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Distinct biogeographic patterns of rhizobia and non-rhizobial endophytes associated with soybean nodules across China

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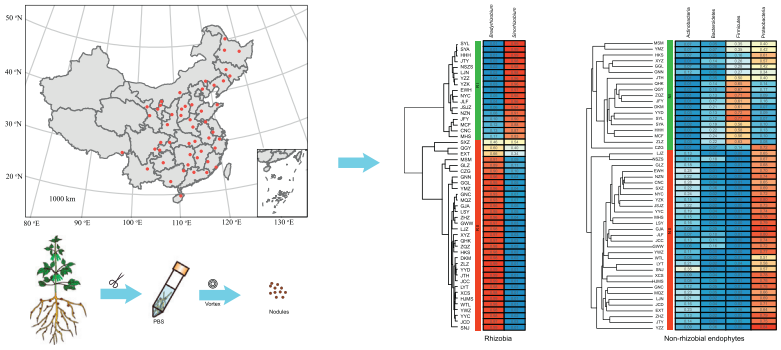
HIGHLIGHTS

- Proteobacteria and Firmicutes dominated non-rhizobial subcommunity.
- Rhizobia and non-rhizobial endophytes displayed distinct biogeographic patterns.
- Non-rhizobial endophytes had a lower dispersal probability than rhizobia.
- Rhizobia and non-rhizobial endophytes grouped separately in association network.

GRAPHICAL ABSTRACT



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ABSTRACT

Both rhizobia and non-rhizobial endophytes (NRE) are inhabitants of legume nodules. The biogeography of rhizobia has been well investigated, but little is known about the spatial distribution and community assemblage of NRE. By using high-throughput sequencing, we compared biogeographic patterns of rhizobial and non-rhizobial subcommunities and investigated their bacterial co-occurrence patterns in nodules collected from 50 soybean fields across China. Dispersal probability was lower in NRE than in rhizobia, as revealed by a significant distance-decay relationship found in NRE, but not in rhizobia, in addition to a significant occupancy–abundance relationship in the entire community. Rhizobial and NRE subcommunities were significantly influenced by different environmental and spatial variables. Moreover, the rhizobial subcommunities were grouped into *Ensifer*- and *Bradyrhizobium*-dominated clusters that were significantly related to soil pH. The non-rhizobial subcommunities were grouped into Proteobacteria- and Firmicutes-dominated clusters that were more influenced by climatic than by edaphic factors. These results demonstrated that rhizobial and non-rhizobial subcommunities are characterized by distinct biogeographic patterns. Network analysis showed rhizobia and NRE as separately grouped and uncorrelated with each other, suggesting they did not share niche space in soybean nodules. In sum, these results broaden our knowledge of how bacteria are distributed and assemble as a community in root nodules.

1. Introduction

Many leguminous plants have the ability to establish binary symbiosis with some diazotrophic bacteria, collectively referred to as rhizobia. These rhizobia induce the formation of root nodules where biological

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nitrogen fixation occurs. Rhizobia have been found in Alphaproteobacterial genera (*Rhizobium*, *Ensifer*, *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Devosia*, *Azorhizobium*, *Allorhizobium*, and *Shinella*) and Betaproteobacterial genera (*Burkholderia* and *Cupriavidus*) (Peix et al., 2014). Together with rhizobia, a great diversity of endophytic bacteria, including those in the genera *Bacillus*, *Pseudomonas*, *Enterobacter*, *Chryseobacterium*, and *Sphingobacterium*, have since been detected inside legume nodules (De Meyer et al., 2015; Leite et al., 2016). Because these bacteria cannot induce nodules or perform biological nitrogen fixation they are called non-rhizobial endophytes (NRE) (Martínez-Hidalgo and Hirsch, 2017). NRE members can provide beneficial services to their host plants, such as plant growth promotion (Tariq et al., 2014), abiotic stress resistance, pathogen protection, as well as nodulation enhancement (Martinez-Hidalgo et al., 2014).

A fundamental goal in community ecology is to understand the factors that determine community distribution patterns. Many studies have explored the biogeographic patterns of rhizobia associated with several plant species, including the common bean (Cao et al., 2014; Verastegui-Valdes et al., 2014; Wang et al., 2016), soybean (Li et al., 2011; Yan et al., 2018; Zhang et al., 2011), cowpea (Chidebe et al., 2018), alfalfa (Donnarumma et al., 2014), and *Caragana* species (Lu et al., 2009). This work has revealed the influence of key environmental factors, such as precipitation, soil nutrient availability and soil pH, on the distribution of rhizobia. Although the literature is rich with studies of NRE's genetic diversity and potential roles (reviewed by Peix et al., 2014), just a few have investigated the spatial distributions of NRE associated with wild legumes, such as the genera *Sphaerophysa salsula* (Deng et al., 2011), *Caragana jubata* and *Oxytropis ochrocephala* (Xu et al., 2014), as well as the subfamily Faboideae (De Meyer et al., 2015), and all these studies relied on culture-dependent approaches and were conducted at regional scales. Hence, the full extent of NRE diversity remains unexamined and our knowledge of NRE biogeographic patterns at larger (i.e., continental) spatial scales is quite limited. Furthermore, biogeographic patterns of rhizobia and NRE have yet to be investigated in the same legume host species. Given the non-symbiotic roles of NRE, we hypothesized that rhizobia and NRE display distinct biogeographic patterns.

Microorganisms in natural ecosystems usually form complex ecological networks through direct (e.g., mutualism and competition) or indirect (e.g., environmental preferences) interactions (Faust and Raes, 2012). Characterizing the interactions among microorganisms—also called co-occurrence patterns—is crucial for better understanding their potential functions or ecological niches (Ju et al., 2014; Steele et al., 2011). Network analysis offers a powerful tool for studying the co-occurrence patterns of microbial communities, as demonstrated by its recent application to various complex environments, such as humans (Faust et al., 2012), oceans (Lima-Mendez et al., 2015), activated sludge (Ju et al., 2014), soils (Ma et al., 2016) and the plant rhizosphere (Fan et al., 2017; Shi et al., 2016). This work has revealed interesting co-occurrence patterns in microbial communities, such as non-random associations, highly connected modules (de Menezes et al., 2015), and relationships between functional groups (Bissett et al., 2013). However, co-occurrence patterns are poorly understood in nodule bacterial communities.

Soybean (*Glycine* max) is a major legume crop grown globally. It originated in China, where it widely cultivated (Li et al., 2008), which provides an excellent opportunity to study the biogeographic and co-occurrence patterns of its nodule bacterial communities at a continental scale. In this study, high-throughput sequencing technology was used to investigate the nodules' bacterial community composition in 50 soybean fields across China. We also used Molecular Ecological Network Analysis (Deng et al., 2012) to construct co-occurrence network for the nodule-dwelling bacteria. Our main objectives were (i) to determine and compare the biogeographic patterns of rhizobia and NRE in soybean nodules; and (ii) to investigate the co-occurrence patterns among bacterial taxa in these nodules.

2. Materials and methods

2.1. Sample collection and preparation

A total of 50 soybean fields across China (Fig. S1) were selected from which to collect soil and plant samples. All the fields were cultivated under conventional agricultural practices, in which chemical fertilizer and pesticide use is permitted yet organic, manure, or compost fertilizers were not used. Samples were collected in all fields at the flowering stage of soybean (May–August 2015). The methods we used for soil and root sampling, characterization of soil physicochemical properties, and climate data collection are described in detail by Zhang et al. (2018). Briefly, in each field, five topsoil samples (0–20 cm) were randomly from a ~100 m^2 plot and pooled as one bulk soil sample. A total of 15–20 randomly selected healthy plants were removed from the soil using a spade. Roots were gently shaken to remove loose soil, combined as one root sample per field. A subset of each soil sample was air-dried for an analysis of its physicochemical properties according to the standard protocols described by Bao (2000), namely soil pH (soil/water = 1:5, w/v), texture, organic matter (OC), total (TN) and available nitrogen (AN), and available phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg). Information on the fields' geographic coordinates, soybean cultivars, and soil and climate factors are provided in Supplementary Table S1.

Roots were placed in a sterile 50-mL tube containing 25 mL of a sterile phosphate-buffered saline solution (PBS, per litre: 6.33 g $NaH_2PO_4 \cdot H_2O$, 16.5 g $Na_2HPO_4 \cdot 7H_2O$, 200 μL Silwet L-77, pH 7.0), and vortexed at the maximum speed for 15 s to remove the rhizosphere soil from the root surfaces. Then the cleaned roots were transferred to a new sterile 50-mL tube with 25 mL of the sterile PBS buffer, and vortexed; this step was repeated until the PBS buffer appeared clear after vortexing. Next, the roots were moved into a new sterile tube and sonicated at low frequency for 5 min (five 30-s bursts, followed by five 30-s rests) to dislodge any attached microbes and to further clean the root exterior surface. Finally, the roots were removed and rinsed in a fresh volume of 25-mL PBS buffer. The efficacy of these procedures for removing microbes from soybean nodule surfaces had been confirmed in our recent study (Xiao et al., 2017).

2.2. DNA extraction, sequencing, and analysis

Nodules were taken from each root sample, and approximately 500 mg of healthy nodules were ground in liquid nitrogen using mortar and pestle. Their total DNA was then extracted using the FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, USA) following the manufacturer's instructions. We used the primers 515F (5′-GTGCCAGCMGCCGCG GTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012) to amplify the V4 region of the bacterial 16S rRNA gene, by following the PCR protocols described in a recent study of ours (Zhang et al., 2018). Paired-end (250 bp) sequencing was conducted on an Illumina HiSeq 2500 instrument at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

Raw sequence data were analyzed using QIIME (Caporaso et al., 2010), and the paired-end sequences merged using FLASH (Magoc and Salzberg, 2011). Those sequences with a length <200 bp, an average quality <25, or containing ambiguous bases were removed. Quality-filtered sequences were then clustered into operational taxonomic units (OTUs) based on 97% similarity using UPARSE (Edgar, 2013). Taxonomic annotation of each OTU was performed by the Ribosomal Database Project (RDP) Classifier (Wang et al., 2007) with the Greengenes database. All OTUs annotated as chloroplast or mitochondria were removed from the OTU matrix table, and singleton OTUs (containing only one sequence) were also removed to avoid possible biases. To correct for sequencing effort across samples, the OTU table was rarefied to 25,571 sequences per sample. Rhizobial OTUs were defined here as taxa that belonged to the genera *Bradyrhizobium*, *Ensifer*, *Rhizobium*, and