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# High diversity of nitrogen-fixing bacteria in upper reaches of Heihe River, Northwestern China

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## Abstract

Vegetation plays a key role to water conservation in southern Qilian Mountains (Northwestern China), the upper reaches of Heihe River. Nitrogen-fixing bacteria are crucial for vegetation protection because they can supply plants with nitrogen source. Nevertheless, little is known about nitrogen-fixing bacteria in this region. In present study, nifH gene clone libraries were established for detecting the difference of nitrogen-fixing bacterial communities between *Potentilla parvifolia* shrub and *Carex alrofusca* meadow in the southern Qilian Mountains. All the identified nitrogen-fixing bacterial clones belonged to Proteobacteria. At the genus level, the *Azospirillum* sp. was only detected in shrub soil while *Thiocapsa* sp., *Derxia* sp., *Ectothiorhodospira* sp., *Mesorhizobium* sp., *Klebsiella* sp., *Ensifer* sp., *Methylocella* sp. and *Peseudomonas* sp. were just detected in meadow soil. Shannon–Wiener index of nifH gene ranged from 1.5 to 2.8 and was higher in meadow soil than shrub soil. Contrarily, the nifH gene copies and CFUs of cultured nitrogen-fixing bacteria ranged from $0.4 \times 10^7$ to $6.9 \times 10^7$ copies $g^{-1}$ soil and $0.97 \times 10^6$ to $12.78 \times 10^6$ $g^{-1}$ soil, respectively. Furthermore, both of them were lower in meadow soil than shrub soil. Statistical analysis revealed that diversity and copies of nifH gene mostly correlated with aboveground biomass in shrub soil. In meadow soil, nifH gene diversity was principally affected by altitude while copies did by soil available K.


## 1 Introduction

Biological nitrogen fixation is considered to be an important nitrogen input in many terrestrial environments and is fundamental to the long term productivity of vegetation, particularly in those without any chemical fertilizer such as alpine ecosystems (Izquierdo and Nüsslein, 2006; Zhang et al., 2006). Nitrogen-fixing bacteria are responsible for nearly a third of the biologically fixed nitrogen in these ecosystems (Izquierdo and Nüsslein, 2006). Hsu and Buckley (2009) found that the variation in nitrogen-fixing





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bacterial community structure has a greater impact on nitrogen fixation rates rather than soil characteristics. Nitrogenase is the enzyme responsible for nitrogen fixation while nifH is the gene that encodes for the iron protein subunit of nitrogenase and is highly conserved among all nitrogen-fixing groups, making it an ideal molecular marker for these microorganisms (Deslippe and Egger, 2006). Cloning and sequencing of nifH gene have provided a large, rapidly expanding database of nifH sequences from diverse environments (Zehr et al., 2003), such as polar and cold soils (Olson et al., 1998; Deslippe and Egger, 2006; Zhang et al., 2006), aquatic habitats (Steward et al., 2004; Moisander et al., 2008; Hamilton et al., 2011), agricultural soils (Coelho et al., 2009; Zou et al., 2011). However, the diversity of nitrogen-fixing bacteria was still poorly described and many microorganisms remain to be discovered (as reviewed in Gaby and Buckley, 2011).

Heihe River Basin is the second-largest inland river basin in the arid regions of northwestern China, which consists of three major geomorphic units: the southern Qilian Mountains, the middle Hexi Corridor, and the northern Alxa Highland (Wang et al., 2009). The southern Qilian Mountains located in upper reaches of Heihe River Basin is hydrologically and ecologically the most important unit, since it functions as the water source that supports agricultural irrigation in the Hexi Corridor and maintains ecological viability in the northern Alxa Highland (Ma and Frank, 2006). However, a recent global-scale analysis by Cleveland and Liptzin (2007) on soil nitrogen indicated that the soil of alpine ecosystems in Qinghai-Tibetan Plateau (including the Qilian Mountains) was mostly limited in nitrogen. Furthermore, just like boreal forest and arctic tundra soils, 95 % of which is organic nitrogen and nitrogen in senescent leaves and they are more difficult for plant to absorb (Xu et al., 2006; Courtney and Harrington, 2010; Jiang et al., 2012). Therefore, studying on diversity of nitrogen-fixing bacteria is needed in such the nitrogen limited regions as the southern Qilian Mountains.

In this study, we examined the nitrogen-fixing bacterial communities associated with *Potentilla parvifolia* shrub and *Carex alrofusca* meadow, which were dominant vegetation types in the southern Qilian Mountains. The objectives were (1) to determine diver-





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sity and abundance of nitrogen-fixing bacteria, (2) to detect the difference of nitrogen-fixing bacterial communities in shrub and meadow soils.

## 2 Materials and methods

### 2.1 Study area and soil samples

Heihe River basin lies between latitude 37° 41′ N and 42° 42′ N and longitude 96° 42′ E and 102° 00′ E and has a drainage area of $14.29 \times 10^4$ $km^2$. The upper reaches of Heihe River basin are located on the north slopes of the Qilian Mountains (Li et al., 2001). Precipitation in the Qilian Mountains is concentrated during the summer and tends to decrease from east to west and to increase with altitude, from approximately 200 mm at low altitudes to 600 mm at high altitudes. Vegetation types are varied in the region and include, from low to high altitudes, desert steppe, drying shrubbery grassland, forest grassland, sub-alpine shrubbery meadow and alpine cold-and-desert meadow (Wang et al., 2009). In the present study, 6 sampling sites along an altitude gradient of 3086 m to 4130 m in one typical valley (Binggou) were selected. At each site, we collected 5 soil samples from each of the 3 quadrats; the samples were mixed together, yielding 3 total samples from each site. The samples were cooled on ice until delivery to the laboratory and further processing. The sample locations, soil physico-chemical properties and vegetation information are given in Table 1.

### 2.2 Biogeochemical properties of the soil

Soil water content, pH, organic C and total N were measured by methods as described previously (Liu et al., 2012). The soil organic matter was determined by loss on ignition while available P and K were measured following $NH_4OAc$ extraction as described previously (Qian et al., 1994) The salt content was calculated as the sum of cations and anions.





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### 2.3 Extraction and purification of DNA from soil

DNA was extracted from three subsamples of 0.51 g from each soil sample using the PowerSoil DNA Isolation Kit (MoBio Inc., Carlsbad, CA, USA) as per manufacturer's instructions.

### 2.4 PCR amplification of the nifH gene fragment

The selected primers nifH-F and nifH-R (5′-AAAGG(C/T)GG(A/T)ATCGG(C/T)AA (A/G)TC CACCAC-3′ and 5′-TTGTT(G/C)GC(G/C)GC(A/G)TACAT(G/C)GCCATCAT-3′) were used to amplify 457 bp nifH gene fragments from the soil DNA. PCR was run on a Sure Cycler 8800 (Agilent Technologies). The PCR amplification conditions were performed exactly as described previously (Rösch et al., 2002). To avoid potential sample biases and to obtain enough PCR products for cloning, three replicate amplifications were carried out for each sample.

### 2.5 Cloning and restriction fragment length polymorphism (RFLP) analysis

The PCR products were purified with a TIANquick Midi Purification Kit (TIANGEN BIOTECH CO., LTD., BEIJING) and cloned into the pMD 18-T vector (TaKaRa Biotechnology Co., Ltd., Dalian) according to the manufacturer's protocols. The plasmids were transformed into competent *E. coli DH5α* by the heat shock method. All white colonies were picked and screened for the desired gene inserts, which were detected with specific primers of M13F/M13R for pMD 18-T vector.

Unique clones were detected by RFLP analysis with two restriction enzymes (Alu I and Hae III). Enzyme digestion and gel electrophoresis of the digested products were performed as described previously (Zhou et al., 2002). The RFLP patterns were analyzed and the resulting clusters were validated visually by comparing the clusters with gel images.



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### 2.6 Sequencing and phylogenetic analysis

To understand the phylogenetic diversity, representative nifH clones of the unique RFLP patterns as determined by cluster analysis were sequenced. A total of 74 nifH clones were sequenced. The nucleotide acid identity of all the sequenced clones were compared by Contig Express software and compared to the Gen Bank database using BLAST software to get the representative which most closely related to the nifH sequences in the database. A phylogenetic tree was constructed using the Neighbour–Joining method with ClustalX (version 1.8) and Mega (version 4.0) software.

### 2.7 Q-PCR analysis of nifH gene

The abundance of nifH gene in shrub and meadow soils was measured using Q-PCR with SYBR green. Primer sequences to amplify fragment of the nifH gene were nifH-F and nifH-R. Standard for measuring the quantity of nifH gene was developed from typical clone known to contain the gene. Plasmid DNA preparation was obtained from the clone by using a Qiagen Miniprep kit (Qiagen, Germantown, MD, USA). The copy number of the target gene in a nanogram of plasmid DNA was determined, and then a serial dilution was prepared from $10^8$ to $10^2$ copies to use as an external standard curve ($r^2 > 0.9$), allowing determination of nifH copy number in each sample of soil DNA.

Q-PCR of nifH was performed in a 10 µL reaction mixture that contained 5 µL of SYBR Green PCR master mix (2 ×) (Applied Biosystems, Foster City, CA, US), 0.4 µL of each primer (10 µM), 3.2 µL of sterile ultrapure water and 1 µL of extracted DNA (∼ 10–25 ng. Amplification was performed in a Stratagene MX3005. The Q-PCR cycling parameters were 30s at 95°, 40 cycles of 95° for 5s, 55° for 30s and 72° for 30s, followed by a dissociation stage of 95° for 30s, 55° for 30s, then a ramp up to 95°.

All soil samples and the standards were assayed in triplicate, the fluorescence signal was used to calculate $C_T$ (cycle threshold) values, using the thermocycler software for




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each machine. The copy number of nifH gene per gram of soil was determined by comparison to a standard curve of $10^2 \sim 10^8$ gene copies in the assay.

### 2.8 Cultivation of nitrogen-fixing bacteria

Ashby medium (Beauchamp et al., 2006) was used to isolate cultured nitrogen-fixing bacteria, CFUs and diversity of which were calculated based on plate count and RFLP analysis of 16S rDNA.

### 2.9 Statistical methods

Coverage of each clone library was calculated with Eq. (1), $n$ was the number of OTUs with unique clone, $N$ was the total clone numbers (Table 1). Cloned genotypes were screened for similarity by restriction fragment length polymorphism (RFLP) analysis. According to genotypes of OTUs and clone numbers of each OTU based on RFLP-clone library approach, Shannon–Wiener diversity index of nifH gene was calculated as Eq. (2), $n_i$ was clone numbers of the $i$-th OTU (Duc et al., 2009). The abundance of nifH gene was examined by using Q-PCR. The standard curve of Q-PCR was Eq. (3) $R$ and efficiency were 0.996 and 105.1 % respectively. Hierarchical Cluster and Correlation analysis were performed using SPSS (version 16.0) software and Canoco (version 4.5) software, respectively. Hierarchical Cluster analysis was used to cluster or separate sites, which were similar or different, based on the characteristics of nitrogen-fixing bacterial community for each site. Correlation analysis was applied to investigate the environmental factors that affected the characteristics of nitrogen-fixing bacterial communities in the Qilian Mountains.

$$[1 - (n/N)] \times 100\% \quad (1)$$

$$H = -\sum p_i \ln p_i (p_i = n_i/N) \quad (2)$$

$$Y = -3.205 \times \log(X) + 36.78 \quad (3)$$

## 2.10 Nucleotide sequence accession numbers

The Gen Bank accession numbers are as follows: KC445661-KC445735.

# 3 Results

## 3.1 Characteristics of nitrogen-fixing bacterial community

Cultured nitrogen-fixing bacterial numbers (CFUs) and diversity ($H_{\text{cultured}}$) are showed in Fig. 1, and CFUs ranged from 0.97 to 12.78 ($10^6$ $g^{-1}$ soil) while $H_{\text{cultured}}$ did from 0.2 to 1.4, both of them were lower in meadow soil than shrub soil along the altitude gradient.

The nitrogen-fixing bacterial community in meadow soil showed higher Shannon–Wiener diversity index ($H_{\text{nif}}$) than that in shrub soil, and $H_{\text{nif}}$ increased from 1.5 to 2.8 with elevating altitude. Figure 2 reveals that abundance of nifH gene (Copies) was more abundant in shrub soil than meadow soil and ranged from 0.4 to 6.9 ($10^7$ $g^{-1}$ soil).

Cluster analysis on nitrogen-fixing bacterial communities in shrub and meadow soil according to their characteristics (CFUs, $H_{\text{cultured}}$, Copies and $H_{\text{nif}}$) showed that nitrogen-fixing bacterial communities derived from shrub soil clustered together while the ones derived from meadow soil clustered. The result of cluster analysis depicted in Fig. 3 indicated that nitrogen-fixing bacterial communities derived from the same soil type (shrub soil or meadow soil) were more similar.

## 3.2 Phylogeny of nitrogen-fixing bacteria

Figures 4 and 5 reveal that some sequences of nifH gene showed close similarity to identified nitrogen-fixing bacteria, but some nifH sequences clustered without any identified diazotrophy species derived from GenBank. Among the identified nitrogen-fixing bacterial clones, the ones affiliated to *Bradyrhizobium* sp., *Sinorhizobium* sp., *Burkholderia* sp., *Dechloromonas* sp. and *Herbaspirillum* sp. settled in both shrub





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and meadow soils while *Bradyrhizobium* sp. was the dominant genus. Table 2 reveals that *Azospirillum* sp. just appeared in shrub soil while *Thiocapsa* sp., *Derxia* sp., *Ectothiorhodospira* sp., *Mesorhizobium* sp., *Klebsiella* sp., *Ensifer* sp., *Methylocella* sp. and *Peseudomonas* sp. existed in meadow soil only. All the identified nitrogen-fixing bacterial clones belonged to Proteobacteria. The $\alpha$ and $\beta$ subgroup existed in both shrub and meadow soil, while the $\gamma$ subgroup just appeared in meadow soil.

### 3.3 Correlations between nitrogen-fixing bacterial community and environmental factors

Figure 6 reveals that in the shrub soil, nifH gene diversity and copies mostly correlated with aboveground biomass of vegetation. The CFUs of cultured nitrogen-fixing bacteria was principally affected by underground biomass. Altitude affected diversity of nifH gene in the meadow soil, whose copies mostly correlated with soil available K. Cultured nitrogen-fixing bacterial diversity was affected by soil water content while CFUs correlated with aboveground biomass.

## 4 Discussion

A high diversity of nifH gene was observed in the southern Qilian Mountains, with a 2.85 value of Shannon–Wiener index, higher than other cold regions, such as Antarctic soil (Jungblut and Neilan, 2010; Niederberger et al., 2012), Arctic soil (Deslippe and Egger, 2006; Izquierdo and Nüsslein, 2006) and Tibetan Plateau alpine soil (Zhang et al., 2006). The Q-PCR results also supported the conclusion. In our study, abundance of nifH gene was $10^7$ copies $g^{-1}$ soil (Fig. 2), which was slightly higher than the previously published results. Niederberger et al. (2012) found that proteobacterial nifH genes ranged from $10^4$ to $10^5$ copies $g^{-1}$ soil in Antarctic wetted soil. Coelho et al. (2009) observed $10^5$ to $10^7$ copies $g^{-1}$ soil of nifH genes in Brazil farmland soil. High diversity and abundance of nifH gene in the southern Qilian Mountains may be because most





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of nitrogen-fixing bacteria have a soil pH optimum close to seven (Tamm, 1991), which was in line with soil pH in the southern Qilian Mountains. The results are depicted in Table 3. Furthermore, the primers nifH-F and nifH-R used in this study, targeting a wide range of nitrogen-fixing bacteria, were different from other studies (Rösch et al., 2002; as reviewed in Kizilova et al., 2012).

Cluster analysis on nitrogen-fixing bacterial communities in shrub and meadow soil showed that the communities derived from same soil type (shrub soil or meadow soil) were more similar. The results are depicted in Fig. 3. Ramette and Tiedje (2007) indicated that samples spatially closer to each other, regardless of their location in the geographic range, tended to support more similar bacterial populations. Additionally, the similarities of soil-inhabiting bacteria could still be detected in the presence of patches of homogeneous plant species. In the southern Qilian Mountains, shrub soil has a lower OTUs and diversity of nitrogen-fixing bacteria than meadow soil. Contrarily, Table 1 reveals that a higher vegetation biomass was observed in shrub soil than in meadow soil. Hsu and Buckley (2009) also found that high vegetation biomass reduced nitrogen-fixing bacterial diversity. Additionally, disturbance will promote diversity of nitrogen-fixing bacteria (Duc et al., 2009). Meadow soil located at higher altitude undergoes more disturbance than shrub soil where carbon and nitrogen availability is higher while moisture and temperature fluctuation is less due to physical protection by the dense surrounding vegetation.

Five major clusters with homology to nifH have been described, cluster I is composed entirely of nifH genes from most Proteobacteria, all Cyanobacteria and certain Firmicutes (Paenibacillus) and Actinobacteria (Frankia); cluster II contains sequences belonging to certain methanogenic Archaea; cluster III is dominated by nifH sequences from anaerobic members of the Bacteria and Archaea including: spirochetes, methanogens, acetogens, sulfate-reducing bacteria, green sulfur bacteria and clostridia; clusters IV and V are composed of nifH paralogues that are not involved in nitrogen fixation and include genes of various functions (Zehr et al., 2003; Palacios and Newton, 2005). Figures 4 and 5 reveal that in this study, all the identified nifH se-





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quences belonged to Proteobacteria and fell into Cluster I, which was consistent with previous studies. Gaby and Buckley (2011) concluded that the most dominant OTUs of nifH belonged to either the Proteobacteria or Cyanobacteria. Chowdhury et al. (2009) demonstrated that the nifH clone library showed a predominance of Proteobacteria in the rhizosphere soil and roots of *Lasiurus sindicus* grass. However, the predominant genera in different extreme environments were distinct (Table 3), among which *Bradyrhizobium* sp. was peculiar to shrub and meadow soils in the southern Qilian Mountains. Furthermore, at the genus level, the *Azospirillum* sp. was only detected in shrub soil while *Thiocapsa* sp., *Derxia* sp., *Ectothiorhodospira* sp., *Mesorhizobium* sp., *Klebsiella* sp., *Ensifer* sp., *Methylocella* sp. and *Peseudomonas* sp. were just detected in meadow soil. One possibility is that plants may select for different nitrogen-fixing bacterial communities (Deslippe and Egger, 2006). The dominance of a native alpine species (e.g. *Potentilla parvifolia* or *Carex alrofusca*) may have controlled soil carbon dynamics through changes in root colonisation and exudation, which, in turn, may have resulted in shifts in nitrogen-fixing bacterial community structure (Bardgett et al., 1998; Gros et al., 2004). Another possibility is that natural selection occurs at distinct locations for nitrogen-fixing bacterial populations, which could originate from the structural complexity and heterogeneity of the habitats considered (Ramette and Tiedje, 2007).

In meadow soil, nifH gene diversity mostly correlated with altitude, which was in line with previous studies. Zhang et al. (2006) found that altitude was one of the key factors influencing nitrogen-fixing bacterial community in alpine prairie soil. Niederberger et al. (2012) demonstrated that temperature which was closely related with altitude may be a driving factor in defining composition of nitrogen-fixing bacterial community in Antarctic soil. Diversity of nifH gene in shrub soil was principally affected by vegetation biomass. The result was also consistent with the previously published results. Hsu and Buckley (2009) found that nitrogen-fixing bacterial community structure differed across the treatments as a function of vegetation biomass in farmland soil. Lovell

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et al. (2001) demonstrated that nitrogen-fixing bacterial community are closely associated with vegetation biomass of *Spartina* in marsh soil.

In this study, we compared levels of nitrogen-fixing bacterial community diversity in shrub and meadow soils in the southern Qilian Mountains, the upper reaches of Heihe River, by analyzing cultivated nitrogen-fixing bacterial isolates and nifH genes amplified from extracted soil DNA. Although cultivation and nifH gene cloning analyses gave contradictory descriptions of nitrogen-fixing bacterial diversity for shrub and meadow soils, the two methods identified qualitatively consistent relationships when levels of abundance (CFUs and copies) were compared. Both methods consistently indicated that nitrogen-fixing bacterial community in meadow soil was distinct from the one in shrub soil. Our data illustrate that while nifH gene cloning and cultivation generally describe similar relationships between nitrogen-fixing bacterial communities in shrub and meadow soils, significant discrepancies can occur.

## 5 Conclusions

In the present study, culture-dependent method, clone library and Q-PCR were applied to analyze communities of nitrogen-fixing bacteria in the southern Qilian Mountains. A high diversity of nifH gene was observed in the study region, with a 2.85 value of Shannon-Wiener index, higher than other cold regions. The Q-PCR results also supported the conclusion. Abundance of nifH gene was $10^7$ copies $g^{-1}$ soil, which was slightly higher than the previously published results. High diversity and abundance of nifH gene may be because most of nitrogen-fixing bacteria have a soil pH optimum close to seven, which was in line with soil pH in the study region. In our study, all the identified nifH sequences belonged to Proteobacteria and fell into Cluster I, which was consistent with previous studies. Plants may select for different nitrogen-fixing bacterial communities. Cluster analysis on nitrogen-fixing bacterial communities in shrub and meadow soil showed that the communities derived from same soil type (shrub soil or meadow soil) were more similar. At the genus level, the *Azospirillum* sp. was only





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detected in shrub soil while *Thiocapsa* sp., *Derxia* sp., *Ectothiorhodospira* sp., *Mesorhizobium* sp., *Klebsiella* sp., *Ensifer* sp., *Methylocella* sp. and *Peseudomonas* sp. were just detected in meadow soil. Among which Bradyrhizobium sp. was predominant to shrub and meadow soils. In meadow soil, nifH gene diversity mostly correlated with altitude while the one in shrub soil was principally affected by vegetation biomass. All the methods consistently indicated that nitrogen-fixing bacterial community in meadow soil was distinct from the one in shrub soil.

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**Table 1.** Site description, soil physicochemical properties, vegetation information and characteristics of clone libraries.

| | | Site1 | Site2 | Site3 | Site4 | Site5 | Site6 |
|---|---|---|---|---|---|---|---|
| Site description | Latitude | 38° 07′ 60″ | 38° 03′ 51″ | 38° 03′ 60″ | 38° 03′ 54″ | 38° 01′ 48″ | 38° 00′ 57″ |
| | Longitude | 100° 10′ 29″ | 100° 15′ 56″ | 100° 12′ 57″ | 100° 12′ 52″ | 100° 13′ 60″ | 100° 14′ 12″ |
| | Altitude $m^{-1}$ | 3086 | 3205 | 3377 | 3602 | 3802 | 4130 |
| Soil physicochemical properties | TOC $g kg^{-1}$ | 163.85 | 132.48 | 105.6 | 134.06 | 157.12 | 16.85 |
| | TN $g kg^{-1}$ | 5.6 | 9.5 | 10.1 | 1.9 | 7.9 | 5.4 |
| | OM $g kg^{-1}$ | 282.47 | 228.39 | 182.05 | 231.11 | 270.87 | 29.26 |
| | pH | 6.89 | 6.97 | 7.71 | 6.43 | 6.36 | 6.61 |
| | Soil water content $g kg^{-1}$ | 406.8 | 518.7 | 233.5 | 362.2 | 489.5 | 337.5 |
| | Available P $mg kg^{-1}$ | 8.14 | 4.26 | 7.30 | 3.24 | 2.08 | 2.82 |
| | Available K $mg kg^{-1}$ | 82.7 | 178.2 | 153.3 | 108.2 | 88.0 | 70.1 |
| | Salt $g kg^{-1}$ | 8.93 | 14.88 | 9.15 | 9.12 | 8.36 | 5.88 |
| Vegetation information | $H_{vegetation}$ | 1.940 | 1.305 | 1.157 | 1.847 | 1.587 | 2.010 |
| | Aboveground biomass $g (m^2)^{-1}$ | 212.50 | 362.50 | 517.50 | 312.50 | 135.00 | 145.00 |
| | Underground biomass $g (0.1 m^3)^{-1}$ | 1734.0 | 3740.00 | 1853.0 | 1037.00 | 1785.00 | 51.00 |
| | Cover degree % | 60 | 65 | 92 | 70 | 70 | 15 |
| | Dominant plant community | *Potentilla parvifolia* shrub | *Potentilla parvifolia* shrub | *Potentilla parvifolia* shrub | *Carex alrofusca* meadow | *Carex alrofusca* meadow | *Carex alrofusca* meadow |
| Characteristics of clone libraries | *n* | 3 | 1 | 1 | 3 | 1 | 5 |
| | *N* | 17 | 20 | 24 | 45 | 76 | 74 |
| | Coverage % | 82.35 | 95 | 95.83 | 93.33 | 98.68 | 93.24 |
| | OTUs | 6 | 6 | 6 | 11 | 11 | 22 |





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**Table 2.** Comparison of nitrogen-fixing bacterial community composition in shrub and meadow soil.

| | Shrub soil | Meadow soil |
|---|---|---|
| Phylum | $\alpha$-, $\beta$-proteobacteria, unidentified cluster (44 %) | $\alpha$-, $\beta$-, $\gamma$-proteobacteria, unidentified cluster (68 %) |
| Genera | *Bradyrhizobium* sp. ⋆[a] | *Bradyrhizobium* sp. ⋆ |
| | *Sinorhizobium* sp. | *Sinorhizobium* sp. |
| | *Burkholderia* sp. | *Burkholderia* sp. |
| | *Dechloromonas* sp. | *Dechloromonas* sp. |
| | *Herbaspirillum* sp. | *Herbaspirillum* sp. |
| | ***Azospirillum* sp.**[b] | ***Thiocapsa* sp.** |
| | | ***Derxia* sp.** |
| | | ***Ectothiorhodospira* sp.** |
| | | ***Mesorhizobium* sp.** |
| | | ***Klebsiella* sp.** |
| | | ***Ensifer* sp.** |
| | | ***Methylocella* sp.** |
| | | ***Peseudomonas* sp.** |

[a] The black pentagram marked the predominant genus in shrub and meadow soils.
[b] The generic names in bold marked the peculiar genera to each soil.





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**Table 3.** Comparison of nitrogen-fixing bacterial communities in different regions.

| Study regions | Average soil pH | Shannon index | Phylum | Predominant genera | Cites |
|---|---|---|---|---|---|
| Antarctica wetted soil | 8.48 | 2.60 | $\alpha$-Proteobacteria, $\beta$-Proteobacteria, $\gamma$-Proteobacteria, $\delta$-Proteobacteria, Cyanobacteria | *Geobacter* sp. | Niederberger et al. (2012) |
| Antarctica microbial mat | 9.90 | 2.11 | $\beta$-Proteobacteria, $\gamma$-Proteobacteria, $\delta$-Proteobacteria, Cyanobacteria, Firmicutes, Spirochaetes, Verrumicrobia, Unidentified cluster | *Azotobacter* sp. | Jungblut et al. (2010) |
| Canadian High Arctic shrub soil | ND | 2.04 | $\alpha$-Proteobacteria, $\beta$-Proteobacteria, $\gamma$-Proteobacteria, Firmicutes, Unidentified cluster | *Rhodopseudomonas* sp. | Deslippe et al. (2006) |
| Arctic tundra soil | 6.70 | 1.97 | $\alpha$-Proteobacteria, $\gamma$-Proteobacteria, $\delta$-Proteobacteria, Cyanobacteria, Spirochaetae, Unidentified cluster | *Rhodopseudomonas* sp. | Izquierdo et al. (2006) |
| Tibetan Plateau Alpine prairie soil | 7.43 | 2.09 | $\alpha$-Proteobacteria, $\beta$-Proteobacteria, $\gamma$-Proteobacteria, $\delta$-Proteobacteria, Unidentified cluster | *Methylocella* sp. | Zhang et al. (2006) |
| Qilian Mountains shrub and meadow soils | 7.04 | 2.85 | $\alpha$-Proteobacteria, $\beta$-Proteobacteria, $\gamma$-Proteobacteria, Unidentified cluster | *Bradyrhizobium* sp. | In this study |





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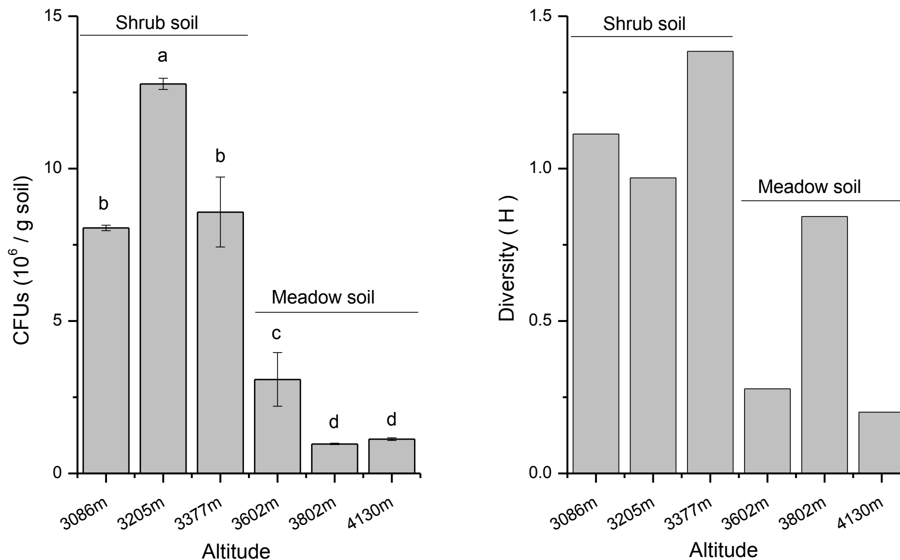


**Fig. 1.** CFUs and diversity of cultured nitrogen-fixing bacteria.





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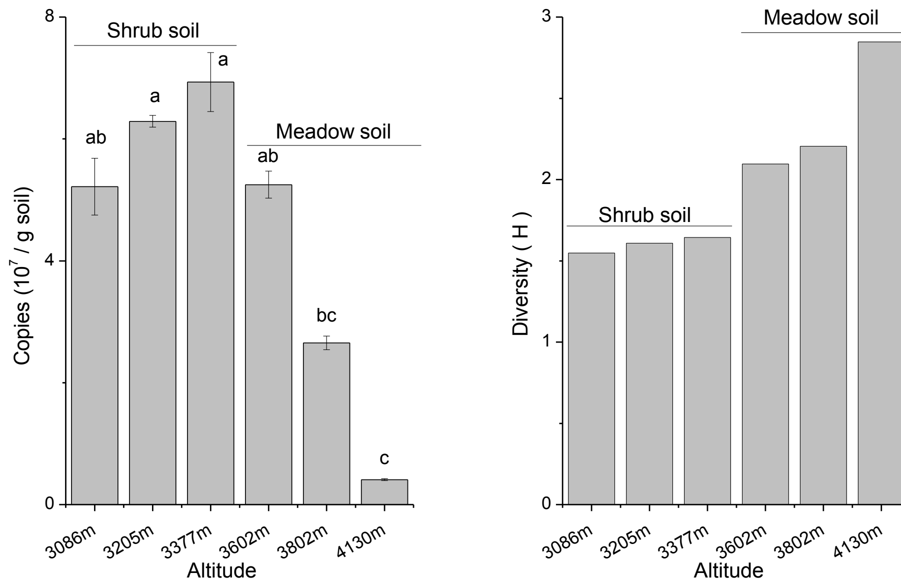


**Fig. 2.** Copies and diversity of nifH gene.





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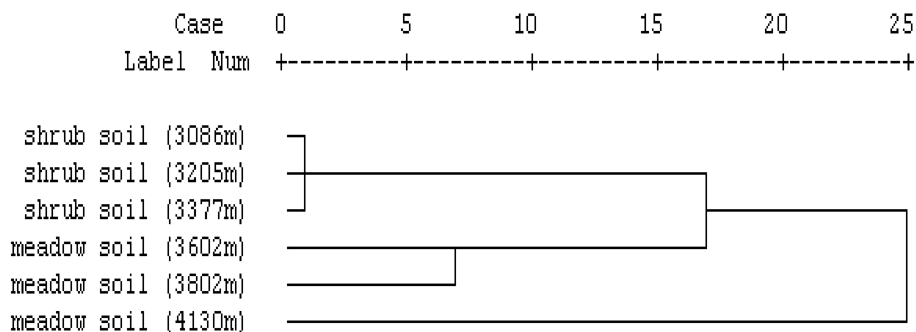


**Fig. 3.** Hierarchical cluster analysis of nitrogen-fixing bacterial communities in the study region.





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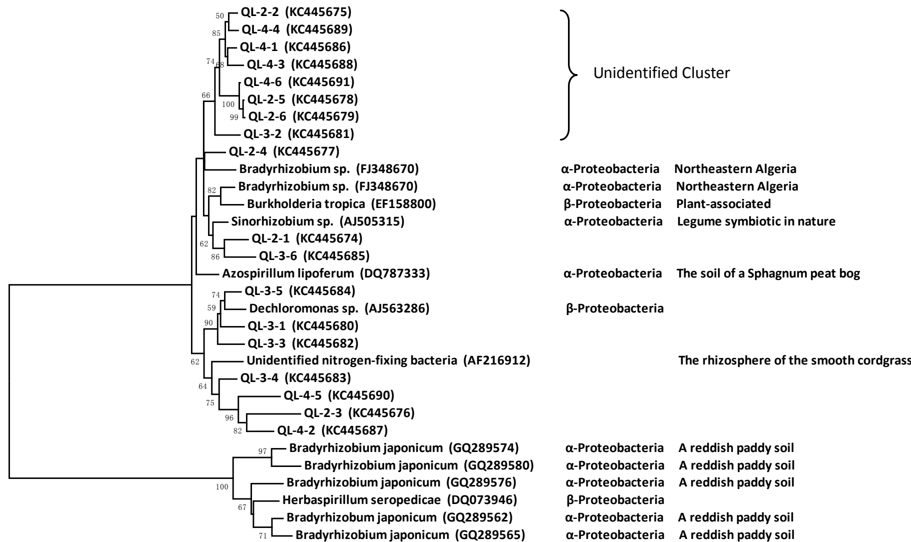


**Fig. 4.** Phylogeny analysis of nitrogen-fixing bacterial community in shrub soil.



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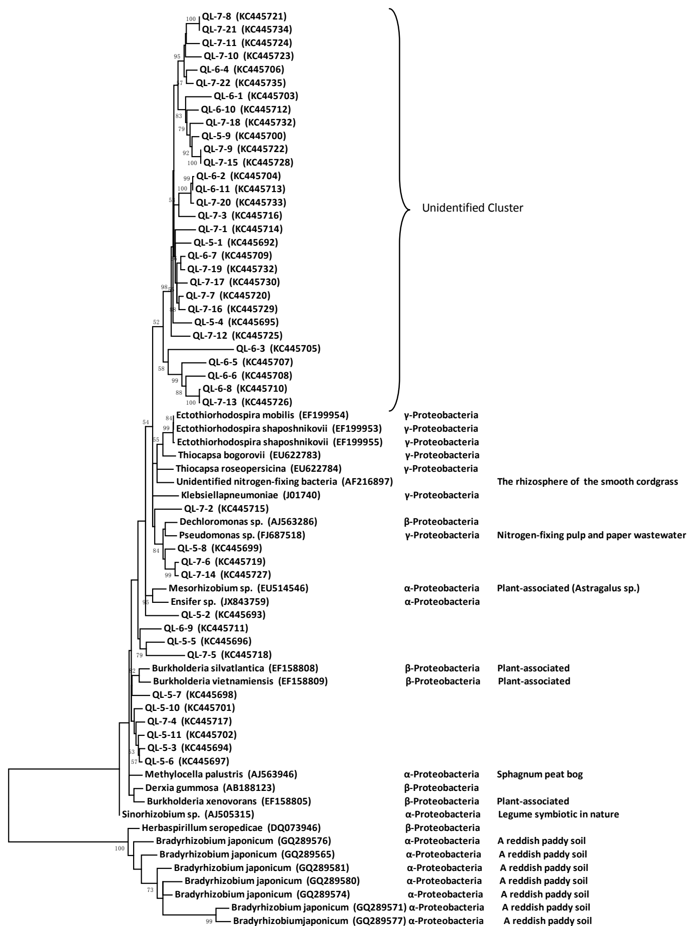


**Fig. 5.** Phylogeny analysis of nitrogen-fixing bacterial community in meadow soil.





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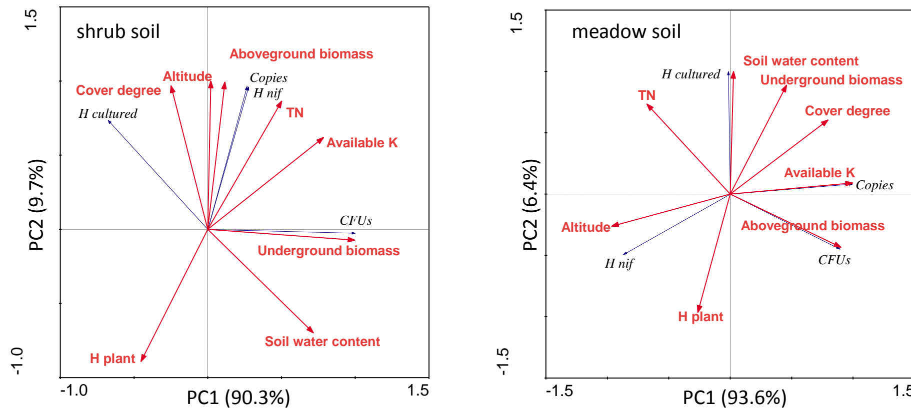


**Fig. 6.** Correlation analysis between nitrogen-fixing bacterial characteristics and environmental factors by RDA.



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