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ORIGINAL ARTICLE

Bacterial community structure associated with the rhizosphere soils and roots of *Stellera chamaejasme* L. along a Tibetan elevation gradient

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Abstract

The effect of altitude on the composition and diversity of microbial communities have attracted highly attention recently but is still poorly understood. We used 16S rRNA gene clone library analyses to characterize the bacterial communities from the rhizosphere and roots of *Stellera chamaejasme* in the Tibetan Plateau. Our results revealed that Actinobacteria and Proteobacteria were dominant bacteria in this medicinal plant in the rhizosphere and root communities. The Shannon diversity index showed that the bacterial diversity of rhizosphere follows a small saddle pattern, while the roots possesses of a hump-backed trend. Significant differences in the composition of bacterial communities between rhizosphere and roots were detected based on multiple comparisons analysis. The community of Actinobacteria was found to be significantly negative correlated with soil available P ($p < 0.01$), while the phylum of Proteobacteria showed a positive relationship with available P ($p < 0.05$). Moreover, redundancy analysis indicated that soil phosphorus, pH, latitude, elevation and potassium positively correlated with bacterial communities associated with rhizosphere soils. Taken together, we provide evidence that bacterial communities associated with *S. chamaejasme* exhibited some certain elevational pattern, and bacterial communities of rhizosphere soil were regulated by environmental characteristics along elevational gradients in this alpine ecosystem.

Keywords Bacterial community · Phylogenetic diversity · *Stellera chamaejasme* L. · Tibetan Plateau · Elevation gradient

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Introduction

The Tibetan Plateau, covering an area of 2.59× 10^6 km^2, is known as the third pole of the earth (Yuan et al. 2014). The plateau is a unique habitat, and it possesses the characteristics of harsh environmental conditions including dry air, low temperature, low oxygen, drought, high solar radiation and poor nutrient soil, which substantially constrain plant growth (Qiu 2008; Yuan et al. 2014). The Tibetan ecosystem is also a particularly vulnerable region that is highly sensitive to climate change, which could greatly impact plant nutrition and ecosystem functioning by inevitably affecting the distribution of microorganisms (Wang et al. 2015). Therefore, it is helpful to study the changes in bacterial community composition within this region for better understanding how the microbe response to the future climate change (Yuan et al. 2014). Some previous studies have been carried out to analyze bacterial communities in soil of grasslands (Yuan et al. 2014; Guo et al. 2015a), Mount Everest and Mount Shegyla (Zhang et al. 2009; Wang et al. 2015). However, as a global biodiversity hotspot, Tibetan Plateau encounters less attention in bacterial communities associated with plant rhizosphere and roots, and their response to environmental changes, especially in medicinal plants.

The rhizosphere of plant sustains a complex microecological system, which can be colonized by a large variety of bacteria (Ulrich et al. 2008). Rhizosphere bacteria play important roles in element cycling in soil ecosystems, and are of very important to plant health and soil fertility (Li et al. 2014; Wu et al. 2015). The composition and diversity of rhizoshpere bacterial communities are directly influenced by plant species, the chemical nature of root exudates, soil properties, and many other factors in this unique ecological niche (Li et al. 2014; Wu et al. 2015). Endophytic and rhizosphere bacteria can be useful for the health, growth and development of plants by direct effects such as the production of phytohormones or providing the availability of mineral nutrients (Lugtenberg and Kamilova 2009; Jin et al. 2014; Wu et al. 2015; Padda et al. 2016; Terrazas et al. 2016), or by indirect effects such as the decomposition of phytotoxic compounds and inhibition of pathogens (Ryan et al. 2008; Wu et al. 2015). Thus, an understanding of the bacterial community structure and diversity in rhizosphere soils and roots of a plant is important to the successful application of biological control strategies and could facilitate the development of soil management techniques to improve plant health and soil fertility (Costa et al. 2006; Wu et al. 2015).

Altitudinal gradients are characterized by tremendous changes in biotic and abiotic factors over a small geographical coordinate range (Yuan et al. 2014; França et al. 2016). It is essential that enhanced knowledge biological elevational composition and diversity patterns to an integrated understanding the influences of climate change on ecosystems (Shen et al. 2013). There have been a great number of recent studies addressing how the composition and diversity of soil microorganisms changes along the elevations (Fierer et al. 2011; Singh et al. 2012; Yuan et al. 2014). Some of these investigations had reported a variety of contrasting diversity elevation patterns, including a pattern of decline (Bryant et al. 2008), a hollow pattern (Singh et al. 2014), and a pattern of increase (Margesin et al. 2009; Djukic et al. 2010). Conversely, others had indicated that the soil microbial communities exhibited no significant elevational gradient in diversity (Männistö et al. 2007; Fierer et al. 2011). These studies have demonstrated that the study of microbial diversity along altitudinal gradients could help explore the response of soil microorganisms to the environment change (Yuan et al. 2014). Despite the available knowledge, we still know very little about the pattern of microbial diversity associated with rhizosphere soil and plant organs or tissues across elevational gradients.

Stellera chamaejasme L. is a toxic perennial herbaceous plant which belongs to the Thymelaeaceae family, it has a wide geographical range from southern Russia to northern China and Mongolia, and southwards as far as the dry regions of the western Himalayas, the Tibetan Plateau and southwestern China (Guo et al. 2015b). As an important pharmacological plant resource, *S. chamaejasme* has been used as a raw material for developing various kinds of pesticides and medicines (Zhang et al. 2013; Cui et al. 2014). Our previous studies on the bacterial communities of *S. chamaejasme* are currently gaining importance, including the recent 16S rRNA gene clone library analyses of the composition of the bacterial communities in rhizosphere, leaves, stems and roots (Jin et al. 2014), and a cultivation-dependent approach describes the composition and characteristics of cultivable bacterial isolates from the rhizosphere and bulk soil (Cui et al. 2015). However, how the bacterial community associated with *S. chamaejasme* changes with elevation gradients is still unknown.

Therefore, in this study, we measured the root endophyte and the directly associated rhizosphere communities of *S. chamaejasme* located in five different elevation levels in the Tibetan Plateau. The paired assessments of rhizospheric and root bacterial communities were obtained from the same root systems and samples with 16S rRNA gene cloning and sequencing methods across the elevation gradient. We are interested in testing the following three hypotheses. (1) rhizospheric and root bacterial diversity might show certain distribution trends along the elevation gradient because of the obvious altitudinal changes in this plateau ecosystem, (2) the composition of bacterial communities between rhizosphere and roots might have divergent patterns due to their inconsistent nutritional features and different physiochemical environmental conditions, (3) environmental factors underlying the elevation gradient drive structure and diversity of bacterial community in rhizosphere of *S. chamaejasme*.

Materials and methods

Study sites and sampling design

Native *S. chamaejasme* samples were collected along the National Highways G318 and G109 in the Tibetan Plateau, China in late July 2012. The global positioning system (GPS) coordinates of the collection point for each sample were recorded using the hand-held GPS position indicator (UniStrong, G138BD). Five sampling sites were chosen along an altitudinal gradient at 3664, 3919, 4243, 4573, and 4741 m above sea level (asl) and an topographical map depicting the geographic locations of study sites was created via the Google Map and Google Earth options (Table 1 and Fig. S1). At each elevation, we defined three plots located about 20–30 m apart depending on the distribution within the vegetation cover as three independent replicates. Within each plot, we selected three healthy looking individual plants were located close together (< 2 m apart), the three plants presented similar sizes and development level and were collected using sterile spades and gloves and composited together as a single sample. Details of rhizosphere soil and root samples collection has been described previously (Jin et al. 2014; Jin et al. 2015). In total, thirty rhizosphere and root samples were collected (5 elevations × 3 replicates, rhizosphere soil and root each had fifteen). Each composite rhizosphere sample was divided into two fractions. One fraction was stored at − 80 °C for following DNA extraction, the other fraction was used for measurement of soil properties. For root samples, they were stored at 4 °C for up to 2 days prior to surface sterilization and then stored at −80 °C thereafter. Root surface sterility procedure and sterility confirmation have been described in a previously published study (Jin et al. 2014).

Soil physical-chemical analyses

The rhizosphere soil samples were carefully ground by hand, thoroughly mixed, airdried at room temperature and sieved through a 2-mm sieve. A soil suspension with a ratio of 1:2.5 (*w*/*v*) for soil and water was prepared and the soil pH was measured with a glass electrode. Total soil organic carbon (OC) and total nitrogen content (TN) were determined by elemental analysis using a C/N analyzer (LECO Corporation, MI, USA), carbon/nitrogen ratio (C/N ratio) was calculated from total C and N. Available phosphorus (P) (using the Bray-1 method) and potassium (K) (using the ammonium acetate extraction method) were determined by an inductively coupled plasma atomic emission spectroscopy (ICP-AES, ICPE-9000, Shimadzu Corporation, Kyoto, Japan), respectively (Lu 2000).

DNA extraction, PCR amplification, and 16S rRNA gene clone library

The total genomic DNA of rhizosphere soil samples was extracted from 0.5 g (fresh weight) of the soil sample using the ZR Soil Microbe DNA Kit™ (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. For the root samples, surface-sterilized roots were cut into approximately 0.5 cm long pieces. Each root sample was pulverized in liquid nitrogen with a mortar and pestle. An aliquot (200 mg) of the resulting homogenate was used for genomic DNA extraction with a Plant DNA Extraction Kit (Tiangen Biotech, China), following the manufacturer's instructions (Jin et al. 2014; Jin et al. 2015). DNA quality and quantity were measured using the NanoDrop ND-2000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). After extraction, the DNA samples were immediately frozen at − 80 °C for further analyses.

Direct amplification of the total DNA extracted from root tissues using universal bacteria primers would produce amplicons dominated by plant mitochondrial and plastidial sequences. In order to inhibit the amplification of plastid products, the total DNA was predigested with the two restriction enzymes of *Pvu*II and *Msc*I for 16 h at 37 °C according to the reference of Shen and Fulthorpe (2015). The digested total DNA was used as template for the 16S rRNA PCR reaction using primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5'-ACG GCT ACC TTG TTA CGA CTT-3′) (Lane 1991). PCR mixture was prepared in a total reaction volume of 50 μl containing 2 μl of DNA template (approximately 50 ng), 5 μl of 10 × PCR buffer (50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-HCl and 1.5 mmol l^{-1} $MgCl_2$; Promega, Madison, WI, USA), 200 μmol l^{-1} of dNTP, 0.2 μmol l^{-1} of each primer, 2.5 U Taq DNA polymerase (Promega, Madison, WI, USA) and dd H_2O. PCR was performed using an Applied Biosystems Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA) with the following PCR conditions: initial denaturation at 95 °C for 1 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) and were quantified with a NanoDrop ND-2000 Spectrophotometer.

The purified amplicons were further cloned into the pGEM-T vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. Around 65 positive clones (white colonies) were randomly picked from the plates of each sample. These plasmid-harboring clones were amplified by PCR with the forward M13F (5′-GTT TTC CCA GTC ACG AC-3′) and the reverse M13R (5'-CAG GAA ACA GCT ATG AC-3′) plasmid-specific primer set to confirm the appropriate size of the insert (approximately 1, 500 bp). Clones containing the plasmid with an insert were sent for sequencing on an ABI

Table 1 Site information and general soil characteristics

	Jinda (JD)	Mozhugongka (MZGK)	Yangbajing (YBJ)	Tanggula (TGL)	Namucuo (NMC)
Latitude	N 30° 01′ 55″	N 29° 46′ 34″	N 30° 03′ 51″	N 30° 16′ 03″	N 30° 51′ 27″
Longitude	E 92° 55′ 56″	E 91° 51′ 49″	E 90° 34′ 46″	E 90° 40′ 56″	E 91° 03′ 27″
Elevation (m a.s.l.)[a]	3664	3919	4243	4573	4741
pH	6.70 ± 0.03	6.22 ± 0.03	6.52 ± 0.01	6.65 ± 0.04	6.82 ± 0.02
Organic C (g kg^{-1})	4.32 ± 0.03	3.98 ± 0.04	2.04 ± 0.01	1.53 ± 0.02	3.12 ± 0.03
Total N (g kg^{-1})	0.31 ± 0.01	0.25 ± 0.01	0.22 ± 0.02	0.12 ± 0.02	0.10 ± 0.01
C/N ratio	13.93 ± 0.37	15.92 ± 0.78	9.52 ± 0.62	13.43 ± 0.89	31.76 ± 0.34
P (mg kg^{-1})	11.04 ± 1.08	5.43 ± 0.55	7.50 ± 0.47	5.96 ± 0.12	7.01 ± 0.31
K (mg kg^{-1})	114.36 ± 4.34	106.71 ± 5.10	211.54 ± 7.22	233.34 ± 3.85	218.03 ± 8.82
MAT (°C)[b]	8.3	7.1	2.5	1.7	− 0.6
MAP (mm)[c]	640.1	515.7	483.1	459.6	414.6
The main accompanying plant species	*Stipa purpurea*, *Poa pratensis*, *Potentilla chinensis*, *Polygonum macrophyllum*	*S. purpurea*, *Kobresia humilis*, *Carex rigescens*, *P. chinensis*	*S. purpurea*, *K. pygmaea*, *C. moorcroftii*	*S. purpurea*, *K. pygmaea*, *K. humilis*	*S. purpurea*, *K. pygmaea*, *K. littledalei*
Soil type	Montane scrub grassland soil	Subalpine meadow soil	Subalpine meadow soil	Alpine meadow soil	Eolian sandy soil

Values are the means (±SD) of three replicates

[a] a.s.l., above sea level

[b] MAT, mean annual air temperature

[c] MAP, mean annual precipitation

3730XL automated sequencer (Applied Biosystems, USA) using the M13 universal sequencing primers at Sangon Biotech Co., Ltd. (DNA Sequencing Service, Shanghai, China).

Sequence analysis and phylogenetic tree construction

16S rRNA gene sequences obtained from sequencing results were performed mainly using the Mothur software (Schloss et al. 2009). We manually corrected aligned sequences with the BioEdit program (version 7.2.5; http://www.mbio.ncsu.edu/bioedit/bioedit.html) and detected and removed chimeras using the Chimera check Bellerophon program v3 (Huber et al. 2004). The candidate sequences within each library were subjected to operational taxonomic unit (OTU) based clustering at a similarity threshold of 97% using the furthest-neighbor algorithm in Mothur software. The "Get.oturep" command in Mothur was used to obtain a representative sequence for each OTU in the dataset and these representative sequences were used for all subsequent analyses. Subsequently, the representative sequences were assigned bacterial taxonomic affiliations based on the closest match to sequences available at the Ribosomal Database Project (RDP) Classifier tool (http://rdp.cme.msu.edu/classifier/) at confidence level of 80% and the BLASTn in nucleotide reference database (http://blast.ncbi.nlm.nih.gov/) (Cole et al. 2014). Rarefaction curves were constructed to estimate the OTU richness by plotting the number of OTUs against the number of clones sequenced using the Mothur application. To characterize the community structures, species richness (Observed OTUs, the relative richness and Chao1 index) and biodiversity indices (non-parametric Shannon's diversity, Shannon's evenness, library coverage and relative number of singletons) were calculated as OTUs at 97% sequence similarity by the Mothur program at the 0.03 level (Schloss et al. 2009).

The 16S rRNA gene sequences were aligned with the reference sequences (the closest matches in the Nucleotide collection database) using ClustalW implemented in MEGA 6.0. Phylogenetic trees were constructed by MEGA 6.0 software using the Neighbor-Joining method, and a bootstrap analysis with 1, 000 replicates was applied to estimate the confidence values of the tree nodes. Bootstra*p* values > 50% are labeled on the tree. The sequences obtained in this study are available in GenBank under accession numbers KX035228 to KX035272 (rhizosphere sample of Jinda, JDrs), KX035273 to KX035321 (rhizosphere sample of Mozhugongka, MZGKrs), KX035322 to KX035369 (rhizosphere sample of Yangbajing, YBJrs), KX035370 to KX035415 (rhizosphere sample of Tanggula, TGLrs), KX035416 to KX035457 (rhizosphere sample of Namucuo, NMCrs), KT759311 to KT759333 (root sample of JD, JDr), KT759334 to KT759370 (root sample of MZGK, MZGKr), KT759371 to KT759395 (root sample of YBJ, YBJr), KT759396 to KT759409 (root sample of TGL, TGLr), and KT759410 to KT759415 (root sample of NMC, NMCr), respectively (Fig. S2 and Fig. S3). For the description of the community, OTUs with the same taxonomy were binned together at the phylum, class and genus level (Bodenhausen et al. 2013).

Statistical analysis

Pearson's correlation analysis of the bacterial abundance and community structure of rhizosphere soil with the determined environmental and soil chemical factors was performed using SigmaPlot 12.5 (Systat Software, San Jose, CA, USA). To test for the significant differences in the composition of bacterial communities from different elevations and different habitats (rhizosphere soil and within roots), we applied Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks followed by all pairwise multiple comparison procedures (Tukey test). We used Spearman rank order correlations to determine the possible correlations between altitudinal variables or habitat variables of the rhizosphere and root-endophytic bacterial communities of *S. chamaejasme*. These above analysis were also completed with SigmaPlot 12.5, and *p* values of less than 0.05 were considered to indicate statistical significance. Principal component analysis (PCA) was used to distinguish the composition of bacterial communities in all rhizosphere soil and root samples from all five elevation gradients based on phylum-level abundances. To reveal the main environmental and soil chemical characteristics which sustain the bacterial groups, redundancy analysis (RDA) was performed to measure environmental and soil chemical parameters that had the most significant influence on bacterial community. The significant correlations of the environmental parameters were examined by a Monte Carlo permutation. Both PCA and RDA analyses were carried out using CANOCO 5 for Windows (Biometris, Wageningen, the Netherlands).

Results

Rhizosphere soil physiochemical properties

The sampling sites encompassed a wide range of rhizosphere soil characteristics of *S. chamaejasme* in Tibet Autonomous Region. Soil pH of each sampling site ranged from 6.2 to 6.8, with the lowest values in the sites located in MZGK and the highest in NMC (Table 1). The organic C varied from 1.5 to 4.3, with the lowest value found at the site of TGL and the highest at the site of JD. The total N, C/N ratios, soil available P, and K ranged from 0.1–0.3, 9.5–31.8, 5.4–11.0, and 106.7–233.3, respectively (Table 1). Among all the factors examined, total N presented a significantly negative relationship with altitude ($CC = -0.987$, $p < 0.01$), and a significant decrease

was observed in total N with increasing altitude. Soil available K (CC = 0.902, $p < 0.05$), on the contrary, showed a significantly positive relationship with altitude (Table 2). No significant relationships with altitude could be detected on soil pH ($p = 0.431$), organic C ($p = 0.187$), C/N ratio ($p = 0.348$), or soil available P ($p = 0.342$) in this study (Table 2).

General description of sequencing result and bacterial community composition

A total of 650 sequences were obtained from the ten clone libraries, and then 21 chimeric sequences were excluded from further analyses. Altogether 629 sequences (316 from the rhizosphere libraries and 313 from the root libraries) were further analyzed, among which 335 different operational taxonomic units (OTUs) (230 from the rhizosphere libraries and 105 from the root libraries) were detected based on 97% sequence similarity. Rarefaction curves (97% identity) in all the five rhizosphere samples did not approach the plateau, indicating a less representation of bacterial diversity, which may increase on repetitive picking positive clones (Fig. S4). Most obviously, the rarefaction curves of the rhizosphere samples were higher than the root samples (Fig. S4).

The 629 classified sequences were assigned to 10 bacterial phyla. Among the identified groups, approximately 66% (416 clones) of the sequences were grouped in the phylum Actinobacteria. The Proteobacteria phylum was ranked second, with about 27% (170 clones) of the sequences, which were distributed as follows: α-proteobacteria (20.3%), γ-proteobacteria (4.6%), β-proteobacteria (1.4%) and δ-proteobacteria (0.6%). Other phyla found were Chloroflexi (16 clones, 2.5%), Firmicutes (9 clones, 1.4%), Gemmatimonadetes (5 clones, 0.8%), Bacteroidetes (5 clones, 0.8%), and Acidobacteria (4 clones, 0.6%). The remaining phyla included Armatimonadetes, Saccharibacteria and Verrucomicrobia, each of which represented less than 0.2% of the total isolated clones. In addition, one clone (0.16%) matched the 16S rRNA genes of uncultured bacterium and could not be grouped with sequences of known bacteria phyla.

Phylogenetic analyses of 316 sequences showed that the bacterial communities in this toxic plant rhizosphere soil consisted of 9 bacterial phyla. Actinobacteria were the most abundant organisms (219 clones, 69.3%), followed by Proteobacteria (65 clones, 20.6%). Other phyla included Chloroflexi, Firmicutes, Gemmatimonadetes, Acidobacteria, Bacteroidetes, and Armatimonadetes, each of which occupied less than 2.9% of the rhizosphere soil clones (Fig. 1, Table S1 and Fig. S2). In general, the bacterial community structures in the rhizosphere soils at different elevations were different at genus level. *Conexibacter* was more abundant in the lower altitudes (JD and MZGK), *Arthrobacter* was more abundant in the middle-higher elevations (YBJ, TGL and NMC), and *Nocardioides* was more abundant in the higher elevations (TGL and NMC), whereas *Streptomyces* was present in most of rhizosphere soils except in JD sample and at a relative higher abundance (Fig. 1, Table S1 and Fig. S2). Some bacterial families only occurred at specific elevations and at relatively higher abundance (> 5 OTUs). For example, Hyphomicrobiaceae and *Solirubrobacter* were only present in the JD sample, *Cellulomonas* only in MZGK sample,

Table 2 Pearson correlations between environmental and soil characteristics and rhizosphere soil bacterial communities for the full sequence set and the two most abundant phyla

Soil properties[a]	Correlation									
	Total bacteria		Actinobacteria		Proteobacteria		Diversity[c]		Elevation	
	CC[b]	*p*	CC	*p*	CC	*p*	CC	*p*	CC	*p*
LAT	− 0.292	0.633	0.116	0.853	− 0.088	0.888	− 0.767	0.130	0.806	0.100
LON	0.565	0.321	− 0.555	0.332	0.658	0.227	− 0.199	0.749	− 0.822	0.088
ElE	− 0.676	0.210	0.543	0.344	− 0.594	0.290	− 0.289	0.637	–	–
pH	0.053	0.933	− 0.413	0.490	0.319	0.600	− 0.638	0.247	0.464	0.431
O. C	0.531	0.357	− 0.338	0.579	0.571	0.315	− 0.394	0.512	− 0.702	0.187
T. N	0.767	0.130	− 0.610	0.275	0.662	0.223	0.279	0.650	*− 0.987*	*0.0017*
C/N	− 0.249	0.686	0.275	0.654	− 0.078	0.901	*− 0.896*	*0.040*	0.539	0.348
P	0.818	0.091	*− 0.979*	*0.0037*	*0.947*	*0.0147*	− 0.297	0.627	− 0.545	0.342
K	− 0.485	0.408	0.272	0.658	− 0.432	0.468	− 0.068	0.913	*0.902*	*0.0362*

Values in italics indicate significant correlations

[a] LAT, latitude; LON, longitude; ELE, elevation; O. C, organic C; T. N, total N; C/N, C/N ratio

[b] CC, correlation coefficient

[c] Diversity, Shannon diversity

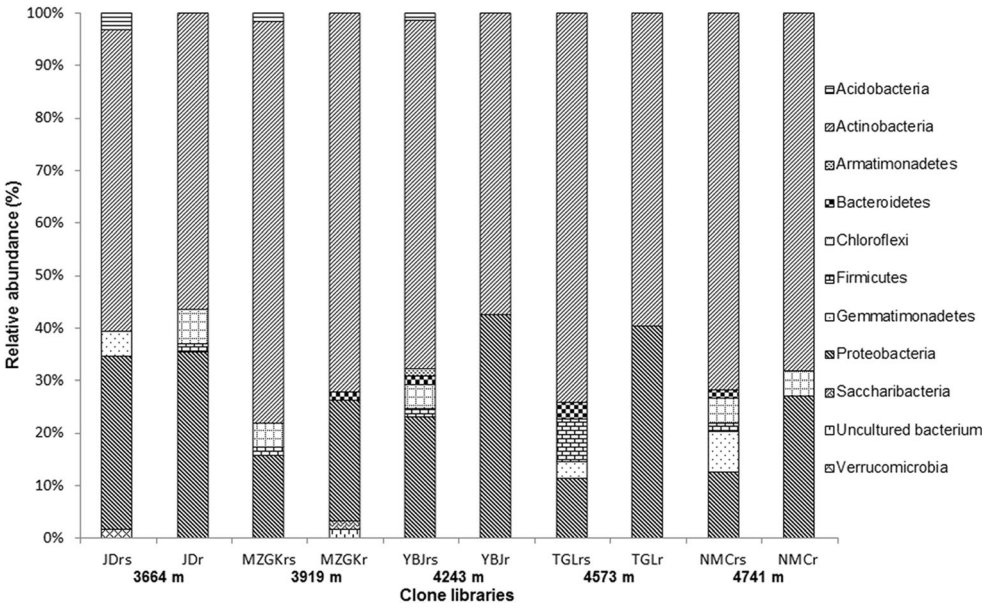

Fig. 1 Relative abundances of the different bacterial phyla in rhizosphere soils and roots along an elevational gradient. Relative abundances are based on the proportional frequencies of those DNA sequences that could be classified at the phylum level

Streptosporangium only in YBJ sample, *Bacillus* only in TGL sample, and *Aeromicrobium*, *Phycicoccus* and *Rhizobium* only in NMC sample. Although there were also some other bacterial families only present in specific elevations, the relatively abundance were lower (< 5 OTUs) (Fig. 1, Table S1 and Fig. S2).

For root samples, similar as the rhizosphere soil samples, the dominant phyla also were Actinobacteria and Proteobacteria accounting for more than 96.5% of the bacterial sequences from root samples. In addition, Chloroflexi, Bacteroidetes, Firmicutes and Saccharibacteria were present in one or two of root samples but at relatively low abundances, and 1 bacterial sequence (0.3%) was uncultured bacterium (Fig. 1, Table S1 and Fig. S3). The dominant bacterial genera were similar among the five root samples from the different elevation levels. Arthrobacter and *Sphingomonas*-associated clones represented the majority of the population at all elevations levels. In addition, *Streptomyces* (> 5 OTUs) was detected only in JD and YBJ samples, and *Modestobacter* only in MZGK sample (Fig. 1, Table S1 and Fig. S3).

Bacterial community diversity

In different altitude areas, Chao 1 and biodiversity indices (relative richness, non-parametric Shannon's diversity and Shannon's evenness) at the dissimilarity of 0.03 for bacteria were obviously higher in rhizosphere soils compared to plant roots. In rhizosphere soils and plant roots, the species richness and diversity indices suggested that the low and medium elevation rhizosphere soils and plant roots contained the higher diversity of bacterial communities than the high elevation rhizoshpere soil and plant root (Table 3). The Shannon diversity index showed that the bacterial communities with more diversity were observed in rhizosphere samples, while the communities associated with root samples were less diverse (Fig. 2). The Shannon diversity index also indicated that root endophytic bacterial diversity of *S. chamaejasme* followed a unimodal pattern with a peak at MZGK, and revealed that the diversity of the root bacterial communities possessed a hump-backed trend with elevation (Table 3 and Fig. 2). The Shannon diversity index of rhizosphere bacterial communities was first increased and then decreased along with the increasing elevation, and takes a "small saddle pattern" with a peak at TGL (Table 3 and Fig. 2). Library overage analysis indicated that the libraries contained from 74.2 to 96.8% of the total number of OTUs that existed in the plant root samples (except the MZGK sample). The libraries of the rhizosphere samples and the root sample for MZGK contained 32.8 to 50.8% of the total number of OTUs, which suggested that additional sampling of the bacterial clones would be needed to reveal the full extent of the diversity (Table 3).

The degree of bacterial species (OTU) overlap and dissimilarity in bacterial community composition within and between habitats

To identify significant differences in the composition of bacterial communities from different elevations and different habitats (rhizosphere soil and within roots), the Kruskal-Wallis one-way ANOVA on ranks followed by all pairwise multiple comparison procedures (Tukey test) was employed (Table 4). No significant differences in the composition of bacterial communities in rhizosphere samples were detected between different elevations ($p > 0.05$). For root samples, there was a significant difference ($p < 0.05$) between MZGK and YBJ samples, a significant difference ($p < 0.05$) between MZGK

Table 3 Statistical analysis of 16S rRNA gene clone libraries derived from rhizosphere and root samples of *S. chamaejasme*, calculated at 97% sequence similarity by MOTHUR program

Dataset[a]		No. of sequences	No. of OTUs[b]	Relative richness (%)[c]	Chao 1	H′	E	LC (%)[d]	Singletons (%)[e]
Rs	JD	61	45	73.77	156.51	3.62	0.95	39.34	60.66
	MZGK	64	49	76.56	276.09	3.69	0.95	32.81	67.19
	YBJ	65	48	73.85	323.61	3.70	0.95	36.92	63.08
	TGL	62	46	74.19	136.37	3.71	0.97	41.94	58.06
	NMC	64	42	65.63	125.11	3.52	0.94	50.00	50.00
R	JD	62	23	37.10	33.65	2.47	0.79	75.81	24.19
	MZGK	61	37	60.66	145.75	3.23	0.89	50.82	49.18
	YBJ	65	25	38.46	44.60	2.42	0.75	74.24	25.76
	TGL	62	14	22.58	18.50	1.66	0.63	83.87	16.13
	NMC	63	6	9.52	6.75	1.04	0.58	96.83	3.17

OTUs, operational taxonomic units; H′, Shannon's diversity index; E, Shannon's evenness index; LC, library coverage

[a] Rs, rhizosphere; R, root; JD, Jinda; MZGK, Mozhugongka; YBJ, Yangbajing; TGL, Tanggula; NMC, Namucuo

[b] No. of OTUs were determined using the MOTHUR software based on the 3% sequence difference

[c] Relative richness, defined as the number of OTUs observed regarding to the number of sequences

[d] Library coverage was calculated using the formula: [1-(singletons/individuals)] × 100

[e] Relative number of singletons regarding to the OTUs occurring only once in the overall clone library

and TGL samples, and a significant difference ($p < 0.05$) between MZGK and NMC samples. For the samples in different habitats at the same elevation, significant differences between rhizosphere and root samples were determined at JD, YBJ, TGL and NMC (Table 4). In addition, we used Spearman's rank-order correlation to evaluate relationships between elevational variables or habitat variables of the rhizosphere and root-endophytic bacterial communities (Table 4). The results showed that the composition of bacterial community of rhizosphere soil in MZGK was positively correlated with TGL sample ($p < 0.05$). Similarly, several significant positive correlations, such as JD and YBJ ($p < 0.01$), YBJ and TGL ($p < 0.0001$), YBJ and NMC ($p < 0.05$), and TGL and NMC ($p < 0.05$) were observed in root samples. And likewise, the composition of bacterial community in rhizosphere soil was positively correlated with that of root sample at MZGK (Table 4).

Furthermore, to reduce the number of variables of the data and maintain as much variance as possible, PCA was used to compare bacterial communities between the different elevations from the same habitat, or between the different habitats at the same elevation. As shown in Fig. 3, the results revealed

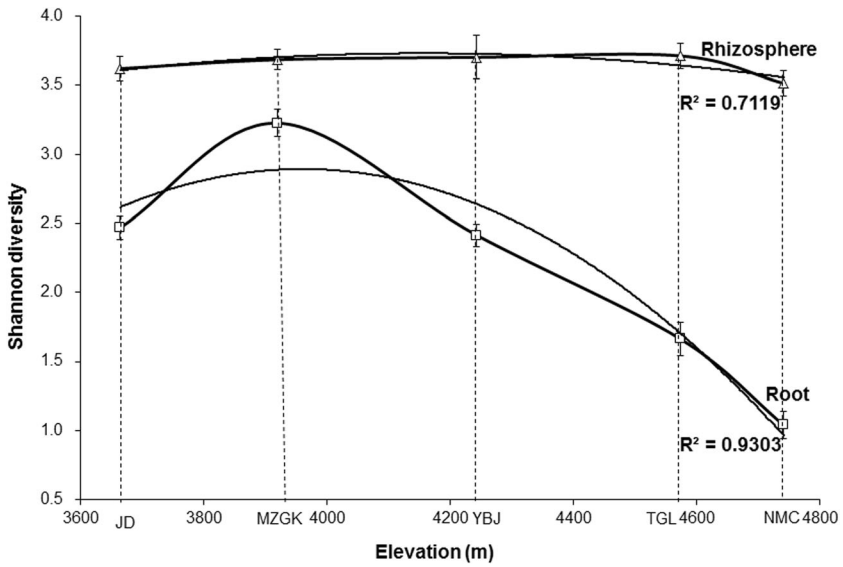

Fig. 2 The relationship between elevation and Shannon diversity index. Data were calculated with sequence similarity threshold of 97%

Table 4 *P* value showing significance of the effects of plant growth elevations and habitats from Kruskal-Wallis one-way ANOVA on ranks (Tukey test) and Spearman rank order correlation for the rhizosphere and root-endophytic bacterial communities of *S. chamaejasme*

Samples[a]	Pairwise comparison[b]	Kruskal-Wallis one-way ANOVA on ranks		Tukey test		Spearman rank order correlation	
		H	*p*	q	*p*	CC[c]	*p*
Rs	JD vs. MZGK	0.453	0.501			0.219	0.095
	JD vs. YBJ	2.109	0.146			0.007	0.956
	JD vs. TGL	0.882	0.348			0.169	0.201
	JD vs. NMC	1.016	0.314			− 0.078	0.557
	MZGK vs. YBJ	0.502	0.479			0.131	0.320
	MZGK vs. TGL	0.007	0.931			*0.279*	*0.032*
	MZGK vs. NMC	0.155	0.694			0.239	0.068
	YBJ vs. TGL	0.175	0.675			− 0.096	0.469
	YBJ vs. NMC	0.323	0.570			0.150	0.256
	TGL vs. NMC	0.030	0.863			0.184	0.161
R	JD vs. MZGK	2.930	0.087			0.0768	0.659
	JD vs. YBJ	0.148	0.700			*0.433*	*0.0096*
	JD vs. TGL	0.961	0.327			0.323	0.058
	JD vs. NMC	3.629	0.057			0.317	0.063
	MZGK vs. YBJ	*5.150*	*0.023*	*3.007*	*< 0.05*	0.148	0.267
	MZGK vs. TGL	*7.064*	*0.008*	*3.397*	*< 0.05*	0.196	0.152
	MZGK vs. NMC	*12.049*	*≤ 0.001*	*4.319*	*< 0.05*	0.229	0.183
	YBJ vs. TGL	0.568	0.451			*0.638*	*≤ 0.0001*
	YBJ vs. NMC	3.131	0.077			*0.349*	*0.0396*
	TGL vs. NMC	0.671	0.413			*0.344*	*0.0431*
Rs vs. R	JD	*6.007*	*0.014*	*2.807*	*< 0.05*	0.0909	0.460
	MZGK	0.192	0.661			*0.274*	*0.024*
	YBJ	*4.019*	*0.045*	2.271	> 0.05	0.179	0.144
	TGL	*6.473*	*0.011*	2.641	> 0.05	0.127	0.379
	NMC	*12.218*	*≤ 0.001*	*3.576*	*<0.05*	0.0515	0.676

Twenty-five pairwise analyses were performed comprising each of the ten possible pairings of the five different elevations and both collected samples (rhizosphere soil and roots) at the same elevation. Values in italics indicate significant differences or significant correlations

[a] Rs, rhizosphere soil; R, root

[b] JD, Jinda; MZGK, Mozhugongka; YBJ, Yangbajing; TGL, Tanggula; NMC, Namucuo

[c] CC, correlation coefficient

that eigenvalues of the first two principal components (PC1 and PC2) closely approached 85.9% and the accumulated contribution ratios of PC1 and PC2 achieved 73.3% and 12.6%, respectively, indicating that PC1 appeared to capture a substantial part of the variation in the structure of the bacterial community. The PCA result confirmed that the bacterial communities could be divided into two groups corresponding with the different habitats. In contrast, the bacterial communities were not clearly differentiated along an elevation gradient (Fig. 3). The PCA results also confirmed that most of the bacterial phyla were negatively associated with PC1. Data points corresponding to each habitat sampled in both elevations tended to create independent clusters indicating a stable bacterial community diversity that could be habitat specific. In addition, the rhizosphere soil samples (except JD sample) were negatively associated with PC1, while the root samples (except MZGK sample) were positively associated with PC1. PC2 clustered the rhizosphere and root samples in two groups. One of the groups, in the upper quadrant, was the JD and YBJ samples, and the four samples have shown positive correlations with PC2. The other group (in the lower quadrant) consisted of the remaining samples were negatively related to PC2 (Fig. 3).

Relationship between bacterial community structure and measured environmental variables

In this study, Pearson correlation analysis was applied to investigate the relationships between the determined environmental and soil chemical parameters and the abundances of bacterial

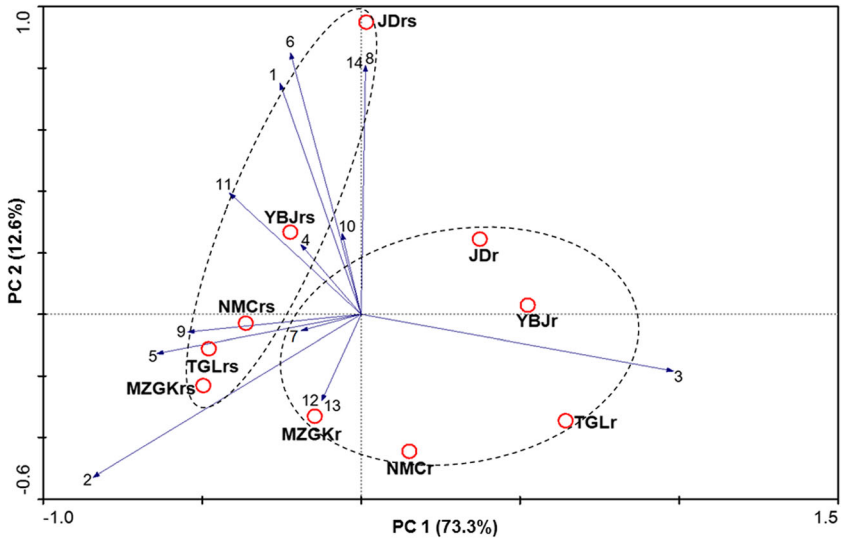

Fig. 3 Principal component analysis (PCA) of bacterial communities associated with rhizosphere and roots of *S. chamaejasme* in the five different sampling sites along an elevation gradient. Each red circle indicates a sampling site. Distances between any two circles on the graph indicate the ecological distance between the community compositions. Ovals drawn with black dashed lines represent the associations of rhizosphere and root samples in different elevations, respectively. Vectors are indicating the importance of each single parameter (single bacterial phyla). 1, Acidobacteria; 2, Actinobacteria; 3, α-proteobacteria; 4, Armatimonadetes; 5, Bacteroidetes; 6, β-proteobacteria; 7, Chloroflexi; 8, δ-proteobacteria; 9, Firmicutes; 10, γ-proteobacteria; 11, Gemmatimonadetes; 12, Saccharibacteria; 13, Uncultured bacterium; 14, Verrucomicrobia

communities from rhizosphere soil of *S. chamaejasme* (Table 2). The results indicated the abundance of bacterial community showed no significant correlations with the measured physicochemical properties ($p > 0.05$) when overall bacterial community composition was considered. However, if we examined the two most abundant bacterial phyla individually, we find that the community of Actinobacteria is significantly negative correlated with soil available P ($p < 0.01$), on the contrary, the phylum of Proteobacteria showed a positive relationship with available P ($p < 0.05$). The remaining pairwise comparisons of bacterial abundance did not show significant correlations with environmental and soil chemical factors ($p > 0.05$) (Table 2).

RDA was performed to measure the influences of different environment and soil geochemical factors on bacterial community assemblages associated with rhizosphere soils and roots (Fig. 4). RDA showed that RDA1 and RDA2 could explain 90.9% of the total variation and the samples dispersed away from each other. The bacterial communities of rhizosphere samples were in first quadrant, second quadrant and third quadrant of graph; whereas, the communities in the root samples were in third quadrant and fourth quadrant in the plot, respectively (Fig. 4). Generally, soil P, pH, latitude, elevation and soil K positively correlated with bacterial communities associated with rhizosphere soils. However, for the bacterial communities in roots of *S. chamaejasme*, the measured environment and soil geochemical properties had no significant influence on bacterial communities. RDA also revealed that soil pH, latitude, K, elevation, C/N ratio, Organic C, longitude, and total N accounted for a large amount of the variation in the distribution of samples along RDA 1, while the soil P content was significant in explaining the variation in bacterial community composition along RDA 2 (Fig. 4).

Discussion

To our best knowledge, no studies have comprehensively examined the bacterial communities from the rhizosphere soils and roots of *S. chamaejasme* simultaneously in the Tibetan Plateau. The rhizosphere and endophyte communities are largely consistent with complete overlap in the dominant phyla regardless of the differences in elevation levels and soil physicochemical characteristics associated with the five different sampling sites. Via multivariate analyses, we also identified that soil P, pH, latitude, elevation and soil K affected bacterial community composition in rhizosphere soils, whereas the measured environment and soil geochemical properties had no significant influence on bacterial communities in roots. This study provided valuable insight into the response of bacterial communities from *S. chamaejasme* in the Tibetan Plateau to elevation changes, and the relationship between environmental properties and bacterial community structure.

Clone libraries showed that the division Actinobacteria (69.3%) and Proteobacteria (20.6%) dominated the rhizosphere samples of *S. chamaejasme*. These two groups were also found to be dominant in root samples in the same region accounting for more than 96.5% of the bacterial sequences. These results are in agreement with some previous studies (Bodenhausen et al. 2013; Wu et al. 2015). Actinobacteria

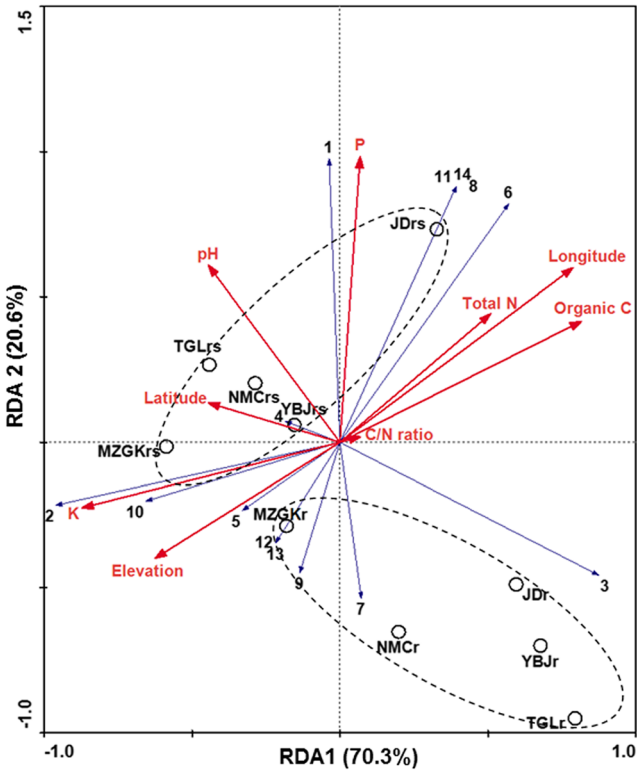

Fig. 4 Redundancy analysis (RDA) ordination biplot showing relationships between bacterial communities and environmental factors based on sequences abundance assigned at phylum level along altitudinal gradients. Black circles indicate sampling sites in the Tibet Plateau. Ovals drawn with black dashed lines indicate samples from the rhizosphere and roots of *S. chamaejasme* in different elevations, respectively. The red arrows indicate the types of environmental variables and their relative effects on bacterial communities, and the blue arrows indicate the different bacterial phylum. The distance between black circles reflects their dissimilarity, and the relative position (in perpendicular distance) of a black circle to a red arrow-line indicates the influence of the specified environmental factor on the bacterial communities in different sampling sites. 1, Acidobacteria; 2, Actinobacteria; 3, α-proteobacteria; 4, Armatimonadetes; 5, Bacteroidetes; 6, β-proteobacteria; 7, Chloroflexi; 8, δ-proteobacteria; 9, Firmicutes; 10, γ-proteobacteria; 11, Gemmatimonadetes; 12, Saccharibacteria; 13, Uncultured bacterium; 14, Verrucomicrobia

and Proteobacteria can actively colonize plant root systems and involved in various important processes, such as decomposing complex molecules (Anderson et al. 2012), producing various antibiotics, bioactive compounds and secondary metabolites, phytohormones (IAA), siderophore as well as solubilize phosphate, fixing nitrogen and promoting plant growth (Palaniyandi et al. 2013; Cui et al. 2015), exhibiting immense biocontrol action against a range of phytopathogens (Anwar et al. 2016). Firmicutes have frequently been found in a wide variety of habitats, which may be due to their ability to switch to a dormant form under stressful conditions (Galperin 2013). However, Firmicutes only occupied 2.5 and 0.3% of the rhizosphere soil and root clones, respectively, and at relatively low abundances in the present study. Intriguingly, members of Firmicutes were found as a dominant bacterial phylum in rhizosphere soil or root samples of *S. chamaejasme* from the Cuiying mountain area in the Yuzhong campus of Lanzhou University detected by cloning and isolation in our previous studies (Jin et al. 2014; Cui et al. 2015).

Of the dominant bacteria obtained in the present study, the genera of *Arthrobacter* and *Streptomyces* were widely distributed in rhizosphere and root samples, and the genus of *Sphingomonas* was associated with roots of *S. chamaejasme* in most of different elevation levels. *Arthrobacter*, being as an overwhelmingly dominant bacterial genus, are found in a variety of environments due to their ability to grow in relatively barren soils in extreme conditions. They are well adapted to life in arid soils, and some *Arthrobacter* species were reported to tolerate drastic environmental stresses for extended periods (Yao et al. 2015). Bacteria in the genus *Streptomyces* are broadly found in a variety of soil environments, and they are effective rhizosphere colonizers of plant root systems. Members of *Streptomyces* are able to tolerate extreme stresses by forming spores (Tamreihao et al. 2016). *Streptomyces* are prolific producers of diverse biologically active secondary metabolites of natural products and are being used to control soil-borne and seed borne diseases of plants (Palaniyandi et al. 2013). *Sphingomonas* species are often found in association with plants (Bodenhausen et al. 2013; Jin et al. 2014). They are known to interact positively with plant roots by producing gibberellins, IAA and siderophores (Khan et al. 2014).

Altitude is relevant to variables that impact on the ecosystem, so it is considered as one of the most important factors that influences microbial diversity (Faoro et al. 2010; Zhang et al. 2015). In this study, endophytic bacterial diversity associated with roots of *S. chamaejasme* follow a unimodal pattern along elevation gradients. The theory of mid-domain effect (MDE) might use to explain the mechanisms that drive this unimodal diversity pattern in our study. The MDE focus on the geometric constriction of species distribution and indicates that species usually accumulate in the middle areas along elevation (Colwell and Lees 2000; Wang et al. 2015). Our result was consistent with this theory that the middle area of MZGK possessed the highest diversity of bacterial species than other areas. In contrast, the diversity of the rhizosphere bacterial communities showed a small saddle pattern with elevation. The Tibetan Plateau is one of the most special regions sensitive to global climate variation and the altitude changing may have a complex impact on below-ground microorganisms (Zhang et al. 2009). The altitude, behaving as a complex climatic and physicochemical gradient, may result in complex alterations of the soil physicochemical factors and, consequently, the bacterial diversity (Faoro et al. 2010; Corneo et al. 2013). The small saddle pattern of rhizosphere soil bacterial diversity of *S. chamaejasme* might be related to environmental factors, and which could be used in the interpretation of elevational diversity pattern in this study. Our observations were in agreement with the most common pattern of species

richness-altitude relationships (Singh et al. 2012; Shen et al. 2015). Taken together, the results support the first of our hypothesis, suggesting that the bacterial communities associated with rhizosphere and root of *S. chamaejasme* indicated certain distribution patterns and possessed different species diversity change trends in the Tibetan Plateau ecosystem.

In addition, the bacterial communities with more diversity were observed in the rhizosphere samples than that in the root samples in all five elevation gradients in the present study. This result is consistent with our previous study demonstrating that the bacterial diversity of the rhizosphere was more diverse than the root of *S. chamaejasme* (Jin et al. 2014). Our findings are also similar with other studies in poplar and *Arabidopsis* (Gottel et al. 2011), and also reported by Rosenblueth and Martínez-Romero (2006). The lower ratios of singletons and doubletons in roots at higher elevation may result in the declining species richness pattern in this study. These results suggest that rare species are relatively less competitive in harsh environments such as high solar radiation, shortage of oxygen, low temperature and drought weather at higher altitudes, and increasing dominance reduces overall diversity (Kan et al. 2007).

Generally, it has been considered that endophytic root bacterial communities were similar with rhizosphere soil, and as a subset of colonists deriving from the surrounding rhizosphere soil. According to this view, the composition of root endophytes will be affected by the surrounding soil and environmental properties, and similar composition patterns of bacterial communities should be observed from the rhizosphere soil and root. In contrast, in this study, significant differences in the composition of bacterial communities between rhizosphere and root samples were detected based on the Kruskal-Wallis one way ANOVA on ranks ($P < 0.05$). Our result is in agreement with the study on bacterial communities within the endosphere and rhizosphere of *Populus deltoides* roots (Gottel et al. 2011). The rhizosphere was regarded as a complex habitat, it supporting a variety of carbon sources, such as amino acids, organic acids and carbohydrates, which are used by microorganisms to obtain energy (Wawrik et al. 2005), while the root might be considered a more stable niche for the bacteria. Thus, the variability in physicochemical environmental conditions and nutrient supply seemed to partly explain the differences in bacterial community composition between the roots and the rhizosphere soils (Compant et al. 2011). Furthermore, PCA plots on the total composition of bacterial communities recovered at each habitat and at each altitudinal gradient reported an apparent habitat-dependent biodiversity, namely, each habitat sampled in both elevations tended to create independent clusters indicating a stable bacterial community diversity that could be habitat specific (Fig. 3). Undoubtedly, it is suggested that PCA further confirmed the authenticity of the results based on the Kruskal-Wallis one-way ANOVA on ranks in our study. Given the above, our findings supported our second hypothesis that the species composition of bacterial communities between rhizosphere and roots presented different patterns because of their variability in physicochemical environmental conditions and available nutrients.

In this study, Pearson correlation analysis indicated that the abundance of total bacterial community did not showed significant correlations with the measured physicochemical parameters ($p > 0.05$). This suggest that the factors regulating the abundance of bacterial community were quite complex, and multiple factors might be involved in shaping the dynamics of bacterial community associated with *S. chamaejasme* in the Tibetan Plateau natural ecosystem. However, the community of Actinobacteria was found to be significantly negative correlated with soil available P ($p < 0.01$), while the phylum of Proteobacteria showed a positive relationship with available P ($p < 0.05$). It is generally known that phosphorus is one of important nutrients required for plant growth, and also the important participants in the activities of plant life. As Actinobacteria have the phosphate solubilizing properties they able to release P from soil minerals play a vital role in supplying P to plants in a more environmentally friendly and sustainable manner (Pragya et al. 2012). Therefore, it is reasonable that we found the community of Actinobacteria was significantly negative correlated with soil P in this study. Wakelin et al. (2012) was also reported that the Actinobacteria communities are intrinsically linked to soil P cycling in pasture soils. Conversely, the α-proteobacteria communities were demonstrated that they had little capacity to solubilize P and relatively infrequently cited as P-solubilizing (Wakelin et al. 2012). Alphaproteobacteria was the dominant subphylum accounting for about 54% of Proteobacteria phylum from rhizosphere samples in our study. So, the Proteobacteria had different relevance with soil P when compared with the Actinobacteria communities. To our knowledge, this was the first report on the links between the bacterial community compositions of *S. chamaejasme* and Soil P.

RDA analysis at the phylum level illustrated the relationships between bacterial community structure and the matrix of environmental variables. Soil P, pH, Latitude, elevation and soil K positively correlated with bacterial communities associated with rhizosphere soils in our study. This result was in line with our third hypothesis that environmental factors play an important role in regulating elevational bacterial distribution pattern in rhizosphere soil of *S. chamaejasme* in the Tibetan Plateau. Our finding could be interpreted by the relevant theory of Bass-Becking hypothesis (Wang et al. 2015), which emphasizes environmental factors upon microbial community compositions, and proposes that elevational microbial community biogeographic patterns are usually structured by variety environmental parameters. Previous studies on bacterial community structures of plant rhizosphere soils have indicated that the rhizosphere soil bacterial composition and

diversity are mainly affected by soil pH (Kourteva et al. 2003; Li et al. 2016), total nitrogen (Kourteva et al. 2003; Sundqvist et al. 2014), organic matter and availability water (Teixeira et al. 2010; Li et al. 2016) and soil available P (Sundqvist et al. 2014). Our result also confirms and expands the conclusion that the environmental factors in regulating distribution patterns of rhizosphere soil microbes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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