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Diversity of nitrogen-fixing, ammonia-oxidizing, and denitrifying bacteria in biological soil crusts of a revegetation area in Horqin Sandy Land, Northeast China

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ABSTRACT

The nitrogen cycle affects ecosystem stability. The occurrence of biological soil crusts (BSCs) in a desertified region significantly affects the nitrogen cycle. BSCs are gradually forming on the sand surface of a sand-fixing plantation in semi-arid Horqin Sandy Land, Northeast China. However, the composition of microbial nitrogen-cycling communities remains unknown. This study aimed to investigate the diversities of nitrogen-fixing, ammonia-oxidizing, and denitrifying bacteria in BSCs of a sand-fixing plantation. Soil samples were obtained from three plantations, namely, *Caragana microphylla* Lam., *Hedysarum fruticosum*, and *Artemisia halodendron*. The bacterial community was characterized by 16S rDNA sequence analysis. Denaturing gradient gel electrophoresis (DGGE), cloning, and sequencing were used to determine the diversities of *nifH*, ammonia-oxidizing 16S rRNA, and *nosZ* genes. Results of 16S rDNA gene phylogenetic analysis indicate that the phyla Proteobacteria, Acidobacteria, Bacteroidetes, and Actinobacteria were dominant groups. Samples from the three plantations displayed similar DGGE profiles for the three genes, suggesting that plantation type had no influence on the dominant population of nitrogen-cycling bacterial communities. Most sequences of *nifH* formed a distinct cluster that branched with known *Azotobacter* diazotrophs. Sequence analysis of the bacterial 16S rRNA gene showed that the ammonia-oxidizing community comprised uncultured *Nitrosomonas* species and *Nitrosospira* members affiliated with *Nitrosospira* cluster 3. Although *nosZ* genes belong to three subclasses of Proteobacteria, almost half are closely affiliated with *Pseudomonas* of Gammaproteobacteria. This study demonstrated that the dominant phylum in BSCs was Proteobacteria.

1. Introduction

Biological soil crusts (BSCs) are ubiquitous assemblages of microorganisms, lichens, mosses, and liverworts. They are more frequently found in plant interspaces and may constitute ≥70% of living covers in arid and semi-arid regions (Belnap and Lange, 2003). BSCs are able to stabilize soil surface, increase fertility, provide resistance to wind erosion, and affect runoff infiltration in arid ecosystems, thereby enhancing soil quality. Therefore, BSC rehabilitation can facilitate the restoration of a degraded ecosystem (Bowker, 2007). Cyanobacteria components of BSCs that are responsible for nitrogen and carbon fixation are well documented (Belnap and Lange, 2003). In addition, the microbial diversity of non-phototrophic bacteria (Gundlapally and Garcia-Pichel, 2006; Nagy et al., 2005), Archaea (Nagy et al., 2005; Soule et al., 2009) and Fungi (Bates and Garcia-Pichel, 2009) present in BSCs have been examined. Studies have shown that many factors such as elevation, soil, and vascular plant community structure influence the species composition of BSCs (Belnap and Lange, 2003). To our knowledge, almost all pertinent studies focus on the microbial communities in BSCs developing in natural environments. By contrast, studies on artificially established plantations in a revegetation area are limited. In desertified areas, shrub or semi-shrub plantations are generally used to restore degraded sand dunes that successfully resulted in the occurrence of species-rich BSCs (Bowker, 2007). Thus, a survey of microbial diversity of BSCs in

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arid or semi-arid areas is necessary to further understand the roles of BSCs in the restoration of a degraded ecosystem.

The Horqin Sandy Land, one of the four sandy lands, is located in the semi-arid agropastoral steppe of northern China. The landscape was historically characterized by an extended forest steppe; however, in recent decades the lands have undergone severe desertification resulting from overgrazing, excessive and intense farming, as well as vegetation devastation by fuel wood gathering (Zhao et al., 2000). Since the 1980s, revegetation has been widely adopted to control desertification and restore the ecological function of degraded ecosystems (Su et al., 2005). Some vascular plants adaptive to sandy environments such as *Artemisia halodendron*, *Caragana microphylla*, and *Hedysarum fruticosum* have been densely planted (usually spaced 1 or 2 m) as sand binders on moving or semi-moving sandy land to abate wind-induced soil erosion and improve the environments in this region. A large revegetation area was gradually formed. Subsequently, BSCs as an indicator of soil health (Bowker, 2007) formed on the surface of sand dunes. Some previous studies have shown the effects of plantations on microclimate, sand-fixation, soil nutrients, and soil enzymatic activities (Cao et al., 2000; Su et al., 2005; Zhao et al., 2007). However, the effect of revegetation on soil microflora composition and diversity is poorly understood. Furthermore, high density vegetations have some adverse effects that result in scarcity of soil water needed during growth (Alamusa et al., 2006; Fu et al., 2001). Therefore, we assume that microbial communities in BSCs under high-density plantations may differ from those under natural sparse vegetations.

Soil microbes involved in nitrogen transformation in BSCs under revegetation plantations should be paid more attention because nitrogen limits primary productivity in arid and semi-arid lands (Evans and Ehlering, 1994), and nitrogen cycles are important for soil fertility and prevention of desertification. Diazotrophs contribute nitrogen to soils by fixing atmospheric nitrogen as ammonia, which is the main nitrogen source in arid and semi-arid areas. Diazotrophic organisms are phylogenetically diverse groups (Zehr et al., 2003). In BSCs, Cyanobacteria and Cyanolichens are important nitrogen fixers in desert ecosystems (Belnap and Lange, 2003). Diazotrophs contain genes encoding the reductase subunit of nitrogenase, *nifH*. *NifH* gene as a molecular mark has been used to explore uncultivated diazotrophic microorganisms in a natural environment. In the case of BSCs, the crust diazotrophic community mainly comprises *Nostoc* spp. and heterocystous Cyanobacteria, and the composition of N_2-fixing species remains stable between poorly developed and mature crusts of BSCs under the Colorado Plateau and Chihuahuan Desert (Yeager et al., 2004).

Ammonia oxidation the limiting step in nitrification is performed by ammonia-oxidizing organisms belonging to ammonia-oxidizing bacteria (AOB) of the beta subdivision of the Proteobacteria (Stephen et al., 1996) and Archaea (Koenneke et al., 2005; Treusch et al., 2005). The most frequently used molecular marker for studying the diversity of AOB is the 16S rRNA gene, followed by *amoA*, a portion of ammonia monooxygenase genes (Junier et al., 2010). 16S rRNA gene reportedly provides higher resolution than *amoA* (Purkhold et al., 2003). Phylogenetic analysis of 16S rRNA gene shows that at least seven distinctive subclusters of the Betaproteobacteria ammonia oxidizers exist, with four belonging to genus *Nitrosospira* and three belonging to genus *Nitrosomonas* (Stephen et al., 1996, 1998; McCaig et al., 1999). Although AOB are well studied in various environments, limited information is known about arid and semi-arid ecosystems. Only one recent study has reported on the occurrence of AOB during rainfall that evoked the nitrogen cycle (Orlando et al., 2010).

Similar to AOB, denitrifying bacteria in an arid environment are less studied. Denitrification involving the reduction of soil nitrate and nitrite to gaseous compounds (NO, N_2O, and N_2) is mediated by microorganisms under anaerobic or oxygen limited conditions, resulting in N loss. High rates of denitrification were detected in arid BSCs (Abed et al., 2013). Denitrifying bacteria are derived from about 50 genera, mostly from the Proteobacteria (Rich and Myrold, 2004). Populations having nitrous oxide reductase gene (*nosZ*) catalyze the final step, i.e., nitrous oxide reduction to N_2. *NosZ* gene is frequently analyzed to study denitrifying bacteria from environmental samples (Dandie et al., 2007; Magalhães et al., 2008; Rich and Myrold, 2004; Rösch et al., 2002).

The complexity of soil microbial communities has provoked scientists to apply molecular techniques for studying the ecology of soil microbial communities. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified genes is not time consuming, low cost, and able to directly determine the nucleotide sequence of bands. DGGE is commonly used to evaluate microorganism diversity (Nakatsu, 2007). The present study aimed to understand microbial community structure of BSCs in artificially established plantations. Accordingly, bacterial community composition and communities involved in nitrogen-cycling bacteria were investigated by 16S rDNA analysis and DGGE coupled with cloning and sequencing.

2. Materials and methods

2.1. Study area description

This study was conducted at the Wulanaodu Experimental Station of Desertification (43°02′N, 119°39′E), Chinese Academy of Sciences, Inner Mongolia Autonomous Region. The station is located in Western Horqin Sandy Land, Northeast China. This region has a temperate semi-arid climate, i.e., dry and windy in winter and spring, warm and rainy in summer, and cool in autumn. The mean precipitation is 340.5 mm, with the majority of the precipitation occurring from May to September. The annual mean open-pan evaporation is about 2500 mm. The annual mean temperature is 6.3 °C, with an absolute maximum of 39 °C and an absolute minimum of −29.3 °C. The soils are cambric arenosols and degraded sand.

C. microphylla Lam., *H. fruticosum*, and *A. halodendron*, the shrub and subshrub species adaptive to sandy and arid soil, were planted usually spaced 1 or 2 m on moving or semi-moving sandy land around Wulanaodu with the aid of straw checkerboards beginning in the 1980s. The checkerboard was composed of 1 m × 1 m squares made of straw. The experimental site was enclosed after seeding. After several years, BSCs develop on the sand surface. The oldest plantation was 28 years old at the time of study. In the growing season, several annual plants (e.g., *Salsola collina*, *Bassia dasyphylla*, *Corispermum thelegium*, *Chenodium acuminatum*, and *Setalia viridis*) were randomly distributed in the glade and under the shrub crown, and the vegetative cover was less than 10%. The mean heights and crown diameters were 1.5 m and 1.2 m × 1.2 m for *C. microphylla*, 0.9 m and 2 m × 3 m for *H. fruticosum*, as well as 0.5 m and 0.5 m × 0.6 m for *A. halodendron*, respectively. Therefore, BSCs were mostly present under the plant canopies (Fig. 1).

2.2. Soil sampling and chemical analysis

Soils were sampled in August, 2011. Crust samples from 28-year-old *C. microphylla* Lam., *H. fruticosum*, and *A. halodendron* plantations (designated as CM, HF, and AH, respectively) were collected. In each plantation, three plots (size 10 m × 10 m) (as three replicates) were set up for sampling. In each plot, 10 subsamples were randomly collected and mixed into one sample. Roots were manually removed, and all samples were sieved (2 mm). The samples were immediately frozen at −70 °C for molecular analysis,