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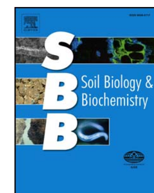

# Co-occurrence patterns of soybean rhizosphere microbiome at a continental scale

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ABSTRACT

Rhizosphere microorganisms play important roles in plant health and growth. The diversity and composition of rhizosphere microbial communities have been well studied, but little is known about their co-occurrence patterns, especially at a continental scale. Herein, we performed a network-based analysis using integrated bacterial and fungal community datasets to delineate the co-occurrence patterns of bulk soil and rhizosphere microbiome and the geographic patterns of network topological features in 51 soybean fields across China. Results showed that the microbial networks differed between bulk soil and rhizosphere in terms of structure and composition. Compared with the bulk soil networks, the rhizosphere networks had fewer links between bacteria and fungi, lower modularity, and smaller average path length; the global, southern and northern networks of rhizosphere showed similar, higher and lower complexity, respectively. The southern-specific networks of both bulk soil and soybean rhizosphere had more links between bacteria and fungi compared with the northern-specific networks. Additionally, the geographic patterns of network topological features differed between bulk soil and rhizosphere habitats, northern and southern regions. Bacterial sub-networks of both bulk soil and rhizosphere were most influenced by soil pH; fungal sub-networks were related to fewer environmental factors and most influenced by soil Mg content. Given that microbial networks may reflect interactions or niches shared among microorganisms, these results provide new insights into the organization of rhizosphere microbial communities.

## 1. Introduction

The rhizosphere is a zone of soil that directly surrounds plant roots; it provides a natural microhabitat for diverse microorganisms and thus has been considered as a hotspot of microbial diversity and activity in soils (Philippot et al., 2013). Rhizosphere microbial communities have a prominent impact on plant nutrition, disease suppression, and abiotic stress resistance (Berendsen et al., 2012; Mendes et al., 2011; Yang et al., 2009). Numerous studies have investigated the diversity and composition of rhizosphere microbial communities and their driving factors, including soil type, plant species, plant genotype, and developmental stage (Chaparro et al., 2014; Peiffer et al., 2013; Reinhold-Hurek et al., 2015). These studies have indicated the effects of selection via environmental factors on rhizosphere community structure and provided important insights into rhizosphere community assembly. The selection process comprises not only environmental variables but also interactions among microbial species (Nemergut et al., 2013). Microbial interactions may be more important than environmental variables in determining community structure (Chow et al., 2014; Lima-Mendez et al., 2015; Steele et al., 2011). However, the interactions among rhizosphere microorganisms at the community level are still far from fully understood.

In natural environments, microorganisms live together to form complex networks through positive (e.g., mutualism), negative (e.g., competition), and neutral (e.g., commensalism) interactions (Faust and Raes, 2012). Network analysis has been used to explore the interactions among microorganisms in various habitats, and non-random co-occurrence patterns were revealed as ubiquitous characteristics of microorganisms in soils (Barberan et al., 2012; Ling et al., 2016; Zhou et al., 2011), lakes (Kara et al., 2013), oceans (Chow et al., 2014; Cram et al., 2015), and human gut (Faust et al., 2012; Greenblum et al., 2012). Network analysis can provide additional information on microbial community ecology that cannot be obtained by traditional analytical approaches. The microbial community can generally be partitioned into modules that consist of highly interconnected microorganisms. Modularity has been interpreted as ecological niche overlap, habitat heterogeneity, and phylogenetic relatedness (Freilich et al., 2010; Olesen et al., 2007). Interestingly, microbial network structures are shaped by

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phylogeny and closely related taxa tend to co-occur more than expected by chance (Barberan et al., 2012; Ju et al., 2014). Additionally, network topological features (i.e., network complexity and modularity) change with environmental conditions (Deng et al., 2016; Ling et al., 2016; Wu et al., 2016) and thus can be used to define microbial communities. Owing to these advantages, network analysis has been considered as a powerful approach in microbial ecology (Zhou et al., 2011).

Mendes et al. (2014) compared microbial association networks in soybean fields based on samples of two different soil types in two years and found the rhizosphere network was less complex than the bulk soil network. In contrast, two recent studies using samples of biological replicates revealed more complex microbial networks in the rhizosphere of wild oat and *Jacobaea vulgaris* than in the bulk soil (Shi et al., 2016; Yan et al., 2017). Presently, it is unclear whether there are differences between the microbial networks of bulk soil and rhizosphere constructed using samples across a broad range of environmental gradients. Moreover, only bacterial communities were analyzed in the above three studies (Mendes et al.; Shi et al., 2016; Yan et al., 2017). Fungi have not been considered in the network analysis, despite their high diversity and essential ecological roles in the rhizosphere (Buée et al., 2009). Recently, Ma et al. (2016) investigated the co-occurrence networks of soil microbiota and revealed the biogeographic patterns of network topological features at a continental scale. However, previous research of rhizosphere microbial communities relied on samples from a small number of locations and thus was not robust to identify the geographic patterns of rhizosphere networks.

In the present study, we used CoNet inference (Faust et al., 2012) to construct integrated co-occurrence networks of bulk soil and rhizosphere microbiota with bacterial and fungal community datasets from 51 soybean (*Glycine max*) fields across China. We aimed to address the following questions: (i) Are the co-occurrence patterns of bulk soil and rhizosphere microorganisms different at a continental scale? (ii) What ecological factors drive the geographic patterns of network topological features, and do these ecological drivers and their contribution differ between habitats (bulk soil and rhizosphere), regions (northern and southern), and kingdoms (bacteria and fungi)?

## 2. Materials and methods

### 2.1. Study area and sampling

Bulk soil and rhizosphere samples were collected from 51 soybean fields across China at the flowering stage of soybean (Fig. S1). In each field, topsoil samples (0–20 cm) were taken randomly from five cores and pooled as one bulk soil sample. Then, 15–20 healthy plants were selected at random and removed from the soil. Roots were shaken gently to remove loose soil, cut off from the plants, and grouped into one sample per field. A subset of bulk soil samples was air-dried and analyzed for edaphic properties using standard soil testing procedures (Bao, 2000), including soil texture (clay, silt, and sand), pH, and organic carbon (OC), total nitrogen (TN), available nitrogen (AN), and macronutrient contents (available P, K, Mg, and Ca). Mean annual temperature (MAT), mean annual precipitation (MAP), potential evapotranspiration (PET), mean annual relative humidity (RH), aridity index (AI), and temperature and precipitation seasonality (TS and PS) were obtained from monthly weather data for 1981–2010. Short-term climatic factors, such as one-, two-, and three-month mean precipitation (SMP1-3) and mean temperature (MMT1-3) ranges were also calculated (Table S1).

### 2.2. DNA extraction, PCR application, and sequence analysis

The rhizosphere samples were prepared as described in Lundberg et al. (2012). Briefly, roots were placed in a sterile 50 mL tube containing 25 mL of sterile phosphate buffered saline solution (pH 7.0) and vortexed at the maximum speed for 15 s to remove the rhizosphere soil from the root surfaces. After the roots were removed, the washing buffer was filtered through a nylon mesh cell strainer into a clean tube. The turbid filtrate was centrifuged (3200 g, 15 min) and the resulting pellet was defined as the rhizosphere compartment. Total genomic DNA was extracted from soils. The V4 region of the bacterial 16S rRNA gene and the fungal internal transcribed spacer 2 region were amplified using primer pairs 515F/806R and ITS3-2024F/ITS4-2409R, respectively (Evans et al., 2014; Orgiazzi et al., 2012). Paired-end sequencing (250 bp) was performed on an Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA). A detailed description for DNA extraction, PCR amplification, sequencing, and processing was provided in the Supporting Information. The bacterial and fungal counts were normalised separately by the trimmed mean of M values (TMM) method in the R package EdgeR (Robinson et al., 2010). This approach simultaneously addresses the differences in library size and biological variability that cannot be detected by common microbiome normalization methods, such as rarefaction and proportions (McMurdie and Holmes, 2014). The raw 16S rDNA sequence data were deposited at the NCBI small read archive dataset under the study number SRP113347 with the run number SRR5859796–SRR5860093, and the raw internal transcribed spacer 2 sequence data under the study number SRP113348 with the run number SRR5859807–SRR5860103.

### 2.3. Network construction

Co-occurrence networks were constructed in CoNet v. 1.0.6 beta (Faust and Raes, 2016) using the Spearman's correlation and Kullback-Leibler dissimilarity (KLD) measures (Lima-Mendez et al., 2015). Bacterial or fungal OTUs that occurred in < 20% (Ju et al., 2014) of the 51 sites and had a sum relative abundance < 0.01% (Ma et al., 2016) in all bulk soil or rhizosphere samples were removed from the network analysis. After this filtering step, a total of 2499 OTUs (1705 bacterial and 794 fungal) in the bulk soil and 1585 OTUs (1072 bacterial and 513 fungal) in the rhizosphere were obtained. To make the bulk soil and rhizosphere networks comparable, the same numbers of OTUs (1072 bacterial and 513 fungal) in the bulk soil were selected based on their relative abundances. Additionally, the 51 sites were separated into northern (26) and southern (25) regions (Fig. S1), and 1062 bacterial and 397 fungal OTUs in each region and habitat were selected to construct region-specific networks. To test whether the choice of the normalization technique impacts the results, networks were also constructed using OTU data normalised by the rarefaction method. For this purpose, the OTU matrices were rarefied to 25,571 and 958 sequences per sample for bacteria and fungi, respectively.

When constructing networks, we first computed the KLD between all OTU pairs; then, we set the dissimilarity threshold to the maximum value of the KLD matrix and the Spearman's correlation threshold to 0.7. For each edge and measure, permutation and bootstrap distributions were generated with 1000 iterations. Measure-specific $P$ value was computed as the area of the mean of the permutation distribution under a Gauss curve generated from the mean and standard deviation of the bootstrap distribution. The $P$ values were merged using Brown's method (Brown, 1975) and then adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Finally, only edges supported by two measures and with adjusted $P$-values below 0.05 were retained. The nodes in the constructed networks represent OTUs and edges represent strong and significant correlations between OTUs. Meanwhile, 1000 Erdös-Réyni random networks in an equal size were constructed as real networks for bulk soil and rhizosphere (Erdős and Rényi, 2012). A set (13) of network topological properties (e.g., degree, modularity, betweenness centrality, and average path length) were calculated for both observed and random networks in the R package igraph (Csardi and Nepusz, 2006). Network visualization were conducted using Gephi (Bastian et al., 2009) and Cytoscape 3.5.1 (Shannon et al., 2003).

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