters we measured only explained 16% to 23% of the variability in community dissimilarity, and therefore were less important than spatial distance. Another potential explanation is that we may underestimate some spatially autocorrelated abiotic or biotic factors that strongly affect community composition (Martiny et al., 2011), which like as biological interactions would limit species' abilities of dispersing, colonizing and surviving become more difficult between any pair of sites. Our results are supported by considerable evidence which suggest that the distributions of many microorganisms are in fact limited by dispersal constraints, which may influence the composition of communities to a greater degree than environmental filtering, particularly at larger spatial scales (Adams, Miletto, Taylor & Bruns, 2013; Talbot et al., 2014; Li et al., 2020). However, from a stochastic aspect, it should be also noted that these enhanced structure turnovers may could be attributed to the potential increasing ecological drift in subsoil. As we know, similar to dispersal limitation and environmental selection, ecological drift would also potentially steepen the slope (Hanson, Fuhrman, Horner-Devine & Martiny, 2012). The ecological drift may significantly play a bigger role when the population size is small and diversity is low (Chase and Myers, 2011). In the current study, the alpha diversity declined along with soil depth, which would potentially promote drift, and would consequently steepen the slope as well.

Our study reveals how prokaryotic communities scale along vertical profiles in a typical grassland soil, by detecting the spatial distribution rates and demonstrating underly mechanisms with the relative contributions of potential factors in shaping microbial community structure and assembly. We found highly regular spatial

scaling results across the communities analyzed, with biodiversity scaling differently with increasing depth and communities displaying higher stability in upper soil strata. This is due to a dominance of deterministic mechanisms in shaping microbial communities in deeper soils. Specifically, because of more difficult dispersal and heterogenous environmental niches in deeper strata that species are rarely associated with higher turnover. Our findings provide a complementary perspective to study the spatial scaling of soil microorganisms from different horizontal spatial scales in the subsoil. However, our work has left some questions unanswered, with further research still necessary to evaluate which species and function(s) underlie the patterns observed, and to test whether such patterns would be consistent for other taxa and ecosystems, as well as to unify microbial ecology and macroecology spanning the entirety of the biodiversity of life and the geographic expanse of the Earth (Shade et al., 2018). These questions especially should be taken into consideration when assessing regional or global microbial gamma diversity.

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Data Accessibility

The raw sequence data from this study were deposited in the SRA at the NCBI database with the assigned study SRP229398 and Biosamples SAMN13220466-SAMN13220696.

Author Contributions

XD, YD and SL conceived and designed the experiments, XD, SL, KF, QH, ZW, YW, DW XP and XW performed the experiments. XD and SL analyzed the data, XD and YD wrote the paper. AE contributed to data

interpretation and manuscript drafting. All authors have reviewed and agreed with the paper.

Table legend

TABLE 1 Significance tests of the differences of dissimilarity between the microbial communities and null model simulations with increasing depth, and overall stochastic and deterministic ratios across soil strata

	Dissimilarity of actual communities	**Dissimilarity of the null expectations**	**F**	***P***	**Stochastic ratio**	**Deterministic ratio**
Upper	0.45±0.07	0.29±0.01	433.94	<0.001***	52.0%	48.0%
Middle	0.47±0.08	0.29±0.02	343.72	<0.001***	46.8%	53.2%
Substratum	0.54±0.09	0.29±0.03	488.29	<0.001***	36.7%	63.3%

Dissimilarity of actual communities and null expectation are significantly differed at the level of 0.001***.

TABLE 2 Results of the multiple regression analysis on matrices analysis (MRM) for the microbial community composition. The proportion of variation in Sorensen dissimilarity matrices that is explained by the remaining variables

	Upper		**Middle**		**Substratum**	
	$R^2 = 0.19$; $P = 0.001$***		$R^2 = 0.24$; $P = 0.001$***		$R^2 = 0.35$; $P = 0.001$***	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
Geographic distance (km)	0.0101	0.007**	0.0150	0.002**	0.0306	0.001***
Whole edaphic parameters	0.0026	0.019*	0.0063	0.001***	0.0060	0.004**
Moisture (%)	0.0004	0.841	0.0061	0.004**	0.0080	0.006**
pH	-0.0009	0.600	0.0012	0.588	0.0020	0.474
TN (mg/kg)	0.0019	0.342	0.0059	0.007**	0.0065	0.025*
$NH4^+$-N (mg/kg)	-0.0011	0.485	0.0035	0.120	-0.0028	0.289
$NO3^-$-N (mg/kg)	0.0018	0.310	0.0041	0.076	0.0060	0.043*
TOC (mg/kg)	0.0049	0.030*	-0.0022	0.379	-0.0027	0.321

Potential factors and community differences are significantly correlated at the level of 0.001***, 0.01**,0.05*.

Figure legend

FIGURE 1 Sampling strategy. Soil strata were divided into Upper (0-20cm), Middle (20-40cm) and Substratum (40-100cm), respectively. The central plot consisted of 17 individual soil cores, while each $1m^2$ plot was composed of 5 cores

FIGURE 2 The community diversity and structure of soil prokaryotes. (a) The relative abundances of the dominant phyla across three strata. (b) Principal coordinate analysis (PCoA) of prokaryotic communities based on Bray-Curtis dissimilarity matrix (n = 77 per strata)

FIGURE 3 Distance-decay and species-area relationships for the prokaryotic community based on different facets of diversity. The left column corresponds to the analysis of taxonomic diversity spatial scaling, with (a) Sorensen dissimilarity, (c) Bray-Curtis dissimilarity and (e) species richness. The right column corresponds to the analysis of phylogenetic diversity spatial scaling, with (b) Unweighted UniFrac matrix, (d) weighted UniFrac matrix, and (f) Faith's phylogenetic diversity (PD)

Supplementary

Table legend

TABLE S1 Soil physicochemical characteristics of different strata used in the study

TABLE S2 Variation of relative abundance of the dominant prokaryotic phyla in soils across different strata

TABLE S3 Dissimilarity tests and multivariate homogeneity of groups dispersions of microbial communities across soil profiles

TABLE S4 Fitness of mmSAR R package for species

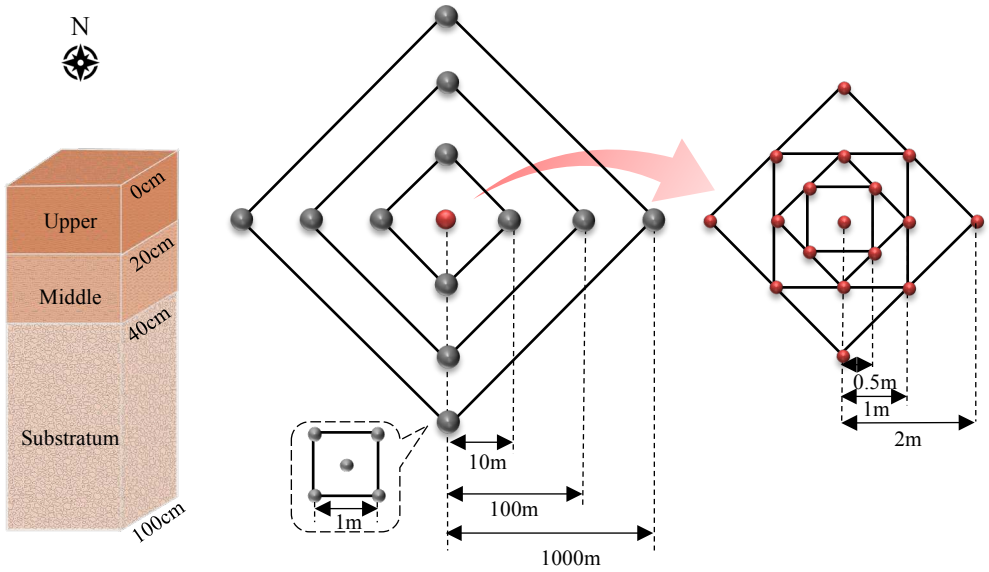
TABLE S5 Results of correlation between the dissimilarity of microbial communities and geographic distance or environmental variables using (partial) mantel test

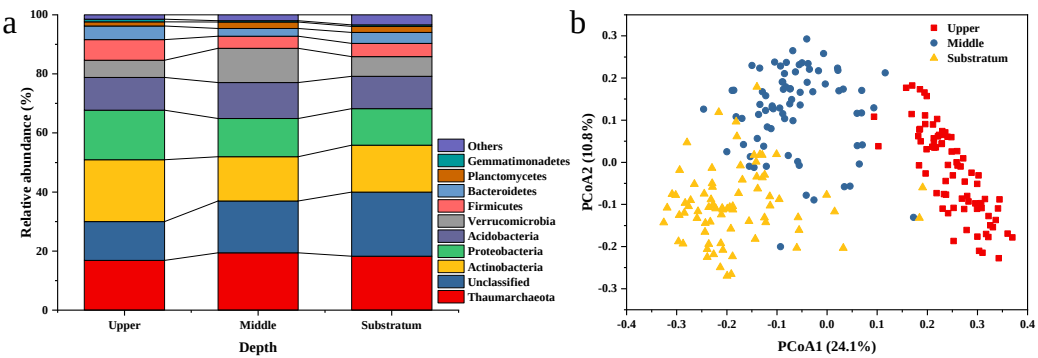
Figure legend

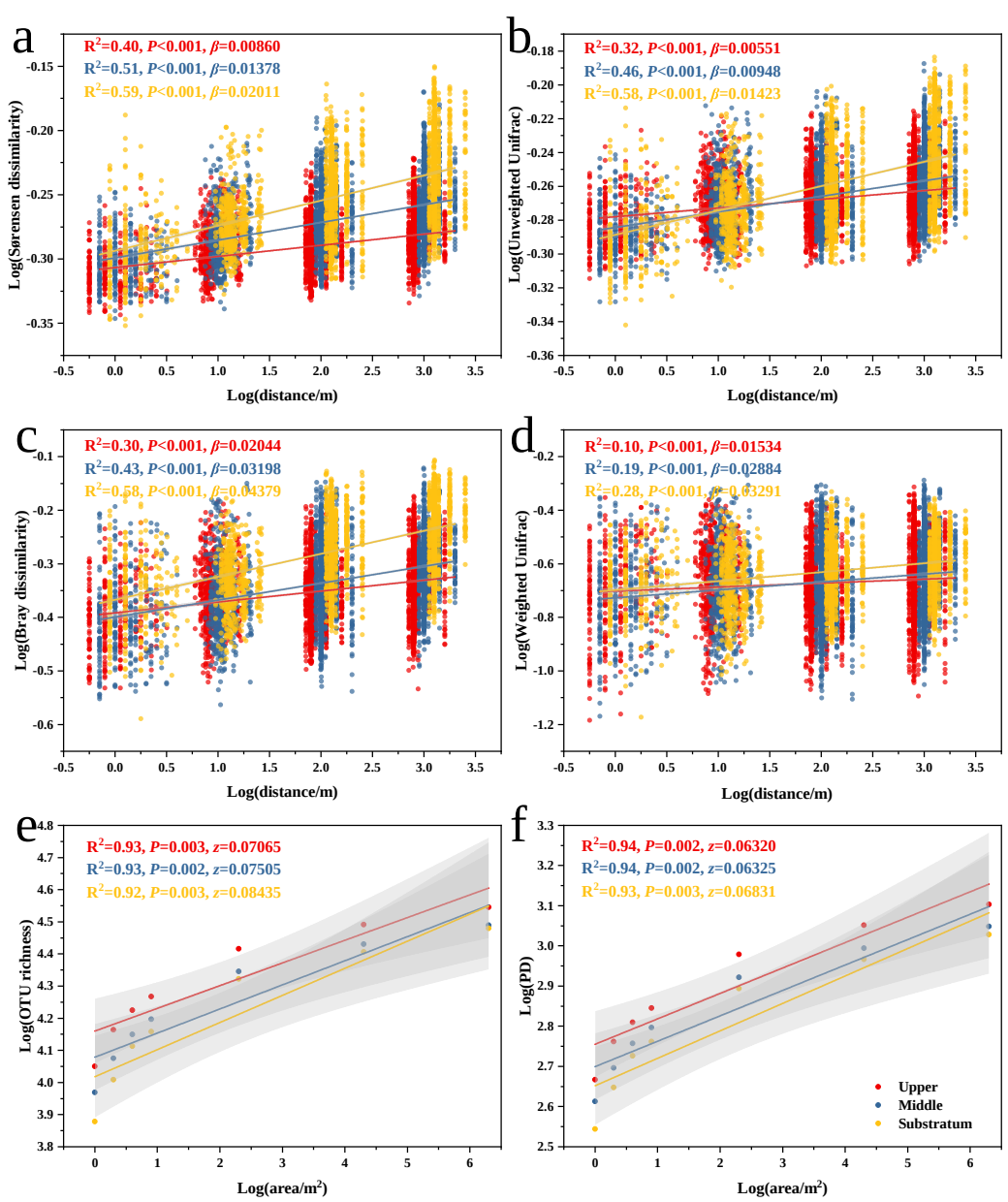
FIGURE S1 Variation in the diversity of soil prokaryotes along soil depth. (a) Number of OTUs; (b) Venn diagram of shared OTUs between Upper, Middle, and Substratum strata

FIGURE S2 β diversity of microbial communities in the different soil depth

FIGURE S3 Determining assembly mechanisms with Stegen's framework. (a) β nearest taxon index (βNTI) at different depths along soil profiles; (b) dynamics of the relative importance of different community assembly processes




a
Relative abundance (%)
100
80
60
40
20
0
Upper
Middle
Substratum
Depth
Others
Gemmatimonadetes
Planctomycetes
Bacteroidetes
Firmicutes
Verrucomicrobia
Acidobacteria
Proteobacteria
Actinobacteria
Unclassified
Thaumarchaeota
b
Upper
Middle
Substratum
PCoA2 (10.8 %)
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
PCoA1 (24.1%)


a
R²=0.40, P<0.001, β=0.00860
R²=0.51, P<0.001, β=0.01378
R²=0.59, P<0.001, β=0.02011
Log(Sørensen dissimilarity)
Log(distance/m)
b
R²=0.32, P<0.001, β=0.00551
R²=0.46, P<0.001, β=0.00948
R²=0.58, P<0.001, β=0.01423
Log(Unweighted Unifrac)
Log(distance/m)
c
R²=0.30, P<0.001, β=0.02044
R²=0.43, P<0.001, β=0.03198
R²=0.58, P<0.001, β=0.04379
Log(Bray dissimilarity)
Log(distance/m)
d
R²=0.10, P<0.001, β=0.01534
R²=0.19, P<0.001, β=0.02884
R²=0.28, P<0.001, β=0.03291
Log(Weighted Unifrac)
Log(distance/m)
e
R²=0.93, P=0.003, z=0.07065
R²=0.93, P=0.002, z=0.07505
R²=0.92, P=0.003, z=0.08435
Log(OTU richness)
Log(area/m²)
f
R²=0.94, P=0.002, z=0.06320
R²=0.94, P=0.002, z=0.06325
R²=0.93, P=0.003, z=0.06831
Log(PD)
Log(area/m²)
Upper
Middle
Substratum