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Distribution of fungal endophytes in roots of *Stipa krylovii* across six vegetation types in grassland of northern China

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ABSTRACT

To explore the biogeographical patterns of endophytic fungal communities on a large scale, we surveyed fungal endophytes in roots of *Stipa krylovii* from six vegetation types in grassland along a 3200 km west–east transect in northern China. Pyrosequencing of samples collected from 18 sites (three sites per vegetation type) revealed that Pleosporales, Hypocreales, Agaricales, and Xylariales were the dominant fungal orders in roots of *S. krylovii*. The dominant genera were *Marasmius*, *Fusarium*, *Acremonium*, *Sarcinomyces*, and *Monosporascus*, and these genera were distributed differently among the six vegetation types. In a variation partitioning analysis, vegetation type, geographical distance, and environmental parameters (mean annual precipitation and air temperature, soil organic carbon, soil total nitrogen, pH, elevation) explained 98.2% of variation in the endophyte fungal community, and environmental parameters explained more variation than did vegetation type or geographical distance. Mean annual precipitation was the major significant factor influencing endophytic fungal communities.

1. Introduction

Endophytic fungi may grow asymptomatically within plants, and have a close interaction with the host plant. They may provide benefits to their host by promoting plant growth and improving resistance to abiotic and biotic stresses, such as drought, salinity, pathogens, and herbivores (Pańka et al., 2013; Thom et al., 2013; Hall et al., 2014; Ma et al., 2015; Lledó et al., 2016). Despite their important roles, little is known about the ecology and evolution of endophytic fungi.

Many factors affect the composition and distribution of endophytic fungal communities. The host species strongly affects the diversity and abundance of endophytic fungi (Zhang and Yao, 2015; Solis et al., 2016). The distribution of endophytic fungi in individual host plants also varies with environmental conditions (Fujimura and Egger, 2012; Zimmerman and Vitousek, 2012), geographical location (Herrera et al., 2010; Langenfeld et al., 2013), and season (Guo et al., 2008; Martins et al., 2016). Recently, the pyrosequencing technique has been used in several endophyte ecology studies (Peršoh, 2013; Zhang and Yao, 2015; Tahtamouni et al., 2016) and may reveal greater fungal diversity compared with traditional isolation methods.

Stipa is one of the most common and widely distributed herbaceous grassland plants in northern China, and it contributes greatly to the ecology and economy of these grasslands. However, little is known about the distribution of the endophytic fungal communities in roots of *Stipa* within various habitats. In this study, we collected *Stipa krylovii* samples from six grassland vegetation types along a 3200 km west–east transect in northern China. We hypothesized that the endophytic fungal community in the roots of *S. krylovii* would differ greatly among different vegetation types due to the effects of environmental conditions and geographical distance. The aims of this study were: (1) to investigate the diversity and distribution of fungal endophytes in roots of *S. krylovii* across six grassland vegetation types, using the MiSeq high-throughput method; and (2) to analyze factors affecting the distribution of fungal endophytes in roots of *S. krylovii* in Chinese grasslands.

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2. Materials and methods

2.1. Sampling sites

The roots of *S. krylovii* were sampled from six grassland vegetation types along a 3200 km west–east transect across the Gansu and Inner Mongolia provinces of northern China in July 2012 (Fig. 1). The six vegetation types from west to east were alpine meadow (AM), subalpine meadow (SM), Gobi desert (GD), desert steppe (DS), typical steppe (TS), and meadow steppe (MS) (Table 1). The climate in these areas is predominantly arid and semiarid continental. The mean annual precipitation (MAP) ranged from 61 to 392 mm, and mean annual air temperature (MAT) from −4.5 to 6.1 °C. The MAP and MAT at each sampling site were determined from the WorldClim database (Hijmans et al., 2005). For each vegetation type, we selected three sites dominated by *S. krylovii* that were at least 500 m apart. Ten roots of *S. krylovii* were randomly sampled at each site, and corresponding soils were collected from around the root samples. The soil was mixed thoroughly and pooled as one composite sample for chemical analyses of soil from each site. The geographical coordinates and elevation of each site were recorded by GPS. Soil samples were sieved through a 2-mm sieve and air-dried for soil property analysis. Root samples of *S. krylovii* were stored at −4 °C.

2.2. Analysis of soil properties

A 10 g subsample of soil was used to determine the pH value in water (1:2.5 soil/water ratio). A subsample was ground in a ball mill and further sieved through a 0.25-mm screen for determining soil organic C (SOC) and total nitrogen (TN). As described by Harris et al. (2001), the soil subsamples for determining SOC were washed with 150 ml 0.5 M HCl to remove carbonate before analysis, and subsamples without HCl treatment were used to determine the TN concentration. The SOC and TN concentrations were measured using an elemental analyzer (2400 II CHN elemental analyzer, Perkin-Elmer, Norwalk, CT, USA).

2.3. Root sample preparation

All roots were surface sterilized to remove surface microorganisms. The roots were rinsed in deionized H_2O, and then sequentially immersed in 75% (vol/vol) ethanol (1 min), 5% $NaClO_3$ (5 min), and 75% ethanol (30 s). Finally, the roots were washed four times in sterile distilled water for 1 min each time. Aliquots (100 μl) of the final wash were plated onto potato dextrose agar to confirm the effectiveness of disinfestation treatments. The samples confirmed to be successfully surface-sterilized were used for DNA extraction.

2.4. DNA extraction, amplification, and sequencing

The surface-sterilized samples were cut into small pieces using sterile scissors under sterile conditions and then ground in liquid N to a fine powder with a sterilized mortar and pestle. Then, DNA was extracted from 0.5 g samples using a QIAGEN DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The purity and concentration of extracted DNA was determined using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA). The ITS1F/ITS2 primer set targeting the ITS1 region was used for amplification (White et al., 1990; Gardes and Bruns, 1993). The reverse primer had a 6 bp barcode unique to each sample. The PCR procedures including the composition of the reaction mixture and the thermal profile were as described by Procter et al. (2014). After successful amplification, PCR products were pooled in equimolar concentrations and subjected to high-throughput sequencing on the Illumina MiSeq platform at Novogene Bioinformatics Technology Co. Ltd (Beijing, China). Raw sequence data have been deposited in the NCBI Sequence Read Archive under accession no. SRP090652.

2.5. Bioinformatic and statistical analyses

Sequences were analyzed with QIIME software. Briefly, low-quality reads were filtered and discarded by QIIME quality filters using the default settings. Sequencing reads were assigned to each sample according to the unique barcode, and then barcodes and primers were removed. Pairs of reads were merged using FLASH. Chimeric sequences were removed using USEARCH software. The sequence reads were clustered into operational taxonomic units (OTUs) defined at 97% sequence similarity, and singleton OTUs (with only one read) were removed. The representative OTU sequences were classified taxonomically through blasting against the UNITE fungal ITS database. After removing non-fungal OTUs, a rarefied dataset down-sampled to the smallest number of reads per sample was obtained, and an OTU table was converted into a suitable input file for alpha and beta analyses.

We calculated OTU richness, Chao1, Fisher's α, and Shannon indices to compare endophytic fungal alpha diversities among vegetation types. A hierarchical clustering heatmap was built for the top 20 classified genera. Both analyses were performed using R version 3.2.3 (R Development Core Team, 2015). Differences in diversity indices among vegetation types were tested by analysis of variance (ANOVA) using SPSS v.19 software (SPSS Inc. Chicago, IL,

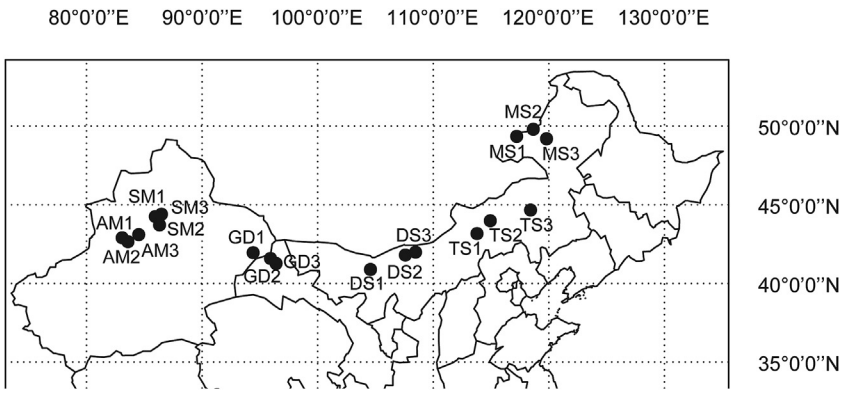

Fig. 1. Map of the study area showing the location of sampling sites.