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RESEARCH ARTICLE

# Distinct mechanisms shape soil bacterial and fungal co-occurrence networks in a mountain ecosystem

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**One sentence summary:** Soil bacterial and fungal co-occurrence networks in a hotspot mountain ecosystem are shaped by both biotic and abiotic factors.

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## ABSTRACT

Understanding microbial network assembly is a promising way to predict potential impacts of environmental changes on ecosystem functions. Yet, soil microbial network assembly in mountain ecosystems and its underlying mechanisms remain elusive. Here, we characterized soil microbial co-occurrence networks across 12 altitudinal sites in Mountain Gongga. Despite differences in habitats, soil bacterial networks separated into two different clusters by altitude, namely the lower and higher altitudes, while fungi did not show such a pattern. Bacterial networks encompassed more complex and closer relationships at the lower altitudes, while fungi had closer relationships at the higher altitudes, which could be attributed to niche differentiation caused by high variations in soil environments and plant communities. Both abiotic and biotic factors (e.g. soil pH and bacterial community composition) shaped bacterial networks. However, biotic factors played more important roles than the measured abiotic factors for fungal network assembly. Further analyses suggest that multiple mechanisms including niche overlap/differentiation, cross-feeding and competition between microorganisms could play important roles in shaping soil microbial networks. This study reveals microbial co-occurrence networks in response to different ecological factors, which provides important insights into our comprehensive understanding of microbial network assembly and their functional potentials in mountain ecosystems.

**Keywords:** co-occurrence network; soil microbial community; network assembly mechanism; altitudinal gradient; Mountain Gongga

## INTRODUCTION

Soil microbiota are abundant and diverse on Earth, and play crucial roles in a wide range of biogeochemical cycles (Fierer 2017; Lladó, López-Mondéjar and Baldrian 2017). Considerable research efforts have been devoted to characterizing microbial species diversity and its responses to known environmental variables, leading to the findings that soil microbiota vary considerably along various ecological gradients (Fierer and Jackson 2006; Tedersoo *et al.* 2014; Barberán *et al.* 2015; Zhou *et al.* 2016). Those studies will in the near future provide opportunities for us to enhance biogeochemical processes via direct manipulation

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of soil environments and microbiome. Successful management of the soil microbiome would require a comprehensive understanding of interplays between ecological factors, including abiotic and biotic factors, and microbial interactions among various community members. However, due to the complexity of the soil microbiome, microbial interactions among their members in a community represent one of the largest knowledge gaps, limiting our understanding of their roles in ecosystem functions (Widder *et al.* 2016).

When living together, microorganisms interact to form a complex ecological network (Faust and Raes 2012). Despite significant roles in ecosystem functions, direct detection and investigation of these interactions are far from straightforward (Faust and Raes 2012; Layeghifard, Hwang and Guttman 2017). Co-occurrence network analysis has recently been used to explore microbial interactions in diverse environments, such as human gut, ocean, bioreactor and soil (Barberán *et al.* 2011; Faust *et al.* 2012; Chow *et al.* 2013; Ma *et al.* 2016; Wu *et al.* 2016; Ma *et al.* 2018). Also, this approach reveals how microorganisms live in a community and occur together in niches, offering new insights into our understanding of microbial assembly and functions (Deng *et al.* 2012; Lupatini *et al.* 2014). In addition, it can provide information on keystone taxa that have the largest influence in a community (Berry and Widder 2014; Widder *et al.* 2014; Banerjee *et al.* 2016b; Schlaeppi and van der Heijden 2018). Therefore, co-occurrence network analysis is a powerful tool to explore microbial interactions, understand microbial assembly and provide keystone taxa of a community.

A limited number of studies have shown that most soil microbial co-occurrence networks are non-randomly assembled and form certain patterns (Barberán *et al.* 2011; Ma *et al.* 2016). Moreover, these patterns vary depending on sampling regions and habitats. For example, microbes in forest soils from northern China had closer relationships but had a lower interaction influence than those from the southern regions (Ma *et al.* 2016), while microbes in soybean fields from southern China had more interactions compared with those of the northern regions (Zhang *et al.* 2018). Even in soil compartments associated with plant roots, microbial co-occurrence relationships reveal contrasting patterns, with more (Shi *et al.* 2016; Yan *et al.* 2016) or less (Mendes *et al.* 2014; Fan *et al.* 2018; Zhang *et al.* 2018) complex interactions in the rhizosphere soil than in the bulk soil. Such differences raise the fundamental question about what the patterns and driving forces controlling diverse network patterns are across different habitats and at large spatial–temporal scales. Some pilot studies have shown that abiotic factors such as soil pH, organic matter, precipitation level, iron and magnesium content may be the key factors shaping microbial networks in forests, grasslands and agricultural fields (Ma *et al.* 2016; Wang *et al.* 2018; Zhang *et al.* 2018), and microbial phylogeny was also found to structure co-occurrence relationships in anaerobic digesters (Nuismer and Harmon 2015; Wu *et al.* 2016). However, microbial network assembly mechanisms remain largely unknown.

The mountain ecosystem represents one of the most important components in terrestrial systems, and provides significant ecological services. Mountain ecosystems harbor a diverse array of habitats along the altitudinal gradient in which a large range of climate, vegetation and soil properties can be found (Sundqvist, Sanders and Wardle 2013). Though altitudinal patterns of species diversity are well-studied (Shen *et al.* 2013; Shen *et al.* 2015; Li *et al.* 2018; Adamczyk *et al.* 2019), microbial network patterns and driving forces have not yet been resolved. Given that microbial interactions may contribute to soil functions more than species diversity (Ma *et al.* 2016), comparing the succession of microbial networks across a diverse range of habitats along the altitudinal gradient may point toward a more comprehensive understanding of microbial assembly in the soil environment. Also, condidering potential microbial interactions and possible mechanisms may help to improve the performance of prediction models for ecosystem function dynamics.

Understanding microbial community assembly mechanisms is a central topic in microbial ecology (Stegen *et al.* 2012; Zhou *et al.* 2014). However, the mechanisms of soil microbial community assembly remain elusive at the higher-order interaction level in mountain ecosystems. In the complex ecological interaction web, the spectrum of interactions primarily includes cross-feeding interactions, by which one organism benefits from the biochemical activity of another one (Sieber, McInerney and Gunsalus 2012; Seth and Taga 2014; Pande and Kost 2017), and competitive interactions, by which community members compete for limiting resources or release toxic products (Cordero *et al.* 2012; Pande *et al.* 2014). Also, co-occurring relationships could result from co-colonization and niche overlap (Faust and Raes 2012; Dohi and Mougi 2018). Microorganisms with similar environmental preferences tend to co-occur in a meta-community (Wiens *et al.* 2010; Wu *et al.* 2016; Morales-Castilla *et al.* 2017), while in a similar way those that have contrasting niche preferences tend to form negative relationships (Faust and Raes 2012). Whether these mechanisms jointly govern the microbial network assembly needs to be further investigated.

Mountain Gongga is the highest mountain of the eastern boundary of Tibetan Plateau (Wu *et al.* 2013) and serves as a hotspot for studying interactions among climate, vegetation, soil and microbial communities (Shen *et al.* 2001; Shen, Liu and Wu 2004; Sun *et al.* 2013; Li *et al.* 2018; Cui *et al.* 2019; Wang *et al.* 2019). Here, we chose this experimental site to reveal the shift of soil microbial network assembly in response to diverse ecological factors, and to explore the possible mechanisms shaping such processes. Given the altitudinal variability in soil microbial communities (Shen *et al.* 2015; Wang *et al.* 2015; Wang *et al.* 2017; Li *et al.* 2018) and fundamental differences in life strategies between bacteria and fungi (Boer *et al.* 2005; Schneider *et al.* 2012; Baldrian 2017), we hypothesized: (i) that the soil microbial network would shift dramatically in response to different habitats along the altitudinal gradients, but bacteria and fungi would show different patterns; (ii) that ecological factors driving such variations in bacterial and fungal networks would be different due to their different niche preferences; and (iii) that multiple mechanisms including niche overlap/differentiation, cross-feeding and competition between microorganisms could shape soil microbial co-occurrence networks. This study provides a comprehensive understanding of microbial network assembly and may have important implications for their potential impacts on soil functions in mountain ecosystems.

## MATERIALS AND METHODS

### Site description and soil sampling

The Mountain Gongga National Nature Reserve is located in the west part of Sichuan Province, Southwest China (ca. 29°01'-30°05' N, 101° 29'-102°12' E). With a peak height of 7556 m, Mountain Gongga is the summit of the Hengduan Mountain Ranges, which constitutes the eastern boundary of the Tibetan Plateau.

The environment in Mountain Gongga is characterized by a diverse range of habitats with an extraordinarily variable topography, climate, vegetation and soil, virtually along the prominent altitudinal gradients (Table S1, see online supplementary material). In July, for example, the mean monthly temperature decreases from 12.7°C at 1600 m to 4.2°C at 3000 m, whereas precipitation increases from 1050 to 1943 mm, respectively (Wu *et al.* 2013). The vegetation types represent the most complete vegetation spectrum along the altitudinal gradients of the subtropical region in China. From lower to higher altitudes (1800–4100 m), the vegetation type exhibits a shift from evergreen broadleaved forests, to deciduous broadleaved forests, to mixed broad-leaved conifer forests, to dark coniferous forests, to subalpine shrubs, and to subalpine shrub meadows.

We collected topsoil samples ($n = 71$, 0–10 cm depth after removing litter layer) from 12 successive altitudinal sites (from 1800 to 4100 m) in the mountain ecosystem in October, 2014 (Fig. S1, see online supplementary material). To eliminate the effect of high spatial heterogeneity on microbial analyses, we collected soils from four to seven independent plots at each altitude with four to five cores per plot pooled as one replicate sample. Visible roots and litter were removed prior to homogenizing the soil samples. Each fresh soil sample, passed through a 2-mm sieve, was divided into two subsamples. One was stored at 4°C for soil abiotic properties measurement and the other was stored at −20°C for DNA extraction. Soil temperature was measured in the field at a depth of 10 cm (T10) using a digital thermometer. In the lab, soil properties including soil pH, total nitrogen (TN), total carbon (TC), $NH_4^+$-N, $NO_3^-$-N and electrical conductivity were measured (Table S2, see online supplementary material), and the mean measured soil properties at each sampling site were calculated. More detailed information is included in the online supplementary material.

## Bacterial and fungal analyses

Soil genomic DNA was extracted using a PowerSoil® DNA Isolation Kit (MOBIO, CA, USA). DNA purity and concentration were analysed with a NanoDrop spectrophotometer (NanoDrop, DE, USA). For sequencing, the V4–V5 region of bacterial 16S rRNA gene primer pairs 515F (5′-GTGYCAGCMGCCGCGGTA- 3′)/909R (5′-CCCCGYCAATTCMTTTRAGT -3′) and fungal Internal Transcribed Spacer 2 (ITS2) primer pairs ITS4 (5′-TCCTCCGCTTATT GATATGC-3′)/ITS3-kyo2 (5′- GATGAAGAACGYAGYRAA-3′) were used (Toju *et al.* 2012; Rui *et al.* 2015). A 12-bp multiplex identifier sequence was added to the 5′ end of primers 515F and ITS4. PCR reactions were conducted based on the method described by Li *et al.* (Li *et al.* 2018). Briefly, an aliquot (10 ng) of high-purity DNA was used as a template for amplification. The PCR amplifications consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C (for both bacteria and fungi) for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. Triplicate PCR reactions were performed per sample and pooled for purification using a DNA Gel Extraction kit (Axygen, CA, USA). Negative controls were performed to check for contamination. An equimolar amount of PCR product from each sample was pooled together, and subjected to Illumina MiSeq sequencing (2 × 250 V2 kit) at the Chengdu Institute of Biology, Chinese Academy of Sciences..

Paired-end sequences were merged using the FLASH tool (Magoč and Salzberg 2011). The sequence data processing, including sequence quality control, Operational Taxonomic Unit (OTU)-based analyses, taxonomy assignment and diversity indices calculation, was performed using the UPARSE pipeline (Edgar 2013). Briefly, sequences with barcode ambiguities, <250 bp in length and with average quality scores <20 were discarded. Bacterial taxonomy was assigned to each unique sequence using the Ribosomal Database Project (RDP) Classifier with a confidence threshold of 80% (Wang *et al.* 2007). The taxonomy of fungal OTUs was assigned using the BLAST method against the UNITE database for QIIME release 7.2 (https://unite.ut.ee/repository.php). Before network construction, the OTU matrices were rarefied to 9500 and 8900 sequences per sample and followed by normalization using total sum scaling (also known as relative abundance) for bacteria and fungi, respectively. To reduce spurious OTUs, we removed OTUs with relative abundances <0.01% of the total number of bacterial or fungal sequences. Because the fungal ITS sequence may not be a suitable marker for phylogenetic analysis (Nilsson *et al.* 2008; Pérez-Izquierdo *et al.* 2017), only a bacterial phylogenetic tree was constructed from the representative sequences using MUSCLE (Edgar 2004). Bactetial phylogenetic diversity (Faith's PD) (Faith 1992), net relatedness index (NRI) and nearest taxon index (NTI) (Webb *et al.* 2002) were calculated using pd, ses.mpd and ses.mntd functions in the *picante* package (Kembel *et al.* 2010).

## Network construction

The meta-network including bacterial and fungal OTUs was inferred based on the Spearman correlation matrix. The false discovery rate (FDR) procedure provided in *multtest* R package was applied to adjust all *P* values for multiple testing (Benjamini, Krieger and Yekutieli 2006). The cutoff of FDR-adjusted *P* values was 0.001. The threshold of correlation value was automatically identified through random matrix theory (RMT)-based algorithms (Luo *et al.* 2006), which have proved to be reliable, sensitive and robust for identifying molecular ecological networks for analysing high-throughput metagenomic data sets (Deng *et al.* 2012; Ma *et al.* 2018; Tian *et al.* 2018; Wang *et al.* 2018). The connections in the network represent positive or negative correlation values greater than the RMT-threshold value. Gephi (https://github.com/gephi/gephi) was used to generate the network image.

In order to depict the differences in topological features associated with bacteria and fungi, we further generated three sub-networks wherein bacteria–bacteria (BB), bacteria–fungi (BF) and fungi–fungi (FF) associations were captured, respectively. Finally, we generated sub-networks for each soil sample from sub-networks BB, BF and FF by preserving the OTUs presented in each sample using the subgraph functions in the *igraph* package. Network-level topological features provided in the *igraph* package were calculated (Csardi and Nepusz 2006). These properties included node-level features, such as betweenness centrality, closeness centrality, clustering coefficient and degree, and network-level features, such as node number, edge number, average degree, average path length, clustering coefficient, cluster number, modularity, diameter, degree assortativity and density. Detailed definitions of these network topological features are described in Table S3, see online supplementary material. Nodes that distributed in more than two-thirds of the samples with high degree (>100) and low betweenness centrality values (< 5000) were recognized as potential keystone OTUs in the meta-network (Ma *et al.* 2016).

### Statistical analysis

We grouped each sub-network (BB, BF and FF) by altitude and used principal component analysis (PCA) to detect possible clusters formed among all the samples. Permutation multivariate analysis of variance (PERMANOVA) was further applied to examine their differences in the topology structure. We used the Wilcoxon rank-sum test to determine the difference in the network-level topological features between the lower and higher altitudes. We used multiple regressions on distance matrices (MRM) in the *ecodist* package to estimate the importance of ecological factors including the environmental and community variables for the network-level topology variations. Respective bacterial or fungal community variables were $\alpha$-diversity (represented by Shannon's index), phylogenetic diversity (measured by Faith's index, bacteria only), and the first (PCoA1) and second (PCoA2) axis value of principal coordinate analysis on the microbial OTU matrix calculated by the pcoa function of the *ape* package. The available environmental variables included space, climate and edaphic properties, such as the longitude, latitude, mean annual precipitation (MAP), mean annual temperature (MAT), soil pH, TC, TN, $NH_4^+$-N, $NO_3^-$-N, electrical conductivity, C:N ratio and T10. The Euclidean distance matrices for network-level topological features and ecological factors standardized with decostand function (standardize method) of the *vegan* package were used in MRM models. We used Spearman's rank correlation test to check the relationships between network topological features and the ecological factors.

### Data accessibility

The original sequencing data are available at the European Nucleotide Archive under accession No. PRJEB26872 (http://www.ebi.ac.uk/ena/data/view/ PRJEB26872).

## RESULTS

### Meta-community co-occurrence network and sub-networks

The majority of bacterial sequences belonged to the phyla Proteobacteria (relative abundance 34.5%), Acidobacteria (30.7%), Bacteroidetes (9.5%), Chloroflexi (5.8%) and Planctomycetes (5.1%). The most abundant fungal phyla were Ascomycota (relative abundance 70.0%), Basidiomycota (24.9%) and Glomeromycota (1.6%). The constructed meta-network captured 17 406 edges, including positive and negative relationships among 1094 bacterial nodes (OTUs) and 1260 fungal nodes (Fig. S2, see online supplementary material). This meta-network roughly followed a scale-free degree distribution (Fig. S3, see online supplementary material), meaning that most nodes had only a few connections and only a few nodes had numerous connections, indicating a non-random co-occurrence pattern.

Because the meta-network included both bacteria and fungi, it was denoted as the sub-network of 'all edges' (All). We then partitioned nodes of sub-network All into two taxonomic groups (bacteria and fungi). Hence, three sub-networks were obtained including BB, BF and FF associations. In the sub-network BB, nodes with high degree values were mainly affiliated with members of Acidobacteria Gp1, Gp2, Gp3, Gp4, Gp5, Gp6 and Gp17, and Planctomycetales, Sphingobacteriales, Xanthomodales, Rhizosbiales, Burkholderiales and Rhodospirillales (Tables S4 and S5, see online supplementary material), including genera *Ohtaekwangia*, *Steroidobacter*, *Terrimonas*, and *Pirellula* (Tables S6 and S7, see online supplementary material). In the sub-network FF, the associations were mainly observed among Helotiales, Agaricales, Pleosporales, Sordariales, Chaetothyriales and Hypocreales (Table S8, see online supplementary material), including genera *Exophiala*, *Mycoarthris*, *Inocybe*, *Cenococcum*, *Veronaea*, *Glutinoglossum* and *Byssocorticium* (Table S9, see online supplementary material). In the sub-network BF, bacterial and fungal OTUs were mainly the same as those in sub-networks BB and FF.

The correlation analysis showed that bacterial genera with high degree values were exclusively correlated with the measured soil characteristics, such as soil pH, $NO_3^-$ and T10 (Fig. 1A). In particular, soil pH had negative correlations with *Rhizomicrobium* and members of Acidobacterial genera Gp1, Gp2 and Gp3, but had positive relationships with members of Acidobacteria Gp4, Gp5, Gp6 and Gp17. Other genera including *Ohtaekwangia*, *Steroidobacter* and *Dongia* showed close relationships with soil electrical conductivity, TC and TN. However, few fungal genera with high degree values were correlated significantly ($P < 0.05$) with the measured variables. These environmental variables except for MAP and altitude showed positive relationships with genera *Exophiala, Mycoarthris, Veronaea, Glomus* and *Geoglossum* but had negative relationships with genera *Fontanospora* and *Purpureocillium* (Fig. 1B). These results suggest that bacteria and fungi in the networks could be constrained by different environmental factors.

### Microbial network structure in diverse habitats

In sub-networks All, BB and BF, the network structure of altitudinal sites at the lower altitudes (1800–2600 m) was significantly ($P < 0.05$) different from that at most higher altitudes (2800–4100 m) (Tables S10, S11 and S12, see online supplementary material), while there were no obvious network structure patterns in sub-network FF (Table S13, see online supplementary material). Further, PCA and PERMANOVA analyses showed that all the samples in sub-networks All, BB and BF were separated into two significantly ($P = 0.001$) different clusters by altitude, namely the lower (1800–2600 m) and higher (2800–4100 m) groups (Fig. 2A, B and C). However, the network structure of sub-network FF did not show such a pattern (Fig. 2D). Also, the network structure of sub-network FF showed significant ($P = 0.001$) difference only between pairs of dark coniferous forest–subalpine shrub and dark coniferous forest–subalpine shrub meadow without differences among other vegetation types (data not shown). These results suggest that topological features of bacterial and fungal networks could have different responses to different habitats.

MRM was used to estimate the importance of ecological factors for the variations of network structure. Collectively, 59.5, 40.2 and 38.9% of the total variations in sub-networks BB, BF and FF were explained, respectively (Fig. 3A). Community variables alone explained 20.4, 16.4 and 29.7% of the total variations, and the communities together with the available environmental variables explained 36.6, 22.5 and 3.6% of the total variations, respectively. This suggests that both measured abiotic and biotic factors shape bacterial networks, while abiotic factors were not as important as biotic factors for fungal network assembly. Further investigation showed that soil pH, PCoA2 and PCoA1 were the three most important variables correlated with the network structure of sub-network BB (Fig. 3B). In the sub-network BF, bacterial PCoA1 and fungal PCoA1 were the two most important variables. In the sub-network FF, however, Shannon's index together with PCoA2 and PCoA1 predominated over other variables in explaining the structure variation.

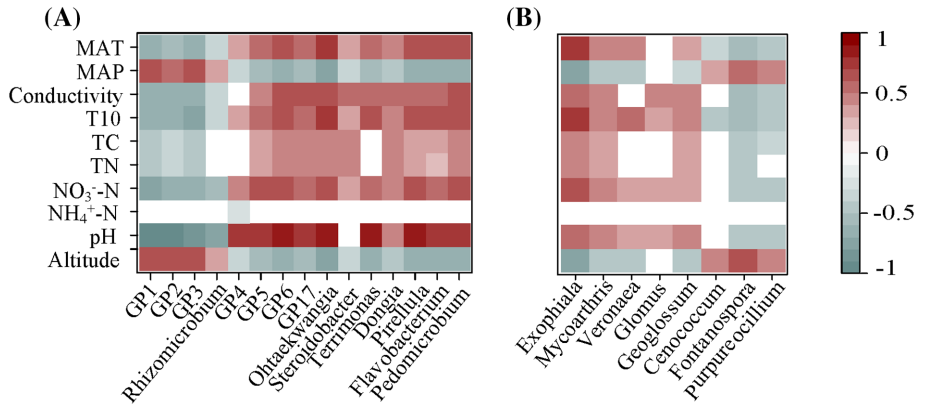


**Figure 1.** Correlations between the measured environmental variables and the relative abundances of microbial genera with high degree value in sub-networks BB (**A**) and FF (**B**). MAT, mean annual temperature (°C); MAP, mean annual precipitation (mm); conductivity, soil electric conductivity ($\mu$S cm$^{-1}$); T10, soil temperature at 10 cm depth (°C); TC, total carbon (%); TN, total nitrogen (%); $NH_4^+$-N, ammonium nitrogen [mg (kg dry wt soil)$^{-1}$]; $NO_3^-$-N, nitrate nitrogen [mg (kg dry wt soil)$^{-1}$]. Spearman's rank correlation tests were performed. Only significant correlations at $P < 0.05$ are colored.

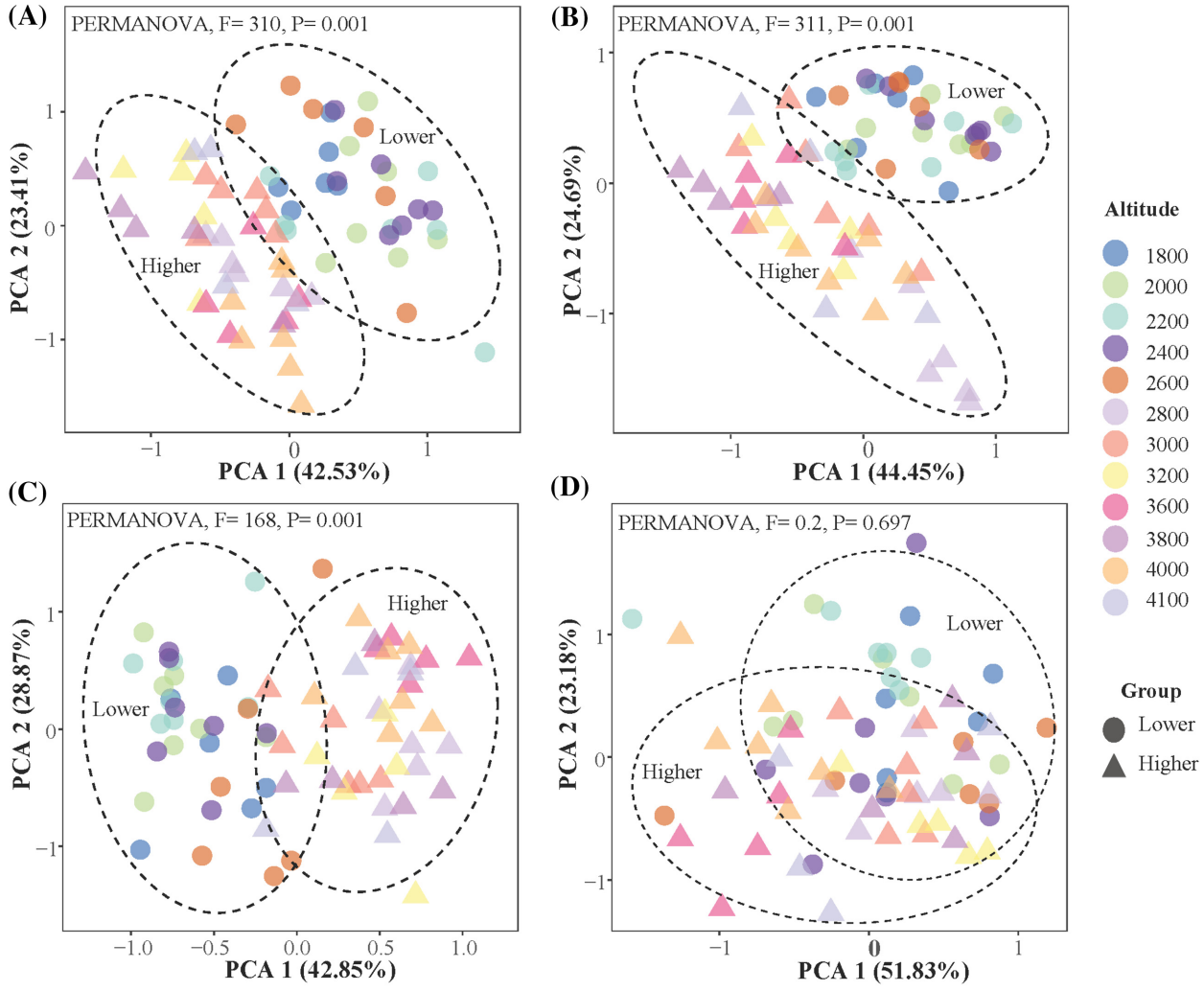


**Figure 2.** Principal component analysis of the network structure of sub-networks All (**A**), BB (**B**), BF (**C**) and FF (**D**). The lower (1800–2600 m) and higher (2800–4100 m) altitudes are circled, respectively. Sub-networks BB, BF and FF included associations only between bacteria–bacteria, bacteria–fungi, and fungi–fungi, respectively. Sub-network All included all the above three types of associations.

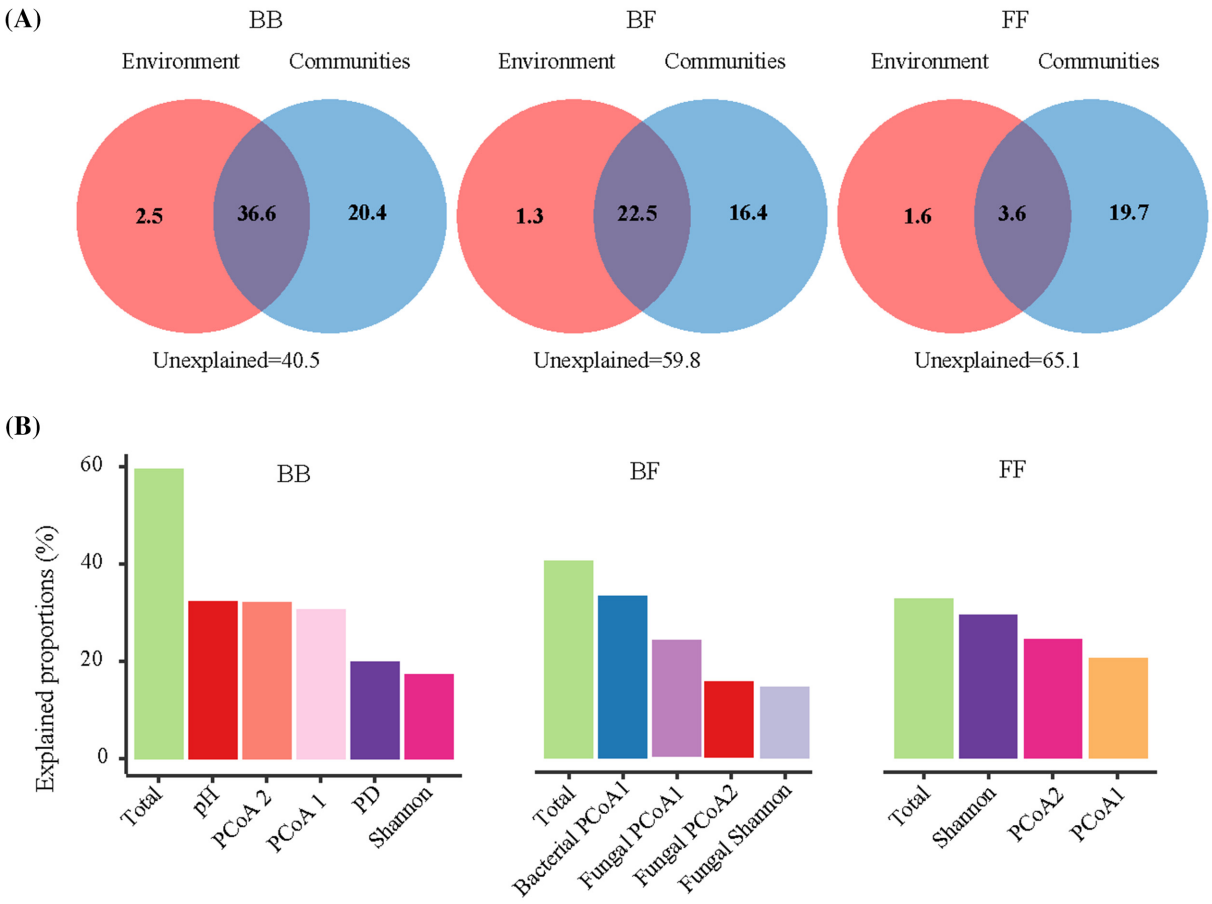


**Figure 3.** The relative contributions (%) of environmental and community composition variables (**A**), and specific variables (**B**) to the variations in network structure of sub-networks BB, BF and FF.

## Microbial topological features along the altitudinal sites

In the sub-network BB, altitudinal succession showed that the topological features including average degree, node number and edge number were higher at the lower altitudes than those at the higher altitudes (Fig. 4A and Fig. S4, see online supplementary material), whereas the average path length had a contrasting trend. In the sub-network FF, however, the most noticeable observation was significantly higher average path lengths at most lower altitudes (1800–2600 m) than those observed at the higher altitudes (2800–4100 m) (Fig. 4B and Fig. S5, see online supplementary material). Most topological features in sub-networks All and BF showed similar trends to those found in sub-network BB (Figs S6 and S7, see online supplementary material). In a network, average degree and average path length describe the complexity level of a network and the distance between any two members in a network, respectively (Table S3). Indeed, the higher the average degree, the more complex the network obtained, and the lower the average path length, the closer the observed relationships among the members. Thus, the above results suggest that bacterial networks have more complex and closer relationships at the lower altitudes, whereas fungal networks have closer relationships at the higher altitudes.

Spearman correlation analysis indicated that topological features of sub-network BB were extensively correlated with community composition and environmental variables. For example, average degree, edge number and node number were positively related to variables such as soil pH, TC, TN, T10, MAT and soil electrical conductivity (Fig. 5A), while they were negatively correlated with PCoA1, altitude and MAP. However, average path length generally showed the opposite trend compared with average degree. In the sub-network FF, Shannon's index ($\alpha$-diversity) was the only variable showing significant ($P < 0.05$) relationships with nearly all topological features (Fig. 5B). The $\alpha$-diversity showed a positive correlation with node number, edge number, average path length, modularity, cluster number and degree assortativity, but was negatively correlated with clustering coefficient and density.

## Potential keystone OTUs in the meta-network

The degree and betweenness centrality for each microbial node were used to infer potential keystone OTUs in the meta-network. Specifically, nodes that were distributed in more than two-thirds of the samples with degree >100 and betweenness centrality <5000 were identified as candidate keystone OTUs (Fig. 6A). Eighty-seven potential keystone OTUs were found from bacterial phyla in our study, which comprised 25.7% of bacterial reads (Fig. 6B, Table S14, see online supplementary material). A large fraction of these OTUs belonged to the phylum Acidobacteria (33 OTUs, ranging from 0.03 to 0.76% in relative abundance), mainly comprising members of Gp6 (8 OTUs), Gp1 (5 OTUs), Gp2

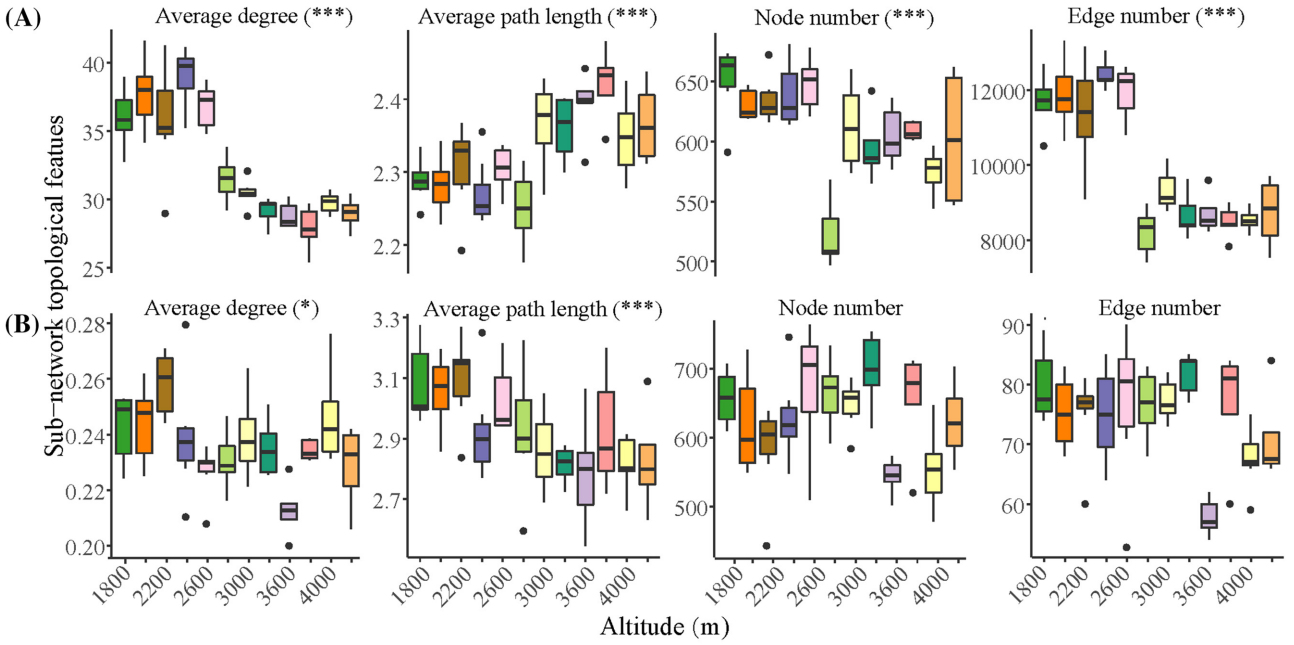


**Figure 4.** The main topological features of sub-network BB (**A**) and FF (**B**). The asterisks in parentheses mean the values are significantly different between the lower (1800–2600 m) and higher (2800–4100 m) altitudes by Wilcoxon rank-sum test. ∗ Significance at $P < 0.05$; ∗∗∗ $P < 0.001$.

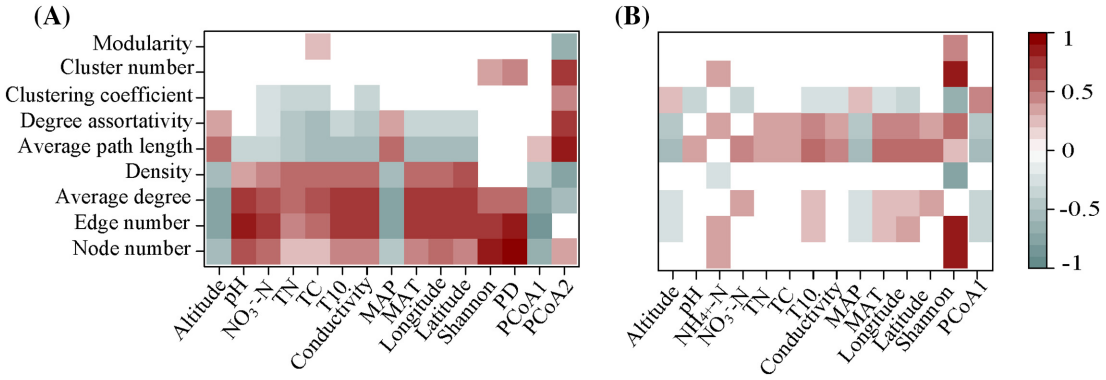


**Figure 5.** Ccorrelations between ecological factors and the network-level topological features of sub-networks BB (**A**) and FF (**B**). Only significant correlations at $P <$ 0.05 are colored.

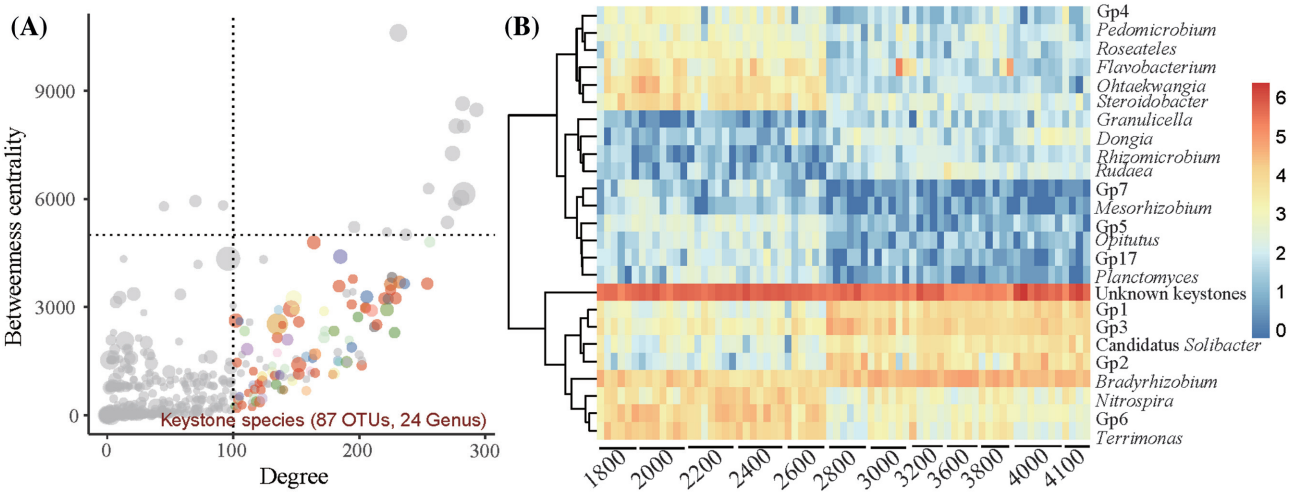


**Figure 6.** Potential keystone OTUs inferred by the betweenness centrality and degree values (**A**) and the altitudinal distributions of these keystone species at the genus level (**B**). Gp1, Gp2, Gp3, Gp4, Gp5, Gp6, Gp7 and Gp17 were unknwon members of Acidobacteria subdivisions. The colors in (B) are ranked by the logarithm of their relative abundances added to 1.

(5 OTUs) and Gp3 (5 OTUs). Keystone OTUs in the phylum Proteobacteria (28 OTUs, ranging from 0.02 to 2.55% in relative abundance) mainly comprised Rhizobiales (6 OTUs, and one genus was *Bradyrhizobium* with high relative abundance of 2.55%) and Xanthomonadales (5 OTUs, represented by genus *Steroidobacter* with relative abundance of 0.84%). The phylum Bacteroidetes had 9 candidate keystone OTUs, mainly encompassing genera *Ohtaekwangia* (3 OTUs, relative abundances of 0.75%) and *Terrimonas* (3 OTUs, relative abundance of 1.24%). The phylum Nitrospirae contained only 1 candidate keystone OTU (genus *Nitrospira*, 1.14% in relative abundance). Other potential keystone OTUs were associated with phyla Chloroflexi (4 OTUs), Planctomycetes (3 OTUs), Actinobacteria (3 OTUs) and Verrucomicrobia (1 OTU).

## DISCUSSION

Understanding microbial network assembly is a promising way to predict the potential impact of environmental changes on ecosystem functions. However, soil microbial network assembly in mountain ecosystems and its underlying mechanisms remain elusive. In this study, we characterized soil microbial co-occurrence networks across 12 altitudinal sites in Mountain Gongga, the highest mountain on the eastern boundary of the Tibetan Plateau. The results showed that bacteria and fungi had distinct network patterns in response to differences in habitats along the altitudinal gradient. Furthermore, driving factors also differed, and multiple mechanisms could play important roles in shaping soil microbial network assembly in this ecosystem. Overall, this study suggests distinct mechanisms for microbial network assembly in mountain ecosystems.

### The shift of microbial networks in response to different habitats

The altitudinal pattern of microbial network structure and topological features demonstrated our first hypothesis that networks for both bacteria and fungi would shift in different manners in response to different habitats. Interestingly, despite differences in habitats along the 12 altitudinal sites, bacterial networks formed two different clusters by altitude, with more complex and closer bacterial relationships at the lower altitudes. Recent studies found more complex and closer microbial relationships in forest soils from northern China compared to southern China (Ma *et al.* 2016), while microbial networks in soybean fields from southern China had more interactions compared with those of the northern regions (Zhang *et al.* 2018). Other studies also revealed contrasting patterns with more (Shi *et al.* 2016; Yan *et al.* 2016) or less (Mendes *et al.* 2014; Fan *et al.* 2018; Zhang *et al.* 2018) complex interactions in the rhizosphere soil than in the bulk soil. Our results combined with those of previous studies suggest that niche differentiation caused by the altitude and soil pH could be the main reasons. Indeed, niche differentiation at the lower altitudes was weaker as environmental conditions were less variable than those at the higher altitudes (Li *et al.* 2018), thus the weaker the niche differentiation, the stronger the microbial interactions would be (Faust and Raes 2012; Ma *et al.* 2016). Another factor resulting in bacterial network difference between the lower and higher altitudes could be soil pH variation. In our study sites, more acidic soil pH at the higher altitudes corresponded to a lower species diversity (Li *et al.* 2018), and low species diversity reduced network complexity, as the Shannon's index showed a positive relationship with average degree (Fig. 5A). Consequently, bacterial relationships at the lower altitudes appeared to be more complex.

Though fungal networks did not differ between the lower and higher altitudes, they had closer relationships at the higher altitudes than at the lower altitudes. Fungal communities are tightly associated with plant communities, such as plant community diversity and composition (Philippot *et al.* 2013; Yang *et al.* 2017; Purahong *et al.* 2018; Adamczyk *et al.* 2019). However, among the six different vegetation types in this study, fungal networks only differed between pairs of dark coniferous forest–subalpine shrub and dark coniferous forest–subalpine shrub meadow without differences among other vegetation types. On the other hand, a recent study at the same site showed that plant diversity decreased with increasing altitude (Li *et al.* 2018). Generally, fungal diversity is known to be positively correlated with plant diversity (Hiiesalu, Bahram and Tedersoo 2017; Yang *et al.* 2017). Therefore, low plant diversity should result in a more homogeneous habitat for fungi and support low fungal diversity. The weaker the niche differentiation, the stronger the microbial interactions would be (Faust and Raes 2012; Ma *et al.* 2016). Indeed, we found that fungal $\alpha$-diversity was negatively correlated with network clustering coefficient and density, thus fungal networks at the hihger altitudes could encompass closer relationships compared to those at the lower altitudes (Faust and Raes 2012).

### Ecological factors driving the network variation

The results obtained supported our second hypothesis that different ecological factors would drive the variations in bacterial and fungal networks, suggesting that both abiotic and biotic factors (e.g. soil pH and bacterial community composition) shaped bacterial networks, while biotic factors played more important roles than abiotic factors for fungal network assembly. A previous study showed that organic matter and iron predominantly shaped microbial networks in forest soils at a continental scale (Ma *et al.* 2016), and mean annual precipitation was the most important factor constraining network structure in semi-arid grassland soils in northern China (Wang *et al.* 2018). However, our results were in line with the widely accepted view that soil pH predominated over other environmental factors in influencing bacterial species diversity (Rousk *et al.* 2010; Cho *et al.* 2018; Li *et al.* 2018). Moreover, a recent study indicated that soil pH was the best predictor for bacterial networks (Zhang *et al.* 2018), which is consistent with our results. We attribute the effect of soil pH on bacterial networks to two factors. First, the shift in soil pH would alter microbial community composition (Rousk *et al.* 2010; Cho *et al.* 2018; Li *et al.* 2018), and as the community composition largely contributes to network construction, this undoubtedly influences the topological features of bacterial networks. Second, soil pH could alter a series of intracellular biochemical pathways, affecting microbial enzymatic activities (Stark, Mannisto and Eskelinen 2014) and changing carbon and nutrient pools in soil environments (Pande and Kost 2017). Therefore, bacterial interactions would be largely shaped by their dependence on these substrates. Nonetheless, we could not exclude the roles of other soil characteristics (e.g. soil temperature, TN and TC) on bacterial network assembly, as they provide microorganisms with physical and nutritional conditions.

As for fungal network assembly, fungal Shannon's index together with community composition rather than abiotic factors were revealed as the main driving factors. Like bacteria, fungal $\alpha$-diversity and community composition should contribute significantly to network assembly since the presence or absence

of community members would not only affect trophic interactions among them but also influence network construction. Though fungal networks only differed between two vegetation types, the effect of fungal community composition and diversity on network assembly could be modified by fungi–plant interactions, since mycorrhizal, plant pathogenic and deadwood-inhabiting fungi were typically associated with different plant species or plant-related factors (Philippot *et al*. 2013; Chen *et al*. 2017; Neuenkamp *et al*. 2018; Purahong *et al*. 2018; Weißbecker *et al*. 2018). However, as we only measured a limited number of biotic and abiotic factors, other unmeasured ones may also impact fungal network assembly, necessitating further studies in the future.

### Mechanisms of soil microbial network assembly

The results also support our third hypothesis that multiple mechanisms would shape soil microbial network assembly. In a co-occurrence network, positive relationships could result from co-colonization, niche overlap and/or cross-feeding, while negative associations could arise from prey–predator relationships, amensalism, differential niche adaptation and/or competition (Faust and Raes 2012; Dohi and Mougi 2018). Our results suggest that soil microbial co-occurrence networks could be primarily due to niche overlap and differentiation. Microorganisms with similar adaptation to a particular environmental factor tend to co-occur (positive relationship) in a meta-community since they share similar environmental preferences (Wiens *et al*. 2010; Wu *et al*. 2016; Morales-Castilla *et al*. 2017). In this study, bacteria and fungi with positive associations were correlated with soil pH as well as other environmental variables such as $NO_3^-$, T10 and soil electrical conductivity (Fig. 1). The niches created by these environmental variables would select microorganisms that share similarity in niche adaptation, thus co-occurring in a network, while those that have contrasting niche preferences tend to form negative relationships in a network. For instance, members of Acidobacteria Gp1, Gp2 and Gp3 (group 1) were negatively correlated with soil pH, whereas members of Gp4, Gp5, Gp6 and Gp17 (group 2) had positive relationships with soil pH. Such opposite niche adaptations have caused negative relationships between these two Acidobacterial groups.

Nonetheless, cross-feeding and competition might shape microbial associations as well. In cross-feeding relationships, one organism benefits from the biochemical activities of another one, or they both benefit from their obligate interactions (Pande *et al*. 2014; Pande *et al*. 2016). However, direct microbial interactions may not occur in a strict species-to-species manner but depend on trophic interactions among various members in a network. For example, metabolites produced by several Rhizobiales were strictly required for *Steroidobacter agariperforans* sp. nov. to grow on polysaccharides (Sakai *et al*. 2014), which agrees well with our network analysis that genus *Steroidobacter* co-occurred with several genera affiliated with Rhizobiales. For this reason, integrating species composition and genomic information in a community may have greater potential to reveal direct microbial interactions (Ma *et al*. 2018). In addition, our phylogenetic analyses with NTI and NRI suggest that bacterial communities are much more phylogenetically clustered at the higher altitudes (Fig. S8), which would increase competition for similar resources between closely related bacterial species (Goberna *et al*. 2014a,b; Pérez-Valera *et al*. 2017). This is in agreement with the increased negative edge proportion at the higher altitudes (Fig. S9), where the environment is harsh and competitive and such negative relationships could be enhanced.

### Potential keystone OTUs involved in soil carbon and nitrogen cycling

Keystone species exert large effects on other community members and play vital roles in maintaining ecosystem functions (Berry and Widder 2014; Williams, Howe and Hofmockel 2014; Ma *et al*. 2016; Banerjee, Schlaeppi and van der Heijden 2018). In this study, the major candidate keystone OTUs were from abundant bacterial phyla, such as genera *Bradyrhizobium*, *Nitrospira*, *Steroidobacter* and members of Acidobacteria Gp6 and Gp1. Genera *Bradyrhizobium* and *Nitrospira* are known for their abilities in nitrogen fixation and nitrification, respectively (Dixon and Kahn 2004; Jetten 2008), which play an important part in biogeochemical cycling whereby inert nitrogen gas is transformed into biological nitrogen to support the growth of other microorganisms and plants. The roles of genus *Steroidobacter* include denitrification (Fahrbach *et al*. 2008) and polysaccharide degradation (Sakai *et al*. 2014). Also, Acidobacteria were shown to decompose organic matter in acidic soils (Naether *et al*. 2012; Rawat *et al*. 2012), and members of Gp1 possibly were adapted to an oligotrophic lifestyle in low-nutrient soils, whereas members of Gp6 preferred to reside in high-nutrient soils (Banerjee *et al*. 2016a; Liu *et al*. 2016). Altogether, these potential keystone OTUs could play significant roles in carbon and nitrogen cycling in mountain soils.

Despite the usefulness of network analysis, there are still some limitations to the present approach. For example, spurious correlations may occur when comparing significant correlations. To tackle this problem, we first discarded low-abundance OTUs, then the FDR method was used to correct possible errors occurring among multiple tests. Moreover, a RMT-based algorithm rather than an arbitrary threshold was applied to generate our networks (Deng *et al*. 2012; Ma *et al*. 2018; Tian *et al*. 2018; Wang *et al*. 2018). Another pitfall in the network construction is the faulty prediction of a relationship between two OTUs because both may be affected by a third one (Faust and Raes 2012). Thus, further experimental verification of microbial interactions is needed in future co-occurrence network investigations (Lima-Mendez *et al*. 2015; Weiss *et al*. 2016).

## CONCLUSIONS

We found that soil microbial networks generally changed in response to differences in habitats along the altitudinal gradient in the mountain ecosystem. Soil bacterial networks differed between the lower and higher altitudes, but fungal networks did not show such a pattern. Bacteria had more complex and closer relationships at the lower altitudes, while fungi had closer relationships at the higher altitudes. Abiotic and biotic factors, i.e. soil pH and community composition, mainly shaped bacterial networks, but biotic factors played more important roles than abiotic factors for fungal network assembly. Further analysis also suggests that soil microbial co-occurrence networks are jointly driven by niche overlap/differentiation, cross-feeding and competition between microorganisms. In addition, some potential keystone OTUs could play significant roles in carbon and nitrogen cycling. These findings provide new insights into our comprehensive understanding of soil microbial network assembly in mountain ecosystems.

## SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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