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Stochastic processes regulate belowground community assembly in alpine grasslands on the Tibetan Plateau

Luyao Kang,[1,2] Leiyi Chen,[1] Dianye Zhang,[1,2] Yunfeng Peng,[1] Yutong Song,[1,2] Dan Kou,[1] Ye Deng [2,3] and Yuanhe Yang [1,2*]

[1]*State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China.*

[2]*University of Chinese Academy of Sciences, Beijing 100049, China.*

[3]*CAS Key Laboratory of Environmental Biotechnology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China.*

Summary

Understanding biogeographical patterns and underlying processes of belowground community assembly is crucial for predicting soil functions and their responses to global environmental change. However, little is known about potential differences of belowground community assembly among bacteria, fungi, protists and soil animals, particularly for alpine ecosystems. Based on the combination of large-scale field sampling, high-throughput marker-gene sequencing and multiple statistical analyses, we explored patterns and drivers of belowground community assembly in alpine grasslands on the Tibetan Plateau. Our results revealed that the distance–decay rates varied among trophic levels, with organisms of higher trophic level having weaker distance–decay pattern. The spatial and environmental variables explained limited variations of belowground communities. By contrast, the stochastic processes, mainly consisting of dispersal limitation and drift, played a primary role in regulating belowground community assembly. Moreover, the relative importance of stochastic processes varied among trophic levels, with the role of dispersal limitation weakening whereas that of drift enhancing in the order of bacteria, fungi, protists and soil animals. These findings advance our understanding of patterns and mechanisms driving belowground community assembly in alpine ecosystems and provide a reference basis for predicting the dynamics of ecosystem functions under changing environment.

Received 14 June, 2021; revised 13 October, 2021; accepted 19 October, 2021. *For correspondence. E-mail yhyang@ibcas.ac.cn; Tel. +86 10 6283 6638; Fax + 86 10-6283 6632.

Introduction

Soil microbiota is the most diverse community which can inhabit almost all environments on Earth (Falkowski *et al*., 2008; Guerra *et al*., 2021). Its community structure is often shaped by environmental factors, biotic interactions, dispersal, diversification and drift (Hanson *et al*., 2012). The hyper-complexity of these ecological processes in the local soil community leads to the large variability of soil microbial communities at a regional scale (Crowther *et al*., 2019). As the 'engine' of the Earth's biogeochemistry, soil microbiota directly drives the turnover of essential elements such as carbon, nitrogen and phosphorus in various ecosystems (Falkowski *et al*., 2008), which are then inextricably linked to soil fertility, plant growth and climate change (Crowther *et al*., 2019; Guerra *et al*., 2021). Given the vital roles of soil microbiota, our knowledge of biogeographical patterns and underpinning processes of belowground community assembly is critical for improving confidence in biogeochemical model predictions, particularly for better predicting ecosystem responses to global environmental change (Crowther *et al*., 2019; Jansson and Hofmockel, 2020).

Over the past decade, biogeographic patterns of the microbial community and the fundamental processes shaping these patterns have been extensively investigated (Nemergut *et al*., 2013; Zhou and Ning, 2017; Xu *et al*., 2020). These studies provide clear evidence that most microbial groups settled in different habitats exhibit distinct biogeographic patterns, and both deterministic and stochastic processes affect microbial community assembly (Dini-Andreote *et al*., 2015; Tripathi *et al*., 2018). The deterministic processes include the selection imposed by environmental factors that could affect organismal fitness and thus shape microbial community structure. For example, extreme acidic or alkaline soil pH conditions could result in more phylogenetically clustered microbial communities, whereas neutral soil pH conditions could lead to less clustered and higher diversity of communities (Kim

et al., 2017; Tripathi *et al*., 2018; Rath *et al*., 2019). Likewise, salinity has been recognized to exert the filtering effect in planktonic lakes (Logares *et al*., 2018) and desert (Zhang *et al*., 2019) or along a salt marsh chronosequence (Dini-Andreote *et al*., 2015). Other factors such as temperature (Kim *et al*., 2017), soil organic matter (Caruso *et al*., 2011; Dini-Andreote *et al*., 2014, 2015) and species interactions (Maestre *et al*., 2010; Jiao *et al*., 2019; Li and Hu, 2021; Lu *et al*., 2021) are also reported to affect the microbial fitness and then shape community structure. By contrast, the stochastic processes including historical contingency (e.g. priority effects), probabilistic dispersal and ecological drift also have been found to affect the microbial community assembly. For instance, priority effects by the initial pioneer species could reduce the establishment success of later taxa and substantially shape community structure (Carlström *et al*., 2019; Cheong *et al*., 2021; Debray *et al*., 2021). Drift interacting with relatively restricted dispersal could lead to greater turnover rates along with geographic distance (Hanson *et al*., 2012). Despite all the work conducted so far, our knowledge of microbial biogeographic patterns and the processes driving these patterns is still limited due to the following two aspects. First, previous studies principally focused on the bacterial and fungal community (Dini-Andreote *et al*., 2015; Powell *et al*., 2015; Tripathi *et al*., 2018), little is known about the protistan and soil animal communities. Protists and soil animals also have extremely high diversity as well as bacteria and fungi, which execute various ecosystem functions such as enhancing microbial loop, altering soil nutrient cycles and promoting the enrichment of beneficial microorganisms in the rhizosphere (Oliverio *et al*., 2020). Their distinct characteristics contrasting with bacteria and fungi may lead to different biogeographic patterns and underlying mechanisms in regulating community structure. For instance, protists and soil animals are generally larger than bacteria and fungi (Keeling and del Campo, 2017). Microorganisms with larger body sizes are more likely affected by the dispersal limitation owing to their lower dispersal ability versus smaller organisms (Farjalla *et al*., 2012; Wu *et al*., 2018). Moreover, protists and soil animals tend to have a smaller population size, the impact of ecological drift such as birth and death could be amplified in small populations (Nemergut *et al*., 2013). In addition, protists and soil animals are generally more metabolically simple and homogeneous, which means that they may have lower metabolic plasticity and exist narrowly in diverse habitats (Keeling and del Campo, 2017), and ultimately lead to stronger environmental filtering (Farjalla *et al*., 2012). Despite these recognitions, there is limited evidence about the potential difference of biogeographic patterns and dominant processes in controlling the community assembly among different trophic levels.

Second, previous studies were primarily conducted on tropical, subtropical and temperate ecosystems (Wang *et al*., 2017; Meyer *et al*., 2018; Jiao *et al*., 2019), our understanding of biogeographical patterns and underlying mechanisms of belowground community assembly in alpine ecosystems is still lacking. In contrast to other ecosystems, alpine ecosystems are characterized by harsh environmental conditions such as low temperature, hypoxia and high radiation intensity, which may induce profound impacts on biogeographical patterns of soil biota and the underlying mechanisms. For example, soil freezing resulting from low temperature, which acts as a physical barrier, can hinder the movement of soil microorganisms (Steven *et al*., 2008). The strength of dispersal limitation may then be stronger and consequently lead to higher variation or species turnover of belowground communities in alpine ecosystems. Furthermore, soil biota colonized in alpine ecosystems tends to be dormant to cope with the harsh environment (Lennon and Jones, 2011), which may promote the resistance of soil biota to environmental stressors and subsequently weaken the effect of environmental selection (Nemergut *et al*., 2013) and reduce the variation or species turnover of belowground communities ultimately (Hanson *et al*., 2012). Nevertheless, due to the harsh physical conditions, biogeographic patterns and dominant processes in controlling the community assembly of soil biota in alpine ecosystems remain poorly understood.

In this study, we examined biogeographic patterns and the underlying mechanisms of belowground community assembly by conducting large-scale field sampling along a 3,500 km transect across alpine grasslands on the Tibetan Plateau (Supporting Information Fig. S1). In total, 147 soil samples were collected and analysed by the *16S* ribosomal RNA (rRNA), internal transcribed spacer 2 (*ITS2*) and *18S* rRNA gene sequencing, the most generally used marker gene for bacteria, fungi and protists respectively. Moreover, the geographical information, climatic factors, plant properties and edaphic factors were also determined. Based on these measurements, we aimed to (i) determine the community structure and biogeographic patterns of soil biota in alpine ecosystems; (ii) estimate the relative contribution of stochastic and deterministic processes in shaping belowground community structure; (iii) examine whether biogeographic patterns and underlying mechanisms of community assembly vary among different types of soil biota.

Results

Alpha diversity and community composition

The sequencing depth of each sample for *16S*, *ITS* and *18S* rRNA gene involved in this study were

126,140 ± 3,664 (mean ± SE), 158,561 ± 4,058 and 165,166 ± 3,172, respectively (Supporting Information Fig. S2), which showed the order of *16S* < *ITS* < *18S* rRNA gene. Bacteria showed higher alpha diversity than fungi, protists and soil animals, while soil animals had the lowest diversity than the other three taxa (Supporting Information Table S1). This trend was opposite to the sequencing depth of the four taxonomic groups, suggesting that the sequencing depth may not be the main reason for the variation of diversity among the four groups. In total, 12,088 bacterial amplicon sequence variants (ASVs) were identified from the reads of *16S* rRNA gene. The most abundant bacterial phyla were *Proteobacteria*, *Actinobacteria* and *Acidobacteria*, which comprised 24.8%, 17.5% and 17.4% of the total reads respectively. *Acidobacteria* and *Alphaproteobacteria* were the dominant groups at the class level, accounting for 15.2% and 14. 6% respectively (Fig. 1A). There were 3,839 fungal ASVs recovered from *ITS* sequences. About 11.1% of the total reads were unclassified at the phylum level. All the identified fungal ASVs belonged to eight phyla dominated by *Ascomycota* (75.0%), which were mainly consisted of *Sordariomycetes* (22.0%) and *Dothideomycetes* (20.6%) at the class level. The *Basidiomycota* and *Zygomycota* accounted for only 6.8% and 6.5% of the total fungal reads respectively (Fig. 1B).

A number of 3,786 protistan ASVs and 870 soil animal ASVs were recovered from the reads of *18S* rRNA gene. Of them, *Cercozoa* was the most abundant protistan taxa, followed by *Ciliophora*, which accounted for 56.8% and 13.4% of the total reads respectively. *Filosa-Sarcomonadea* was the dominant group within *Cercozoa*, which contributed to 42.2% of total protistan reads (Fig. 1C). The most abundant taxa within the soil animal community was *Nematoda*, which comprised 88.2% of

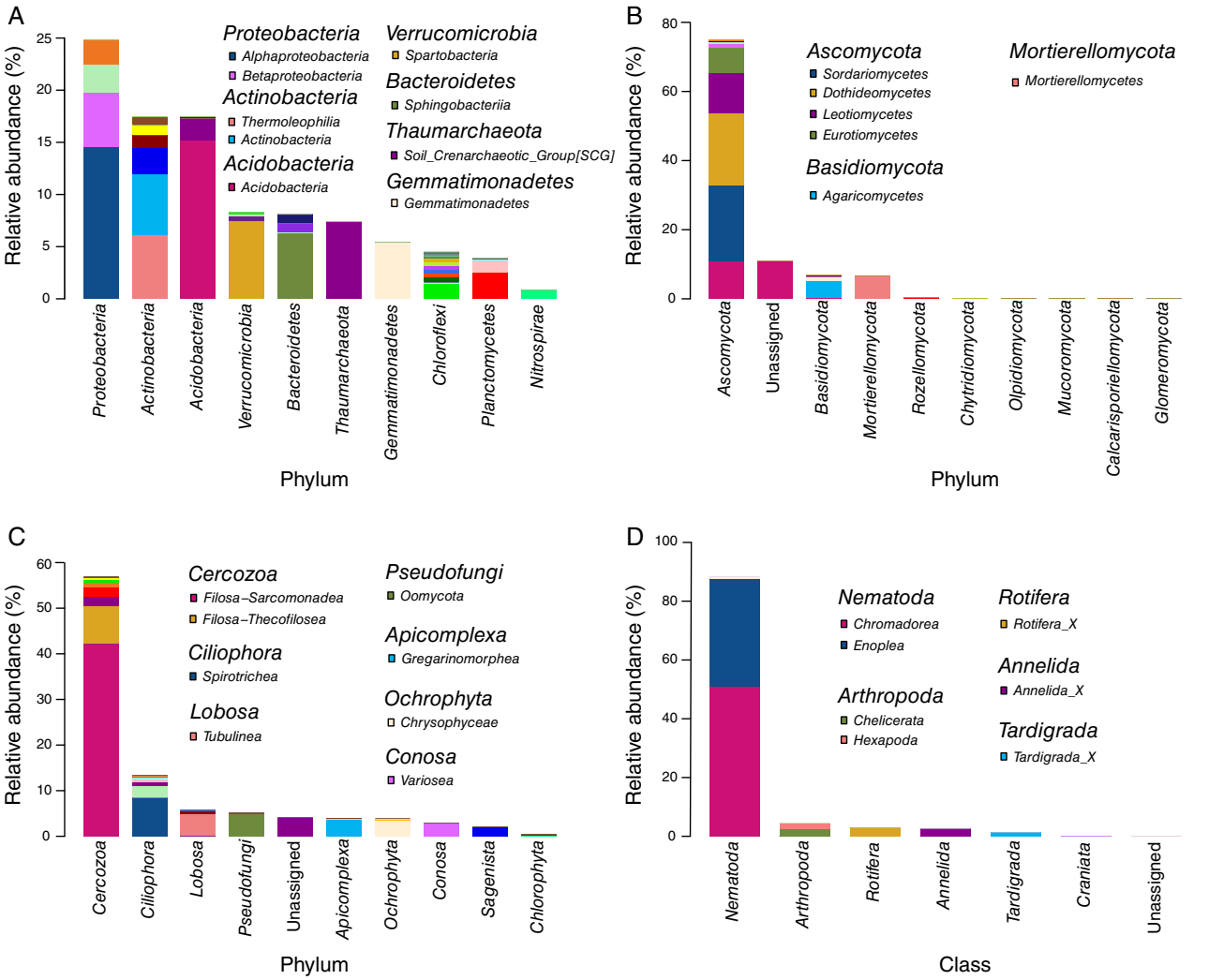

Fig. 1. The taxonomic composition of the dominant phylotypes within phylum or class in (A) bacteria, (B) fungi, (C) protists and (D) soil animals. [Color figure can be viewed at wileyonlinelibrary.com]

total reads, and was mainly consisted of *Chromadorea* (51.0%) and *Enoplea* (36.8%) (Fig. 1D).

Variations and species turnover of community structure

The variation of bacterial communities across Tibetan alpine grasslands was the lowest, while those of protists and soil animals were higher than bacteria and fungi (Fig. 2A). The community similarity versus geographic distance for each soil organism displayed a significant distance–decay relationship (DDRs), but the slopes of distance–decay curves estimated by linear regression models varied among different taxonomic groups (Fig. 2B). To be specific, the slopes of bacterial and fungal DDRs were −0.84 (Mantel $r = -0.40$, $p < 0.001$) and −0.78 (Mantel $r = -0.47$, $p < 0.001$), respectively, which were significantly steeper than those of protists (slope = −0.51; Mantel $r = -0.44$, $p < 0.001$) and soil animals (slope = −0.55; Mantel $r = -0.34$, $p < 0.001$) (Fig. 2B; Supporting Information Table S2).

Community assembly of different taxonomic groups

Redundancy analysis (RDA) showed that there were weaker relationships among the four organismal community structures (Supporting Information Fig. S3), reflecting weak trophic interactions among the four taxonomic groups. Variation partition analysis illustrated that both environmental and spatial variables had significant effects on the community assembly for four taxonomic groups. The overall variations explained by environmental and spatial variables ranged from 16.5% to 28.7% (Fig. 3A). The ratios of species sorting to dispersal limitation were 0.53, 0.08, 0.25 and 0.19 for bacteria, fungi, protists and soil animals, respectively, which indicated that the relative strength of dispersal limitation was higher than species sorting in regulating community assembly (Fig. 3B). Within the selected variables, the aridity index was the most important factor to improve the model fit during the variable selection (Supporting Information Table S3). Correspondingly, the distance-based RDA (dbRDA) also revealed that the aridity index had an important effect on the belowground community structure (Fig. 4).

Null model analysis revealed that the β-nearest taxon index (βNTI) values for the four taxonomic groups mainly fell within the range of −2 to 2 (Supporting Information Fig. S4), which confirmed the important role of stochastic processes in regulating belowground community assembly. Moreover, the results of partitioning the pairwise comparisons based on Bray–Curtis-based Raup–Crick (RC_{Bray}) values indicated that the community assembly of different taxonomic groups was

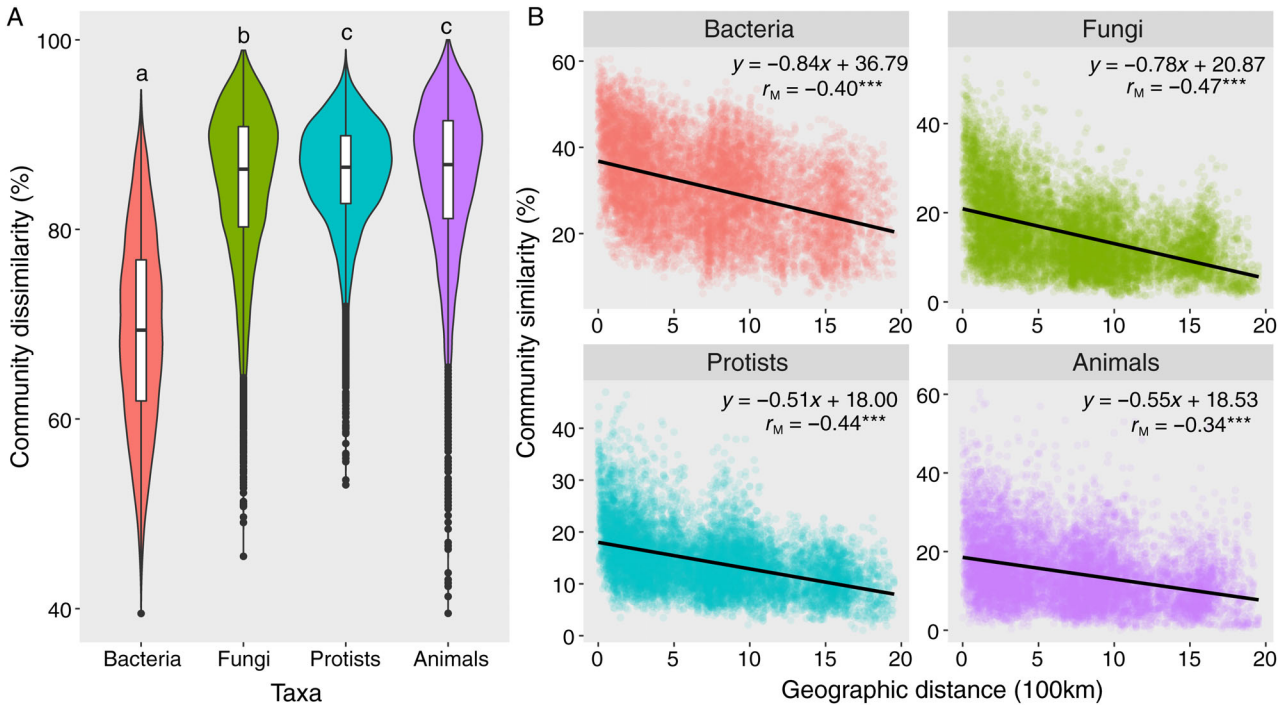

Fig. 2. General patterns of beta-diversity of belowground communities.
A. The variation (Bray–Curtis dissimilarity) of community structure among soil samples for four groups. The different lower-case letters mean the values are statistically different ($p < 0.05$) among four taxonomic groups.
B. Distance–decay curves showing community similarity (1 − Bray–Curtis dissimilarity) along the geographic distances among sampling sites. Solid lines indicate the ordinary least-squares linear regressions. Mantel test is used to examine the significance. Asterisks denote significant correlation (***$p < 0.001$). [Color figure can be viewed at wileyonlinelibrary.com]

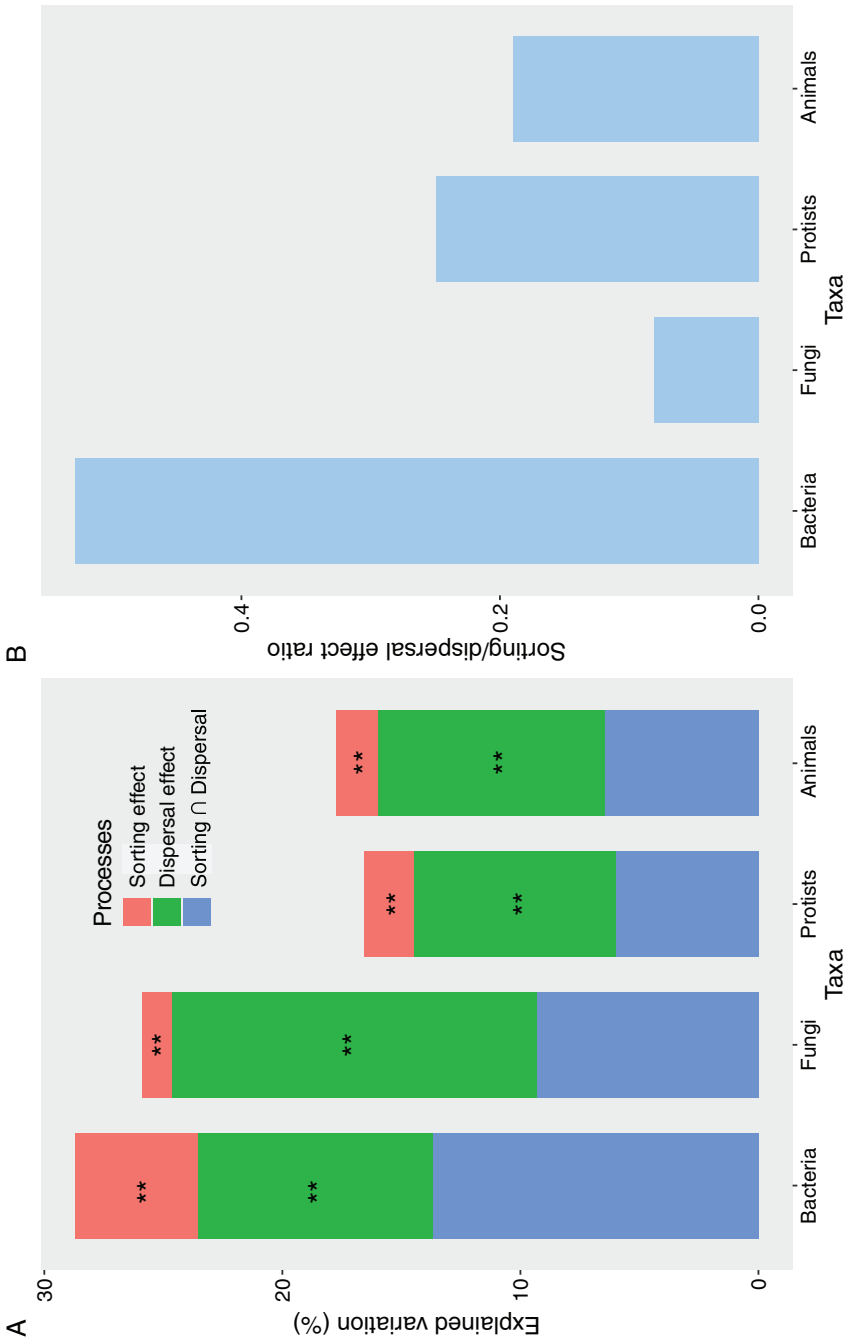

Fig. 3. Summary of the results derived from the variation partitioning analysis.
A. The relative effects of species sorting, dispersal limitation and their interaction.
B. The values of sorting/dispersal effect ratio. Asterisks indicate significant effects (**$p < 0.01$). [Color figure can be viewed at wileyonlinelibrary.com]

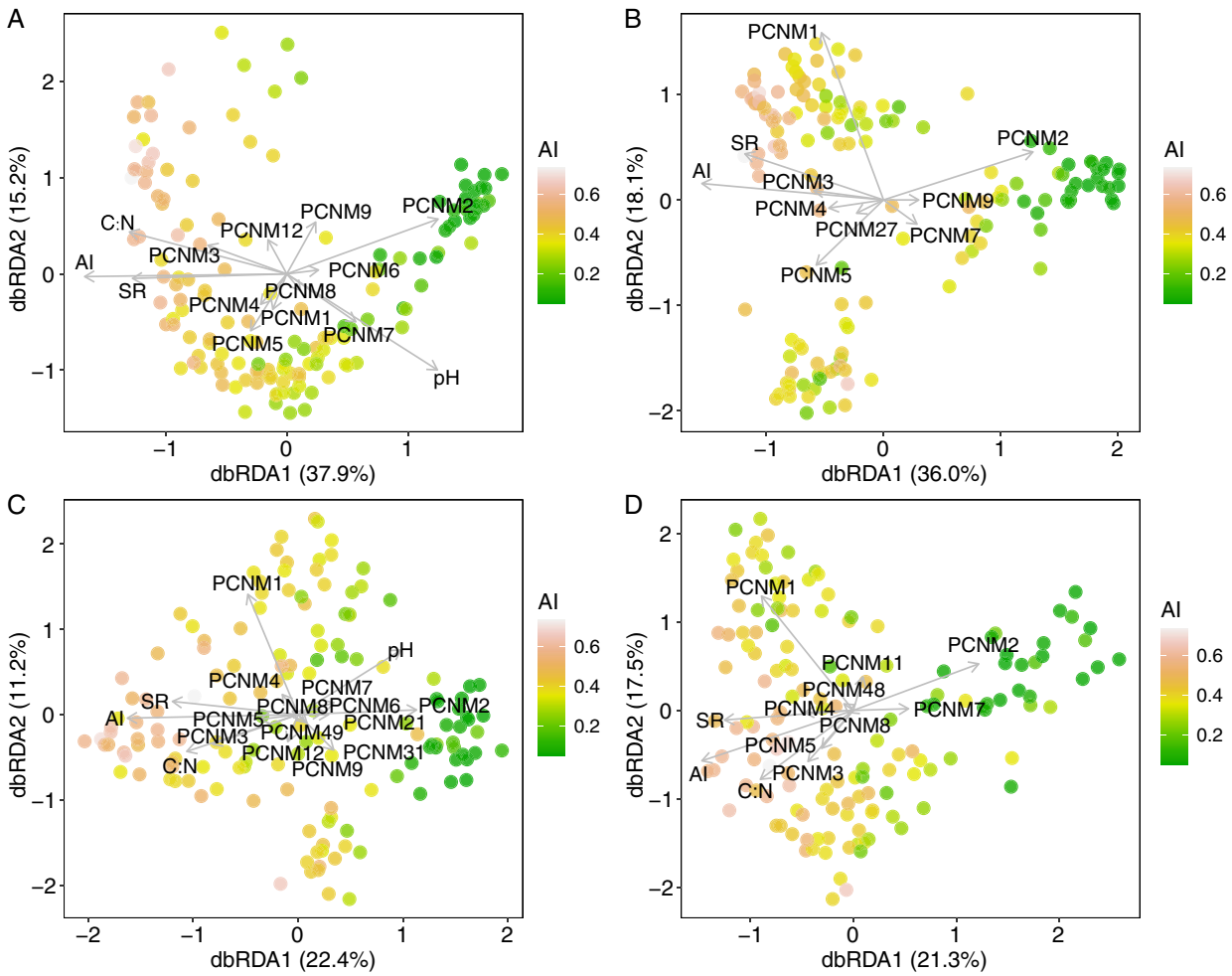

Fig. 4. Distance-based redundancy analysis (dbRDA) showing the relationships of belowground communities (response variables) with environmental and spatial factors (explanatory variables) for (A) bacteria, (B) fungi, (C) protists and (D) soil animals.
Only those variables with VIF < 20 and $p < 0.05$ are kept in the final plot. Points are coloured according to the value of the aridity index. AI, aridity index; C:N, the ratio of soil organic carbon to total nitrogen; SR, plant species richness. [Color figure can be viewed at wileyonlinelibrary.com]

mainly driven by dispersal limitation and drift. Specifically, the relative contribution of dispersal limitation ranged from 25.7% to 72.5%, increasing in the order of soil animals, protists, fungi and bacteria (Fig. 5). In contrast, the relative importance of drift varied from 4.3% to 61.6%, which exhibited an opposite trend with the dispersal limitation (Fig. 5). In addition to these stochastic processes mentioned above, the heterogeneous selection had a non-neglectable contribution in structuring the belowground communities, which ranged from 6.0% for soil animals to 24.6% for fungi (Fig. 5).

Discussion

Convergence and divergence of belowground community composition in alpine grasslands with other ecosystems

Our results showed that the dominant groups of soil bacterial communities in Tibetan alpine grasslands were *Proteobacteria*, *Actinobacteria* and *Acidobacteria* (Fig. 1A), which was consistent with a recent investigation about the global atlas of the dominant bacteria in soil (Delgado-Baquerizo *et al*., 2018). The taxa within *Proteobacteria*, *Actinobacteria* and *Acidobacteria* are hyperdiverse in morphology and metabolism, which enable them to be broadly distributed in various habitats (Mukhopadhya *et al*., 2012; Lewin *et al*., 2016). On the contrary, our results revealed that the *Sordariomycetes* and *Dothideomycetes* within *Ascomycota* accounted for 42.7% of the fungal individuals collectively (Fig. 1B). Unlike this result, Tedersoo *et al*. (2014) found that *Agaricomycetes* within *Basidiomycota* was the most abundant fungal taxa in global soils based on 365 soil samples from natural ecosystems. Such a difference may result from the wondrously broad habitat tolerance of *Sordariomycetes* and *Dothideomycetes*, which could make them widely distributed in extreme habitats such as cold deserts and high-altitude palaeolithic rocks (Selbmann *et al*., 2005; Sterflinger *et al*., 2012).

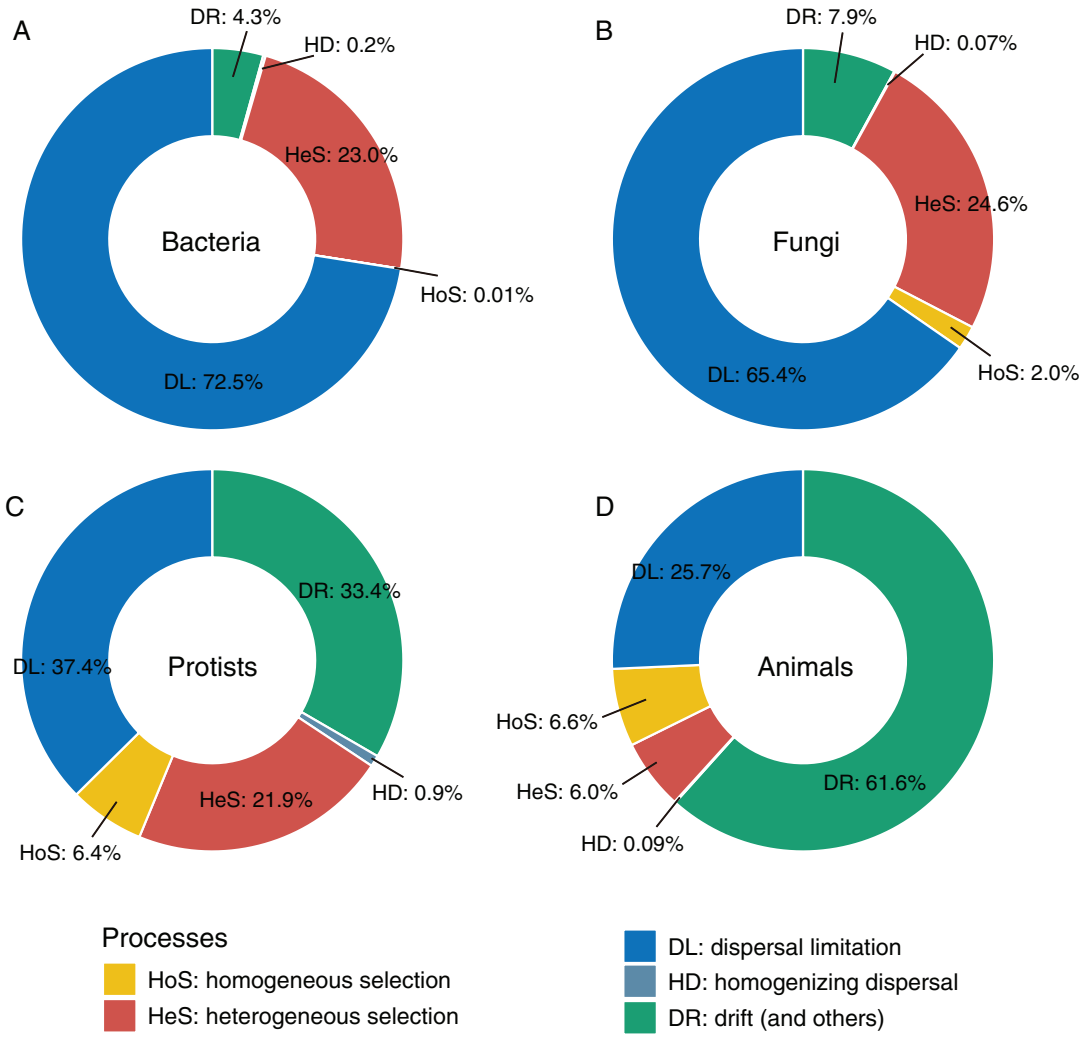

Fig. 5. Summary of the relative contribution of each process driving the community assembly within each taxonomic group based on null model analysis: (A) bacteria, (B) fungi, (C) protists, (D) soil animals. [Color figure can be viewed at wileyonlinelibrary.com]

The high relative abundance of *Filosa-Sarcomonadea* (42.2%) within *Cercozoa* observed in Tibetan alpine grasslands (Fig. 1C) was in line with a recent global survey conducted by Oliverio *et al*. (2020). *Filosa-Sarcomonadea* was built to unite cercozoan heterotrophic, free-living and naked (amoebo-) flagellates, which was very diverse and abundant in terrestrial and freshwater systems (Cavalier-Smith, 1993). Moreover, our results illustrated that soil animal community was primarily composed of *Nematoda* (88.2%) (Fig. 1D). Likewise, a recent national-scale analysis of soil biodiversity across Wales, UK also reported that the *Nematoda* was the most abundant taxa in soils (George *et al*., 2019). *Nematoda* was found to have diverse feeding habits, which could promote the chance of its survival among various habits (Yeates *et al*., 1993) and thus lead to a high abundance in soil.

Spatial patterns of belowground communities in alpine grasslands and their comparisons with other ecosystems

The DDR rates of belowground communities observed in Tibetan alpine grasslands were remarkably steeper than that of deserts, desert grasslands, typical grasslands, tropic forests and agricultural soils (Supporting Information Table S4). The steeper slopes observed in our study indicated that the species turnover of the belowground communities in alpine ecosystems could be higher than other ecosystems. Such a difference may be ascribed to the physical barrier caused by soil freezing on the Tibetan Plateau. It has been reported that the area-averaged frozen days near-surface soils are 269 days per year for the whole Tibetan Plateau (Li *et al*., 2012), and the distribution areas of both permafrost and

seasonally frozen ground account for 96% of the total area on the plateau (Zou *et al*., 2017). This long-term and large-area soil freezing could enhance the effects of physical barriers and hinder the migration of soil biota and would thus elevate the species turnover rates along with the spatial (distance) gradients (Steven *et al*., 2008).

Our results also showed that the species turnover rates of the bacterial and fungal community were higher than those of protists and soil animals (Fig. 2B; Supporting Information Table S2). This discrepancy among different taxonomic groups might arise from their different inherent characteristics. Bacteria and fungi with inactively motile may tend to keep frozen due to the low fluidity of water resulting from soil freezing (McCoy and Hagedorn, 1979; Bollag and Stotzky, 1992; Steven *et al*., 2008; Bottos *et al*., 2018), particularly for the Tibetan Plateau which frequently experiences cold conditions. This effect may reduce the possibility of being transported via wind and thus lead to strong dispersal limitation across our study area. Compared with bacteria and fungi, protists and soil animals have stronger active dispersal ability due to their diverse modes of locomotion (Lee *et al*., 2000; Keeling and del Campo, 2017), which may alleviate the effects of dispersal limitation resulting from soil freezing, and then flatten the slopes of DDRs. Another reason resulting in the discrepancy among taxonomic groups may be partly related to difference in sampling effort. It has been reported that distance–decay rate could be steeper when a community was sampled more thoroughly (Meyer *et al*., 2018).

Effects of deterministic and stochastic processes on belowground community assembly

Our results indicated that the contribution of species sorting was relatively weaker than the dispersal limitation on the community assembly (Fig. 3B). The minor effects of environmental variables were supported by several studies conducted on the Tibetan Plateau (Chu *et al*., 2016; Yang *et al*., 2017; Xue *et al*., 2019). The weak environmental effects may be due to the dormancy strategies of belowground communities in alpine ecosystems. It has been reported that soil biota colonized in alpine ecosystems utilize dormancy as the fundamental metabolic strategy to cope with environmental stresses (Darcy *et al*., 2011). This stronger resistance of soil biota to environmental stresses resulting from dormancy strategies could then weaken the selection processes (Nemergut *et al*., 2013). In addition, the non-living microbes detected by the DNA-based sequencing may also induce weaker environmental effects due to their inactive responses to environmental selection (Carini *et al*., 2016; Lennon *et al*., 2018; Locey *et al*., 2020).

Our results also revealed the prominent role of stochastic processes (dispersal limitation and drift) in shaping belowground communities (Fig. 5; Supporting Information Fig. S4). Ecological drift and dispersal limitation could strengthen the DDRs (steepen the slope) (Hanson *et al*., 2012), which was confirmed by the steeper slopes of distance–decay curves observed in our study (Fig. 2B). The higher contributions of these two processes may be explained by the following three possible mechanisms. First, as mentioned above, soil biota on the Tibetan Plateau may tend to be dormant to cope with the harsh environment, which is conducive to the resistance to environmental stressors and consequently weaken deterministic processes (Nemergut *et al*., 2013). Second, the long-term and large-area soil freezing on the Tibetan Plateau (Li *et al*., 2012; Zou *et al*., 2017) could enhance the physical barrier effects and thus increase the dispersal limitation. Third, the constant freeze–thaw cycles could result in considerable disturbances and then likely induce the periodic decimation of local microbial populations (Darcy *et al*., 2011), which may ultimately strengthen the ecological drift.

Our results further demonstrated that the relative contribution of dispersal limitation declined while drift increased in the order of bacteria, fungi, protists and soil animals (Fig. 5). This finding partly exceeded our expectations. Bacteria and fungi are expected to be less affected by the dispersal limitation, because microorganisms with a smaller body size are less likely affected by the dispersal limitation owing to their stronger dispersal ability versus larger organisms (Farjalla *et al*., 2012; Xu *et al*., 2020). Several possible reasons may account for this finding. Bacterial and fungal dispersal is typically a passive process, which transport via wind, water and hitchhiking (Nemergut *et al*., 2013). This passive dispersal process may be hindered on the Tibetan Plateau due to soil freezing which acts as a physical barrier to some extent. Generally, the availability of continuous water pathways could facilitate microbial movement throughout the soil (McCoy and Hagedorn, 1979); however, the low fluidity of water resulting from the increasing ice content could reduce the mobility of microbes, especially for cells that are not actively motile (Bollag and Stotzky, 1992). In contrast to bacteria and fungi, protists and soil animals can propel themselves to some degree (Keeling and del Campo, 2017), and this active motile could alleviate the physical barrier caused by soil freezing. Furthermore, it has been demonstrated that the contribution of ecological drift results from stochastic differences in population size, birth and death rates may vary among different taxonomic groups (Stegen *et al*., 2013). In our study, protists and soil animals have smaller population sizes than bacteria and fungi (Supporting Information Table S1) and are therefore,

generally more influenced by ecological drift, since any slight negative disturbances in their abundance could induce their decimation on a local scale (Pedrós-Alió, 2006; Nemergut *et al*., 2013).

Although the stochastic processes play a dominant role in the belowground community assembly, the contribution of deterministic processes especially for the heterogeneous selection in regulating belowground communities should not be neglected (Fig. 5). Results of the dbRDA demonstrated that the aridity index was the main driver in shaping the community composition (Fig. 4). Aridity has been reported to affect soil microbial community structure via several mechanisms. Increases in aridity can exclude the species which are vulnerable to drought, then alter the compositional and functional diversity (Wang *et al*., 2014; Maestre *et al*., 2015; Fernandes *et al*., 2018) and ultimately lead to stronger niche-selection effects (Chase, 2007). By contrast, increases in precipitation can promote the magnitudes of variation of the stochastic processes compared with the deterministic processes, and subsequently affect the assembly of soil bacterial community (Zhang *et al*., 2016). Therefore, heterogeneity resulting from aridity may act as an environmental filter, which expects to lead to compositional variations among the belowground communities (Delgado-Baquerizo *et al*., 2020).

Conclusions

Based on the combination of large-scale field sampling, high-throughput marker-gene sequencing and multiple statistical analyses, we found that the dominant taxa of fungal community in Tibetan alpine grasslands was distinct, while those of bacterial, protistan and soil animal communities were similar compared with other ecosystems. Such a divergence and convergence of community composition may lead to a specific soil functionality in the alpine grasslands. Our results also revealed that the variation and turnover of belowground community structure varied among trophic levels. Organisms with higher trophic level tended to have higher community variation but weaker distance–decay pattern. Our results further demonstrated that the stochastic processes, which mainly consisted of dispersal limitation and drift, primarily shaped the community assembly of soil biota in alpine grasslands. This finding implies that the belowground communities in Tibetan alpine grasslands may be sensitive to global change. It has been reported that the degree of variation of the stochastic processes was greater than that of deterministic processes under several environmental changes, such as climate warming, nitrogen deposition, increased precipitation and their combinations (Zhang *et al*., 2016). With the trend of warming and humidification on the Tibetan Plateau (Kang *et al*., 2010; Su *et al*., 2013), changes in the stochastic processes may directly determine the belowground biodiversity and ecosystem functions in the future. Therefore, further studies are encouraged to explore the impact of changes in the relative importance of stochastic and deterministic processes on the belowground community assembly in alpine ecosystems under the context of global change.

Experimental procedures

Study area

The Tibetan Plateau is the largest and highest plateau in the world. Mean annual temperature (MAT) and precipitation (MAP) across the entire plateau spans from −3 to 5°C and 84.3 to 593.9 mm respectively (Ding *et al*., 2016). Accordingly, the mean annual aridity index varies from 0.05 to 0.75 with a mean value of 0.35. Its average annual radiation intensity ranges from 6,000 to 8,000 MJ m^{-2}, ranking it second after the Sahara worldwide (Wang and Qiu, 2009). Vegetation types of the plateau are mainly comprised of alpine steppe and alpine meadow. The dominant plant species in alpine steppe are *Stipa purpurea* and *Carex moorcroftii*, while those in alpine meadow are *Kobresia pygmaea*, *Kobresia parva* and *Kobresia littledalei* (Zhang *et al*., 1988). The soil types are categorized as Xerosols and Cambisols for alpine steppe and alpine meadow, respectively, according to the soil classification system of the Food and Agriculture Organization (Wu *et al*., 2003).

Field sampling

A total of 147 sites were chosen along a 3,500 km transect across the Tibetan alpine grasslands during July and August (near the phase of maximum aboveground biomass, AGB) in 2013 and 2014 (Supporting Information Fig. S1). At each site, five 1 m × 1 m quadrats along the diagonal line of a 10 m × 10 m plot were established. Plant species richness was determined within each quadrat of the plot, and the aboveground vegetation within each quadrat was clipped at ground level. Plant samples were then transported to the laboratory and oven-dried at 65°C for 48 h and weighed to determine AGB. Three replicate soil cores were excavated at the depth of 0–10 cm along a diagonal line across the plot. For details about field sampling, see Ding *et al*. (2016). The three cores were sieved using a 2 mm mesh to discard the roots and gravels and then mixed. The composite soils at each sampling site were subsequently separated into two parts: one was air-dried for the characterization of soil

physicochemical properties; the other was kept at −80°C for the subsequent DNA extraction.

Soil physical and chemical analyses

Soil physical and chemical factors measurements were described previously (Ding *et al.,* 2016). Specifically, a particle size analyser (Malvern Masterizer 2000, UK) was used to measure the soil texture after discarding organic matter and calcium carbonates with 30% hydrochloric acid and 30% hydrogen peroxide respectively. Soil pH was analysed by a pH meter (PB-10, Sartorius, Germany) after suspending soil with 2 M KCl according to the soil: water ratio of 1:2.5. Total soil carbon (TC) and nitrogen (TN) content were measured by combustion at 950°C using an elemental analyser (Vario EL III, Elementar, Germany). Soil inorganic carbon (SIC) concentration was quantified using a carbonate content analyser (Eijkelkamp, Giesbeek, The Netherlands). The difference between TC and SIC was calculated as the soil organic carbon (SOC) content. Soil carbon:nitrogen (C:N) was indicated by the ratio of SOC to TN. The acid-dissolved molybdenum, antimony and scandium colorimetry were used to measure the total phosphorous (Dick and Tabatabai, 1977).

Climate data set

The original MAT and MAP data were derived from direct observations of the 73 weather stations during 1985–2014, which were provided by the China Meteorological Data Sharing Service System (http://cdc.nmic.cn/home.do). These data sets were retrieved from the spatial interpolation using the Cokriging method with a spatial resolution of 10 km × 10 km for each sampling site using ArcGIS 10.0 (Environmental Systems Research Institute, Inc., Redlands, CA, USA). During the spatial interpolation, altitude was treated as a covariant to characterize the topographic effects (Ding *et al*., 2016). In addition, the aridity index of our 147 sampling sites was extracted from the CGIAR-CSI Global-Aridity and Global-PET database (http://www.cgiar-csi.org; Zomer *et al*., 2008). We used aridity index rather than precipitation to indicate water availability since it was obtained by dividing mean annual precipitation by mean annual potential evapotranspiration and considered both water input (precipitation) and output (evapotranspiration) (Delgado-Baquerizo *et al*., 2020).

DNA extraction, amplification and Hiseq sequencing

Soil sample of 0.5 g was used to extract the DNA following the manufacturer's instructions of the FastDNA® SPIN Kit for Soil (Q-Biogene, Carlsbad, CA, USA). This sample volume has been used for DNA extraction of eukaryotic microbes such as protist (Huang *et al*., 2021) and soil metazoa (Wu *et al*., 2011; Shen *et al*., 2014). Moreover, previous studies reported a relatively consistent trend of alpha diversity among trophic levels despite different sample volumes across various studies (Supporting Information Table S5). This consistency highlights that the sample volume would not alter the trend of alpha diversity among the four groups, especially for the bacteria and metazoa. After DNA extraction, the 260/280 and 260/230 nm absorbance ratios were used to assess its quality, which were obtained using the NanoDrop® ND-2000 UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). PicoGreen (Life Technologies, Grand Island, NY, USA) with a FLUOstar OPTIMA fluorescence plate reader (BMG LabTech, Jena, Germany) was used to quantify the DNA concentration. The extracted DNA was kept at −80°C until being used for sequencing library preparation.

Amplicon libraries were created to determine the soil biota using primers for rRNA marker genes. Specifically, primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the hypervariable V4 region of the bacterial *16S* rRNA gene (Caporaso *et al*., 2011). *ITS2*rRNA gene targeting fungi was amplified by the primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCT CCGCTTATTGATATGC-3′) (White *et al*., 1990). The V4 region of the *18S* rRNA gene was used to detect the eukaryotic organisms with primers TAReuk454FWD1F (5′-CCAGCA(G/C)C(C/T)GCGGTAATTCC-3′) and TAReukREV3R (5′-ACTTTCGTTCTTGAT(C/T)(A/G)A-3′) (George *et al*., 2019). All primers were synthesized by Invitrogen (Carlsbad, CA, USA). Polymerase chain reaction (PCR) was operated by BioRad S1000 (Bio-Rad Laboratory, Irvine, CA, USA). Each sample was amplified in triplicate with a 50 μl reactions comprise of 25 μl 2× Premix Taq (Takara Biotechnology, Dalian Co. Ltd, China), 1 μl each primer (10 mM) and 3 μl DNA (20 ng μl^{-1}) template in a volume of 50 μl. The PCR conditions for the *16S* rRNA gene were as follows: initialization at 94°C for 5 min, 30 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 8 min. For the *ITS2* rRNA gene, initial denaturation was at 98°C for 30 s, 32 cycles at 98°C for 10 s, 56°C for 20 s and 72°C for 30 s, with a final extension at 72°C for 8 min. For the *18S* rRNA gene, initialization was at 95°C for 5 min, 30 cycles at 94°C for 30 s, 47°C for 45 s and 72°C for 1 min, with a final extension at 72°C for 5 min. The length and concentration of the PCR products were detected by 1% agarose gel electrophoresis. Then the PCR products from three amplifications of each sample were pooled before purification with EZNA Gel Extraction Kit (Omega, Norcross, GA, USA) and quantification with

a Quant-iT dsDNA HS Assay Kit (Invitrogen). Finally, the PCR products were sequenced on an Illumina Hiseq 2500 platform (Illumina, San Diego, CA, USA) and 250 bp paired-end reads were generated subsequently.

Bioinformatic analyses

The quality of the paired-end row reads was checked by FastQC v.0.11.9 (Andrews, 2010). Paired-end reads were merged and relabelled using the *-fastq_mergepairs* command in USEARCH v10.0.240 (Edgar, 2010). Then quality filtering was proceed using the *-fastx_filter* command with a maximum expected error threshold of 0.01 in VSEARCH v2.15.2 (Rognes *et al*., 2016). Barcodes and primers were removed at the same time. Filtered reads were subsequently dereplicated with VSEARCH using the *-derep_fulllength* command. Final unique reads were denoised and clustered into ASVs using the unoise3 algorithm (Edgar *et al*., 2016) implemented in USEARCH, the chimera sequence and singleton were removed during ASV clustering. Here we used ASV rather than operational taxonomic unit, because the ASV can control errors sufficiently and permit more robust downstream analyses (Callahan *et al*., 2017). The most abundant sequence within each ASV was defined as the representative sequence and extracted for further annotation. The Silva v123 (for *16S* rRNA gene, https://www.arb-silva.de/), Unite v7.1 (for *ITS* rRNA gene, http://unite.ut.ee/index.php) and PR2 v4.13.0 (for *18S* rRNA gene, https://pr2-database.org/) database were used to annotate taxonomic information for each representative sequence with the confidence threshold to ≥0.6 using the *-sintax* command of Vesearch (Gao *et al*., 2017). Approximately, maximum-likelihood trees were constructed using FastTree (Price *et al*., 2010) with a midpoint root after that representative sequences were aligned by MUSCLE (Edgar, 2004). In this study, all eukaryotic taxa, except vascular plants (*Streptophyta*), fungi and invertebrates (*Metazoa*) from the *18S* ASV table were classified as protists. Soil animals were defined as invertebrates (*Metazoa*) (Delgado-Baquerizo *et al*., 2019). ASVs identified as non-bacteria ASVs in the *16S* ASV table, non-fungi ASVs in the *ITS* ASV table, as well as ASVs identified as fungi, plants and non-soil animals in the *18S* ASV table were removed. Afterwards, we got four ASV tables for bacteria, fungi, protists and soil animals ultimately. To ensure the even sampling depth within each group of soil organisms, the four ASV abundance tables were rarefied at the minimal sequence number per sample. DNA sequences involved in this study were submitted to the NCBI Sequence Read Archive (SRA) database under the BioProject number PRJNA731395.

Statistical analyses

Taxonomic composition and alpha diversity. To determine the belowground community composition, we collapsed ASVs to their respective taxonomic ranks (phylum and class for bacteria, fungi and protists, while class and order for soil animals) using the tax_glom function in the 'phyloseq' package (McMurdie and Holmes, 2013). Average relative abundances of all taxonomy groups across all samples were calculated and shown by stacked barplot. Alpha diversity indices including richness, Shannon–Wiener and Simpson were determined using the diversity function in the 'vegan' package (Oksanen *et al*., 2020).

Variation of community and DDRs. We adopted two indexes to characterize the beta diversity of the belowground communities in Tibetan alpine grasslands. The first type of beta diversity (i.e. the dissimilarity) is the notion of variation, which means the variation of community structure among a number of samples within a given spatial scale (Vellend, 2010; Anderson *et al*., 2011). The second type of beta diversity (i.e. the slope of DDRs) is the species turnover, which is used to detect the variation of community structure along a spatial or environmental gradient (Anderson *et al*., 2011). The turnover rate (or slope) is used to examine how much of the changes in the variation of communities (Δy) is explained by space (distance) or the environment (e.g. ΔpH), which may be different along different gradients such as pH and geography (Harrison *et al*., 1992; Anderson *et al*., 2011). Notably, as observed in previous studies (Monroy *et al*., 2012; Jiao *et al*., 2019; De Gruyter *et al*., 2020), one group may have a higher variation of community structure but lower turnover along a specific gradient and vice versa.

Variation of community composition was determined based on Bray–Curtis distances using the vegdist function in the 'vegan' package. Post hoc with Tukey's test was used to compare the significance in the variation of community composition among four taxonomic groups. The spatial species turnover rates were defined as the slopes of DDRs, which were measured by the ordinary least squares regressions between community similarities and geographic distances. Geographic distance matrixes were calculated using Euclidean distance based on sampling site coordinates, which was the straight line distance between sampling sites. Community similarities were determined by one subtracting dissimilarity of the Bray–Curtis metric. A steeper distance–decay slope indicates that community structure may have a higher species turnover rate along the spatial gradient (Anderson *et al*., 2011). The statistical significance of DDRs was evaluated by the Mantel test using the mantel function in

the 'vegan' package. Then Emtrends function in the 'emmeans' package (Lenth *et al*., 2018) was adopted to estimate the significant difference of the slopes of DDRs among four taxonomic groups.

Multivariate statistical analysis. We used the principal component axes determined by RDA to indicate the community structure and then detect the trophic interactions based on the correlation between PC1s (Ramette, 2007; Nguyen *et al*., 2021). We then performed variation partitioning analysis to evaluate the relative importance of environmental and spatial factors in regulating belowground community assembly through the following three steps. Firstly, the dbrda and vif.cca functions in the 'vegan' package were used to check the collinearities among environmental and spatial variables based on the variance inflation factors (VIFs). Spatial variables were determined by principal coordinates analysis of neighbour matrices (PCNM) with geographic factors using the pcnm function in the 'vegan' package. Then variables with VIF > 20 were removed to avoid the impact of collinearity (O'brien *et al*., 2016; Chen *et al*., 2019). Subsequently, environmental and spatial variables were selected using a forward selection procedure following the Kaiser–Guttman rule (Jackson, 1993). Scripts for this procedure were modified according to Wu *et al*. (2018). Furthermore, variation partitioning analysis (also named two-way permutational multivariate analyses of variance; McArdle and Anderson, 2001) was conducted to assess the relative importance of environmental and spatial factors in regulating the belowground community assembly. The strength of species sorting was defined as the individual environmental fraction without spatial component, while the strength of dispersal limitation was characterized by the pure spatial fraction without environmental component (Wu *et al*., 2018; Jiao *et al*., 2019). The relative importance of species sorting versus dispersal limitation was estimated using the ratio of species sorting to dispersal limitation. The explained variance fractions were adjusted accounting for the number of factors and sample sizes. The permutation test was executed to test the significance of each component, except for the interaction fractions (environmental and spatial variables) and residuals, which cannot be examined statistically (Legendre, 2008).

Community assembly analysis. A framework proposed by Stegen *et al*. (2013) was carried out to evaluate the importance of several ecological processes in controlling community assembly. The Stegen model has provided a useful tool to explore the underlying mechanisms of community assembly for bacteria (Dini-Andreote *et al*., 2015; Tripathi *et al*., 2018; Wu *et al*., 2018; Jiao *et al*., 2019), fungi (Bahram *et al*., 2016; Jiao *et al*., 2019), protists (Wu *et al*., 2011; Zinger *et al*., 2011) and soil animals (*Metazoa*) (Liu *et al*., 2020; Vass *et al*., 2020). As done by Stegen *et al*. (2013), we selected the 1,000 most abundant ASVs (all ASVs for soil animals) to conduct the null model. This step could avoid the influences of sequencing errors such as singletons and was not likely to greatly affect the results because all community-level analyses were abundance-weighted (Stegen *et al*., 2013). The random expectations of null model were obtained by 999 randomizations. To estimate the phylogenetic turnover between samples, β-mean nearest taxon distance (βMNTD) was measured by the comdistnt function of the 'picante' package (Kembel *et al*., 2010). Then the β-nearest taxon index (βNTI) was calculated as the number of standard deviations of the observed βMNTD from the null distribution of βMNTD. A significant deviation indicates heterogeneous (βNTI values > 2, significantly more than expected phylogenetic turnover) or homogeneous (βNTI values < −2, significantly less than expected phylogenetic turnover) selection, which represents the deterministic process. βNTI values falling within the range of −2 to 2 represent stochastic processes that include homogenizing dispersal, dispersal limitation and drift. Subsequently, a Bray–Curtis-based Raup–Crick (RC_{Bray}) was calculated with $RC_{Bray} > 0.95$, $|RC_{Bray}| < 0.95$ and $RC_{Bray} < -0.95$ being interpreted as dispersal limitation, homogenizing dispersal and drift respectively. All statistical analyses were performed in R 3.6.2 (R Core Team, 2019). R codes for the statistical analyses, metadata, the ASV tables and corresponding taxonomic classifications are available at https://github.com/kangluyao/Assembly-of-soil-microbiota-in-alpine-grasslands.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supplementary Information.