

Archaea Enhance the Robustness of Microbial Co-occurrence Networks in Tibetan Plateau Soils

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Microbial co-occurrence is a ubiquitous ecological phenomenon, yet we know surprisingly little of the relative importance of bacteria, fungi, and archaea for structuring their co-occurrence network, especially in natural ecosystems. Here, we present a visualization of the soil microbial co-occurrence network pattern based on a sampling from across the eastern Tibetan Plateau. We found more intra-kingdom than inter-kingdom links within the network, and most of these links were positive. Compared with module hubs and network hubs, the connectors had more connections with environmental variables, and among the microbial groups the archaea built more connections than did bacteria or fungi, which pointed to the greater ecological importance of the archaea in constructing the overall network. Omitting the archaea resulted in a lower natural connectivity, suggesting this group is crucial for enhancing the robustness of the microbial co-occurrence network. Taken together, our results suggest that the archaea play a critical role in constructing soil microbial co-occurrence networks in the Tibetan plateau, and possibly also in other similar climate change-sensitive regions.

Abbreviations: OTU, operational taxonomic unit.

Core Ideas

- **Connectors had more correlations with environments than module and network hubs.**
- **Archaea built more connections than bacteria and fungi.**
- **The absence of archaea resulted in a lower natural connectivity.**
- **Archaea are more important in constructing soil microbial co-occurrence network.**

Soil microorganisms including bacteria, fungi, and archaea are key below-ground components of natural terrestrial ecosystems (Fierer, 2017). To ensure carbon sequestration, nutrition cycling and food security, the study of various kinds of microbial interactions, such as cross-feeding, co-aggregation, co-colonization or niche overlap and construction among individuals, has proceeded swiftly given its urgency (Kylafis and Loreau, 2011; Faust and Raes, 2012; Weiss

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et al., 2016). This flurry of research presupposes microorganisms are coexistent and could form stable ecological networks during these vital processes. Fortunately, recent monumental advances in molecular techniques and bioinformatics now enable an unprecedented level of investigation into the complicated ecological networks of soil microbes. An emerging approach, called co-occurrence network analysis, provides a powerful opportunity to investigate the interactions among microorganisms and their relationships with their surrounding environment (Faust et al., 2015). For example, using co-occurrence network analysis, Faust et al. (2015) identified a node in the microbial network of a tundra soil which could represent soil pH and that built links with members of Alphaproteobacteria and Acidobacteria. Furthermore, this approach lets us see and better understand the positive and negative impacts, or even non-impact, of the species involved or those non-participating in the network (Faust and Raes, 2012; Peura et al., 2015), and to identify those key species imperative in the network's construction.

Within a network there are some operational taxonomic units (OTUs) which are highly connected among themselves, yet they have fewer connections with OTUs outside their group; these groups have been defined as a module (Horvath and Dong, 2008; Oldham et al., 2008). There are many reasons for why a module is formed; for example, performing specific functions (Guimera and Amaral, 2005), ecological niche overlap (Faust et al., 2015), environmental selection (Deng et al., 2015), electronic transfer (Mackelprang et al., 2017), quorum sensing (Bareia et al., 2018), or coevolution (Toju et al., 2017). Once formed, the OTUs in the module can fulfill different roles in the network, functioning as network hubs, connectors, or module hubs and peripherals. Network hubs are those nodes having more links with other nodes within and between the modules; connectors are the nodes having more links with other nodes between the modules; and module hubs are the nodes having more links with other nodes within the module. A peripheral has few or almost no links with any of the other nodes. The hubs and connectors, so defined, are not only related to the network's construction, and hence structure, but they are often associated with soil properties (Deng et al., 2015). For example, connectors were found to have a strong relationship with soil pH in cropland soil (Fan et al., 2018). Therefore, investigating the disparate roles of microbial taxa within the network should further our understanding of their functions in the soil ecosystem.

To perform nutrient cycling and energy exchange—in the form of fixation of nitrogen from the atmosphere, decomposition of ground litter or redox reactions—a robust microbial co-occurrence network is a fundamental prerequisite. Because the suite of microbial ecological functions cannot be performed by just one species, microorganisms communicate mutually by forming a complex ecological interaction network (Bascompte, 2009; Pande and Kost, 2017). However, the relative importance of bacteria, fungi, and archaea in constructing such a microbial co-occurrence network remains largely unknown. Because natural connectivity reflects the robustness—the capacity of resisting

disturbance—of complex networks in the field of physics (Wu et al., 2010; Peng and Wu, 2016), its investigation and application to ecology could help us to better understand microbial co-occurrence network patterns.

The Tibetan Plateau is the youngest ($\sim 2.4 \times 10^8$ years old), largest ($\sim 2.0 \times 10^6$ km²) and highest (~ 4000 m above sea level, on average) land plateau in the world. Due to its extreme environmental conditions (low oxygen, dry and cold weather) (Yang et al., 2008), unique soil microbial co-occurrence patterns are expected in this region. Moreover, as the Tibetan Plateau begins to experience the effects of rapid climate change (Genxu et al., 2002), its microbial interactions may also shift in strength or change in composition in response to ongoing and future environmental change. Using the co-occurrence network approach, here we asked two questions: (i) What environmental variables are mostly correlated with the microbial co-occurrence network in Tibetan Plateau soils; and (ii) Which microbial kingdom (bacteria, fungi, or archaea) is most important for enhancing the robustness of the co-occurrence network?

MATERIALS AND METHODS

Sample Information

Details on soil sampling, DNA extraction, and soil characterization were described in our previous study (Shi et al., 2016). Briefly, in the 2011 growing season, 94 soil samples had been collected from 36 sites across the eastern Tibetan plateau (Supplemental Fig. S1). In each site, we sampled three plots, with five to seven cores collected per plot and then subsequently mixed to form a single composite plot sample. From each, soil DNA was extracted from 0.5 g of soil using the PowerSoil kit (MO BIO laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The V4-V5 hyper-variable regions of bacterial 16S rRNAs were amplified using the primer set of F515: 5'-GTGCCAGCMGCCGCGG, R907: 5'-CCGTC AATTCMTTTRAGTTT (Lane et al., 1985). The V3-V5 hyper variable regions of archaeal 16S rRNA were amplified using the primer set of Arch344F: 5'-ACGGG-GYGCAGCAGGCGCGA, Arch915R: 5'-GTGCTCCCC-GCCAATTCCT (Yu et al., 2008). The fungal internal transcribed spacer 2 (ITS2) rDNA region was amplified using the primer set of ITS3: 5'-GCATCGATGAAGAACGCAGC, ITS4: 5'TCCTCCGCTTATTGATATGC (White et al., 1990). The high-throughput sequencing process and analysis of soil bacteria, fungi, and archaea has been described already by Ladau et al. (2018), Yang et al. (2017), and Shi et al. (2016), respectively. All the pertinent soil variables and location information in our study are given in Supplemental Table S1.

Network Construction

The microbial co-occurrence network was inferred from the Sparse Correlations for Compositional data (SParCC) (Friedman and Alm, 2012) correlation matrix constructed with the WGCNA package (Langfelder and Horvath, 2012). To reduce the rare OTUs in the data set, we removed those OTUs with relative abundances <0.01% of the total number of archaeal, bacte-

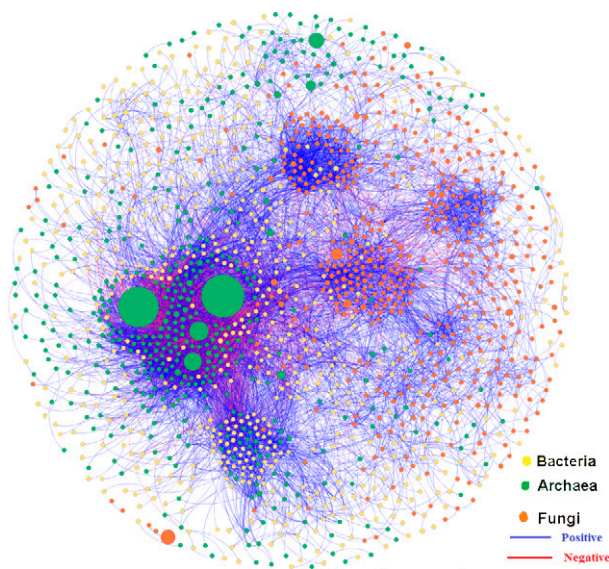


Fig. 1. Whole co-occurrence network of bacterial operational taxonomic units (OTUs), archaea OTUs, and fungal OTUs in the soils of the eastern Tibetan Plateau.

rial, and fungal sequences, respectively. The nodes in the network thus represent OTUs, while the edges connecting these nodes represent correlations between OTUs. We adjusted all P-values for multiple testing by applying the Benjamini-Hochberg false discovery rate (FDR) control procedure (Benjamini et al., 2006), as implemented in the “multtest” R package. Based on the obtained correlation coefficients and their FDR-adjusted P-values, we constructed the co-occurrence networks. The a priori cutoff for the FDR-adjusted P-values was set to 0.001.

Network properties were calculated with the “igraph” R package, and we generated network images with Gephi (<http://gephi.github.io/>) and Cytoscape (<http://www.cytoscape.org/>). Based on their roles in the network structure (Poudel et al., 2016), we defined the nodes as network hubs (z -score > 2.5; c -score > 0.6), module hubs (z -score > 2.5; c -score < 0.6), connectors (z -score < 2.5; c -score > 0.6) or peripherals (z -score < 2.5; c -score < 0.6). The algorithms used are based on investigative methods for metabolic networks (Guimera and Amaral, 2005). Network connectivity was used to convey the robustness of the network and it was estimated using the ‘attacking’ nodes in the static network (Peng and Wu, 2016). To confirm whether or not the generated network structure was randomly constructed, random networks with equal numbers of nodes and edges as the empirical network were generated, and the topological indices were summarized on the level of 999 iterations based on the Erdős-Rényi model in Supplemental Table S2.

Data Availability

All the soil bacterial, archaeal, and fungal sequences data have been deposited, respectively, in the DDBJ Sequence Read Archive under accession number DRA001226, in the European Molecular Biology Laboratory (EMBL) under accession number ERP009034, and in the European Nucleotide Archive under accession number PRJEB16010.

RESULTS

General Information about the Network

By applying the co-occurrence network analyses to the bacterial, fungal, and archaeal sequences data, we constructed the whole microbial network of Tibetan soils (Fig. 1), which contained 1827 nodes and 26 108 edges in all (Table 1). Other topological features of the network are shown in Supplemental Table S2. Importantly, there were more intra-kingdom links than inter-kingdom linkages (Supplemental Table S3), and most of these links were positive (Table 1; Supplemental Table S3). In particular, the archaea (15310 links) formed more connections than either the bacteria (11458 links) or fungi (8299 links) (Supplemental Table S3).

Role of Different Kingdoms in the Network

The Z-C scatterplot of all OTUs in the entire network showed the relative role of soil bacterial, fungal, and archaeal groups in its construction (Fig. 2A). The bacteria (13 nodes) and archaea (8 nodes) had more module hubs than did fungi (1 node), while the fungi (6) had more network hubs than either bacteria (2) or archaea (2) (Supplemental Fig. S2). In terms of connectors, the bacteria (161) formed more nodes than the archaea (112) or fungi (77). Generally, the hubs and connectors were mainly Proteobacteria (bacteria), Crenarchaeota (archaea), and Ascomycota (Fungi) at the phylum level (Supplemental Table S4).

Relationship between Network Topological Features and Environmental Factors

To investigate the relationships of hubs and connectors to soil variables, network links were built (Fig. 2B). In total, we obtained 350 connectors, 19 module hubs, and 10 network hubs (Supplemental Table S5); however, the connectors built many more links (750) with the soil variables than did network hubs (7 links) and module hubs (19 links) (Supplemental Table S5). The network hubs were positively correlated with soil moisture, total nitrogen, and total carbon but were negatively correlated with MAP (mean annual precipitation). The module hubs were positively correlated with soil moisture, total carbon, and SOC. The connectors were most negatively correlated with soil pH

Table 1. Topological features of the soil microbial network of the eastern Tibetan Plateau.

	Nodes	Edges	Density	Transitivity	Degree	Node betweenness centrality	Transitivity	Positive	Negative
								— % —	
Bacteria	1827	26108	0.0076	0.365	19.7	1236.4	0.355	77.50	22.50
Archaea					45.7	631.1	0.439		
Fungi					25.9	1455.8	0.392		

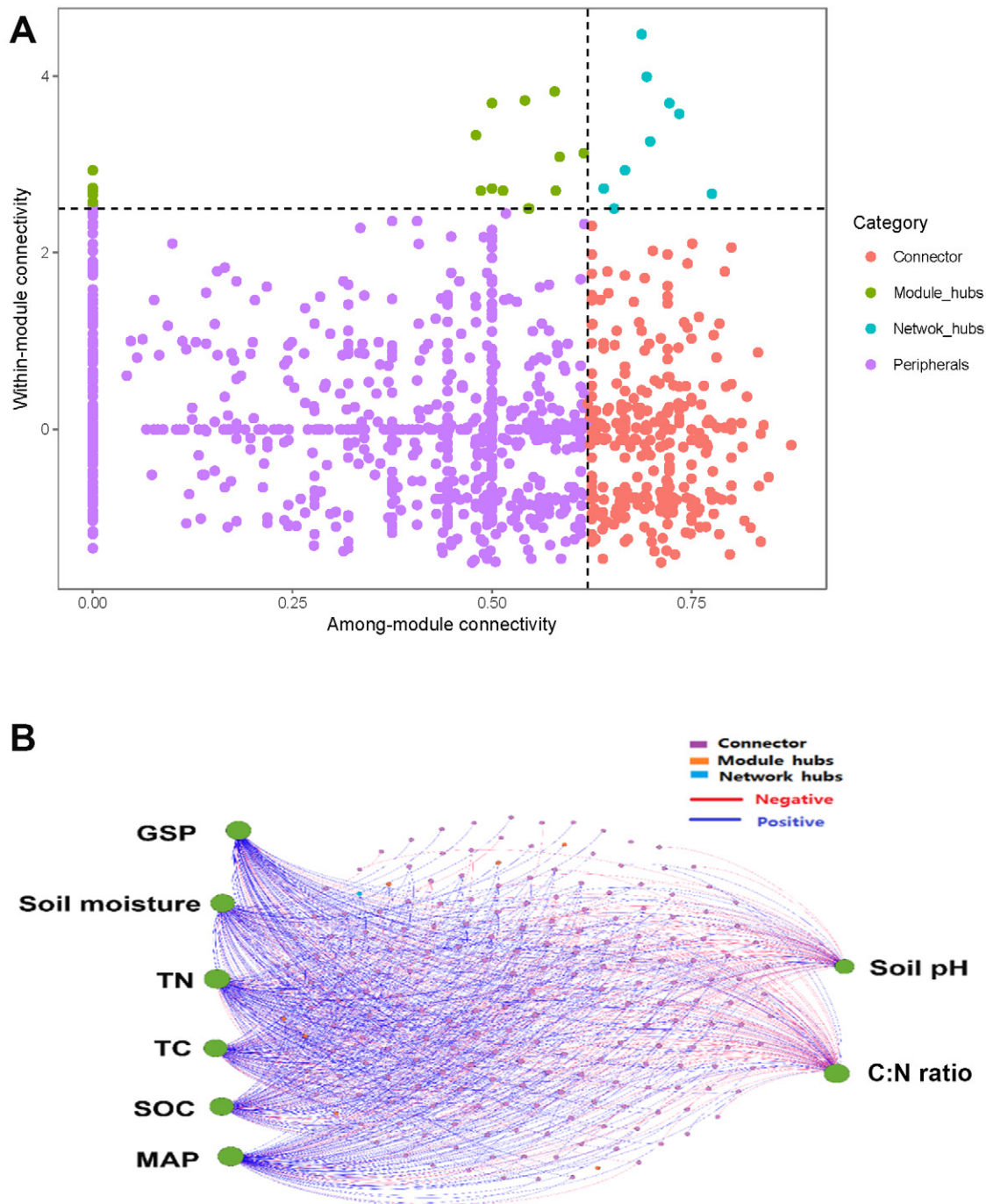


Fig. 2. Z-C plot distribution of the OTUs involved in the whole network (A), and its visualized links between soil and climatic factors, network hubs, and modular hubs and connectors (B). GSP, growing season precipitation; TN, total nitrogen; TC, total carbon; SOC, soil organic carbon; MAP, mean annual precipitation

and C to N ratio yet they were positively correlated with GSP, MAP, moisture, TN, TC, and SOC (Supplemental Table S5).

Relative Importance of Bacteria, Fungi, and Archaea in Constructing the Soil Microbial Network

To reveal the relative importance of soil bacteria, fungi, and archaea in building the entire microbial co-occurrence network, the correlation frequency (real links/possible links) between bacteria, archaea, and fungi was calculated (Table 2). With the archaea absent, the correlation frequency was lower than that with

fungi or bacteria absent from the network (Table 2). Further, the archaea had more within (intra-kingdom, 6.09%) and between (inter-kingdom, 1.13%) links than did bacteria (1.28% within; 0.99% between) and fungi (3.87% within; 0.52% between) (Table 2; Supplemental Table S5). At order level of archaea, there were 7734 links involving Nitrososphaerales (Supplemental Table S6). To verify the importance of archaea in constructing the microbial network, the network robustness (as expressed by natural connectivity) analysis was conducted. Although natural connectivity gradually decreased as more nodes of OTUs were removed, the

Table 2. The correlation frequency (i.e., real links/possible links) between bacteria, archaea, and fungi groups based on an analysis of their absence and presence in Tibetan Plateau soils.

Absence	Presence	Correlation rates			
		(real/possible)	Bacteria	Archaea	Fungi
		%			
Bacteria	Archaea	2.72	0	6.09	0.26
	Fungi			0.26	3.87
Archaea	Bacteria	1.26	1.28	0	0.17
	Fungi		0.17		3.87
Fungi	Bacteria	2.15	1.28	0.74	0
	Archaea		0.74	6.09	

connectivity values generated with the archaea absent were consistently lower than those with bacteria or fungi omitted (Fig. 3).

DISCUSSION

More Connections were found between Intra-kingdom Species

Microorganisms prefer to coexist through building complex ecological networks (Faust et al., 2015), since most cannot live independently and often survive depending on each other's extracellular metabolites (Pande and Kost, 2017). In this study, we found that intra-kingdom (within bacteria, archaea, or fungi) species built more connections than did inter-kingdom (between bacteria, archaea or fungi) species, suggesting that intra-kingdom OTUs can more easily form a mutualistic community. We also show that most connections between OTUs were positive in the network. Such positive connections are often observed in natural ecosystems (Faust et al., 2015), and perhaps not surprising, given the fact that the lives of microbes highly depend cross-feeding, co-aggregation, co-colonization, or niche overlap and construction (Kylafis and Loreau, 2011; Faust and Raes, 2012). Furthermore, we found the archaea as group had more connections than either bacteria or fungi in the eastern Tibetan soils. Archaea are known to have evolved means of persisting in barren and harsh environments (Eme et al., 2017), and the living conditions in soils of the Tibetan Plateau are challenging for microbes, due to its high elevation, low oxygen, high UV-light (ultraviolet radiation) and rapidly changing weather (Shi et al., 2016). Hence, it is reasonable to infer this tough environment promotes more intra-kingdom links for archaea than it would for bacteria and fungi.

Role of Nodes in the Network

In this study, the bacteria and archaea had more nodes related to module hub and connector functions, while the fungi had more nodes related to network hubs' function. The reason for this might be that fungi with hyphae have a stronger ability to connect nodes among and within a module (Klein et al., 2016), while the small size and short generation times of bacteria and archaea probably limit their range of communication or fix it in a local space (Fenchel and Finlay, 2004). Ascomycota are the largest phylum of fungi and have been found to widely distributed in soils (Fierer, 2017), and the mycelium its members develop may explain why this particular phylum had the ability of link-

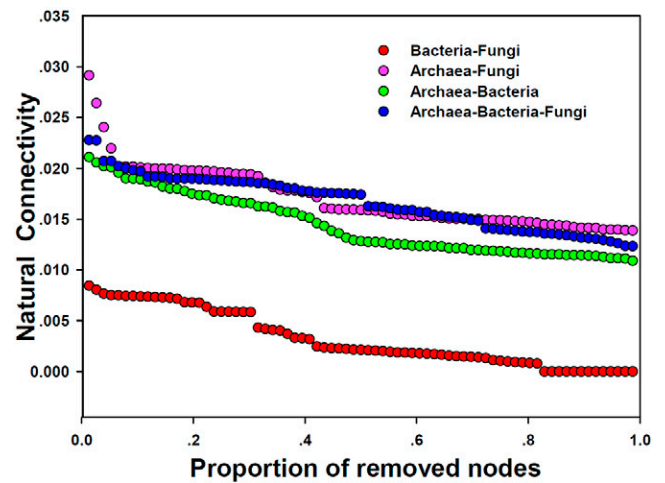


Fig. 3. Network robustness analysis of containing bacteria-fungi, archaea-fungi, archaea-bacteria, and archaea-bacteria-fungi microbial groups in soils of the eastern Tibetan Plateau.

ing nodes both within and between the modules in our study. The two bacterial nodes featuring a network hub function belonged to Acidobacteria and Gammaproteobacteria, which are commonly distributed in soil environments (Delgado-Baquerizo et al., 2018). Nitrososphaerales, which had most within connections in our microbial network, also presented evidence for network functions, again suggesting the importance of archaeal taxa in constructing the eastern Tibetan Plateau's soil network.

Relationships between the Network and Environmental Variables

It is believed that an understanding of these hubs and connectors in the network and the environmental variables that could influence can better guide our manipulations of soil microbial communities in natural ecosystems (Shade and Handelsman, 2012; Toju et al., 2018). In this context it would be useful to identify environmental factors affecting a network's topological features, because should the primary roles of these features become changed or disturbed, it may lead to community-level changes (Toju et al., 2018). We found that connectors had the most negative links with soil pH and C to N ratio, and the most positive links with GSP, moisture, TN, TC, SOC, and MAP. In prior work, we had reported that soil C to N ratio and soil pH were the main factors influencing bacterial and fungal community assembly (Jing et al., 2015; Yang et al., 2017; Ladau et al., 2018), while soil moisture and soil C to N ratio most influenced the archaeal communities (Shi et al., 2016) in Tibetan Plateau soils. Therefore, we suggest that soil pH and C to N ratio and moisture are key factors driving not only microbial co-occurrence network construction but also its resulting microbial community structure. For a tundra soil, Faust et al. (2015) found that a cluster in its microbial network containing mainly Alphaproteobacteria was positively correlated with pH, while a cluster containing mainly Acidobacteria was negatively correlated with soil pH. Similarly, Deng et al. (2012) found that soil pH and temperature contributed significantly toward determining the microbial co-occurrence network in a

long-term warming experiment. For a forest soil, Ma et al. (2016) the nodes' attribute value was correlated with soil pH, soil organic matter, soil nitrogen, and iron. Our study, however, is the first to investigate how nodes with different functional roles are correlated with environmental variables such as soil pH.

Although the relationship between soil C to N ratio and the connectors in soil microbial network has not been reported, it has been found that C to N ratio was a good predictor for the relative abundance of some individual taxa in soils (Delgado-Baquerizo et al., 2018). For example, strong negative correlations were found between the relative abundance of Crenarchaeota and soil C to N ratio in Amazon soils (Khan et al., 2019). In our study, the relative abundance of Actinobacteria and Proteobacteria also presented negative correlations with soil C to N ratio in Tibetan Plateau soils (Ladau et al., 2018). It has been also found that the higher relative abundance of Ascomycota could be explained by higher soil C to N ratios (Zhou et al., 2017). In our study, the phyla such as Crenarchaeota, Actinobacteria, Proteobacteria and Ascomycota were the dominant compartments of the connectors in the microbial network, which might be the reason why we found connectors had the most negative links with C to N ratio.

Soil Archaea Exhibited a Pivotal Role in Constructing the Ecological Network

A prominent finding of our study is that archaea exhibited a pivotal role in constructing the microbial co-occurrence network in Tibetan soils (Fig. 3). The archaea were characterized by topological functions because not only do they occupy a key position in the tree of life (Pace, 2006), but they also formed greater link numbers in the network compared with those of bacteria and fungi (Supplemental Table S6). Additionally, we observed very many links (7734) within Nitrososphaerales (Supplemental Table S7), probably due in part to their high relative abundance (>85%) in the study region's archaeal community (Shi et al., 2016). Recently, it was proposed that high abundances determined the functional role of microbes in complex communities (Rivett and Bell, 2018), which could explain why omitting Archaea dramatically affect the natural connectivity of the soil microbial network in our study. Further, Nitrososphaerales contains the genus Nitrososphaera, which has an ammonia oxidizing function and plays an important role in nitrogen cycling (Auguet et al., 2012; Shi et al., 2016). Due to the low temperatures and N limitation in Tibetan Plateau soils (Yang et al., 2008), it is reasonable to speculate more communications are favored among microbial species related to nitrogen cycling.

CONCLUSION

Intra-kingdom links exceeded inter-kingdom links in the microbial network of Tibetan Plateau soils, and their connectors had more correlations with the environmental variables. Compared with the bacteria and fungi, the archaea built more connections and without them network had a lowered natural connectivity, suggesting this group is vital for maintaining the microbial ecosystems in Tibetan Plateau soils. In the future,

large-scale culture-based studies could reveal the biological mechanisms of microbial interactions, thus deepening our understanding of how these three life domains respond to environmental changes in this cold region.

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SUPPLEMENTAL MATERIAL

Supplemental material is available with the online version of this article.

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