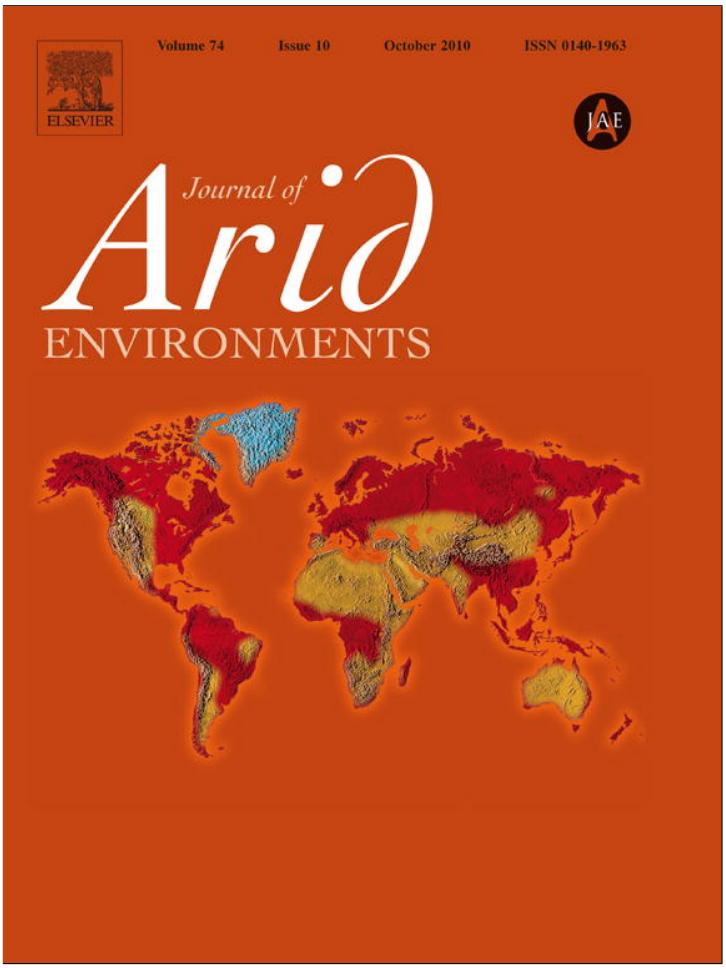

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Molecular characterization and *in situ* detection of bacterial communities associated with rhizosphere soil of high altitude native Poaceae from the Andean Puna region

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ABSTRACT

Puna is a harsh bio-geographical region in South America that characterized by distinct plant diversity, high aridity and irradiance. In the present study, rhizospheric bacterial communities were analyzed along an altitudinal gradient in this region while considering the different photosynthesis pathways (C_3/C_4) of the native grasses. This analysis included cultivation-dependent and -independent approaches such as PCR-DGGE analysis of the 16S rRNA genes and FISH. DGGE revealed that the band richness differed along the altitudinal gradient, but that it did not differ significantly among plants with different metabolic pathways. Overall, we found that *Bacillus* and *Pseudomonas* were the dominant phylotypes based on the DGGE analysis. Additionally, nearly 80% of the species identified by PCR were also identified using the cultivation method. FISH analysis revealed that the Gammaproteobacteria and Actinobacteria were the dominant bacterial groups at most sites, followed by Archaea. Finally, low bacterial diversity was detected in samples collected from all heights, possibly due to the harsh environment. Overall, evaluation of the results obtained using the two different approaches revealed that the culture method is efficient for screening of the bacterial community in Puna rhizospheric soils. Additionally, we discuss possible effects of Puna rhizospheric bacteria on the protection of native grasses and nutrient capture.

1. Introduction

Soil is a highly complex and heterogeneous environment that serves as a major reservoir of organic carbon and supports a wide variety of microbial growth. Plant roots interact with the soil through the rhizosphere, which has higher levels of microbial biomass and activity than bulk soil. Rhizospheric microbial communities are influenced by the plant exudates, roots as mechanical support and competition for nutrients. Reciprocally, plants are affected by rhizospheric microbial communities through their participation in the fast soil nutrient cycle, water dependence and efficient to capture volatile exudates (Buscot and Varma, 2005).

The composition of root exudates changes according to plant species, and different plant rhizodepositions drive the selection of microbial communities associated with the rhizosphere (Duineveld et al., 1998, 2001; Landi et al., 2006). Moreover, organic compounds present in rhizodeposits fluctuate with time, environmental features, plant age and developmental stage (Duineveld et al., 2001), root location and management practices (Paterson and Sim, 1999, 2000; Yang and Crowley, 2000).

Specific plant effects on the bacterial communities of rhizospheres have been observed in members of different plant families including *Chrysanthemum*, Asteraceae (Duineveld et al., 2001), *Brassica*, Brassicaceae; *Solanum*, Solanaceae; *Fragaria*, Rosaceae (Smalla et al., 2001); *Bromus*, *Hilaria*, *Stipa* (Kuske et al., 2002), *Alopecurus*, *Anthoxanthum*, *Arrhenatherum*, *Holcus*, Poaceae; *Plantago* Plantaginaceae; and *Geranium*, Geraniaceae (Zul et al., 2007). However, Poaceae *Lolium*, *Anthoxanthum* and *Agrostis* have not been found to affect the compositions of rhizosphere bacterial

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communities, which were instead influenced by soil features (Singh et al., 2007). Furthermore, environmental and soil characteristics have been found to influence the bacterial community diversity in the rhizospheres of other plant families (Dunbar et al., 1999; Kuske et al., 1997; Wieland et al., 2001).

Soils ecosystems are characterized by high levels of microbial diversity (Torsvik and Øvreås, 2002), but these levels differ among ecosystems. Some authors consider the tropics to have a richer diversity than boreal or temperate environments, while others suggest that deserts have similar or higher diversities than other systems (Hunter-Cevera, 1998). Among microbial communities, the soil bacterial diversity also changes across ecosystem types (Fierer and Jackson, 2006). Furthermore, there is very little knowledge available regarding the rhizospheric bacterial communities of desert plants (Chowdhury et al., 2009).

Bacterial populations play an important role in carbon and nitrogen cycling in deserts systems. In nutrient-limited soil, plant demand may exceed soil sources; thus, bacteria are essential to nutrient translocation from the soil to the roots. In addition, bacterial nutrient movements are coupled with moisture content in soil, and soil water content is a crucial factor in arid regions (Dhillon and Zak, 1993).

Puna is a harsh bio-geographical region in South America that is subject to desert conditions such as intense radiation, low relative humidity and very low annual precipitation (Cabrera, 1976). This Andean environment also has unique properties that determine the biodiversity of the region. Although the diversity of plants and animals in the region has been studied (Cabrera and Willink, 1980; Morrone, 2001), only a few studies have been conducted to evaluate microbial communities in this region (Lugo et al., 2008).

Previously, we explored the diversity of bacteria and arbuscular mycorrhizal fungi (AMF) in rhizosphere soil samples of Puna native grasses by the isolation method. A low number of different taxa were observed, and sequencing of the isolates revealed 97–99% similarity with sequences currently available in databases that are described as typical bacteria recovered from soil environments (Lugo et al., 2008). However, culture-based methods exclude non-culturable microorganisms (Wagner et al., 1993) because they select rapid growing bacteria with high growth rates or organisms that are best adapted to the growth medium used for cultivation (Garland et al., 2001; Liesack et al., 1997).

Molecular fingerprinting techniques such as DGGE (denaturing gradient gel electrophoresis) (Muyzer et al., 1993) or T-RFLP (terminal restriction fragment length polymorphism) (Osborn et al., 2000) allow analysis of large numbers of samples, which is essential for the evaluation of spatial and temporal variations in the bacterial rhizosphere community. Additionally, DGGE fingerprints of PCR-amplified 16S rDNA genes allow assessment of the diversity of community assemblages. Accordingly, PCR-DGGE has been used to study the dominant bacterial communities in the rhizosphere of many plants in a wide variety of regions to understand the microbial–plant interactions involved in cultivation practices and many others applications (Berg et al., 2002; Gundlapally and Garcia-Pichel, 2006; Smalla et al., 2001).

Additionally, FISH (Flourescence *in situ* hybridisation) enables *in situ* quantification of distinct bacterial groups, which reduces the biases associated with PCR (Hunter-Cevera, 1998; Wintzingerode et al., 1997). However, FISH is limited by the number of probes that can be used as well as possible mismatches of specific probes. Thus, a substantial proportion of the cells can remain unidentified when general probes are used (Amann et al., 1995).

In this study, we analyzed the bacterial community composition while considering altitude and the metabolic type (C_3/C_4) of the plants. We applied two complementary approaches (culture dependent and independent approaches) to assess rhizospheric bacterial diversity in Puna native grasses. All methodologies used targeted 16S rDNA extracted from the rhizosphere soil of native grasses (culture-independent approach) and from bacteria grown on R2A agar medium (culture-dependent approach), which was analyzed by PCR-DGGE and FISH.

2. Materials and methods

2.1. Sampling area and measurements

The Puna region was described previously as a highland that extends from the North West of Argentina to Southern Perú. The sampling area was located in the floristic district known as "Jujeño" or "Puna Seca", and varied in altitude from 2000 to 4400 m above sea level (masl). The study area is subject to an intense solar radiation, low relative air humidity (10–15%) and large daily thermal differences (16–20 °C), which results in a desert type climate (Martínez Carretero, 1995). The soils in the area are superficial and immature, very poor in organic matter, sandy and rocky (Cabrera, 1976) Aridisols and Entisols (Martínez Carretero, 1995).

Five sampling sites were determined along an altitudinal gradient between 3320 and 3870 masl in the Puna mountain landscape. The sampling sites were located at 22°51′35.2″, 65°13′43.71″, 23°00′06.8″, 65°22′07.3″, 22°52′14″, 65°14′25.51″, 22°53′34.1″, 65°16′0.06″ and 22°53′23.3″ 65°14′56.1″ in Jujuy and Salta provinces (Argentina). The physicochemical soil features of the sites were as follows: hygroscopic humidity (0.74–1.56%), pH (5.9 and 7.1), organic matter (0.4–2%), phosphorous (1.11–6.85 ppm), free carbonates (0.1–0.35%) and oxidizable carbon (0.22–1.15%) (Lugo et al., 2005; Strasser et al., 2005).

Rhizosphere soil (soil that immediately surrounds and is affected by the roots of a plant) samples were collected from each grass species in the 5 sampling sites (Table 1). Specifically, 5–6 of the most abundant C_3 or C_4 grass species were sampled from each site. Rhizosphere soil from 5 individual grasses belonging to each species at each sample site were mixed and then divided into aliquots that were used for DNA extraction and the cultivation of soil bacteria on R2A agar medium. In addition, an aliquot was fixed in 3.7% formaldehyde (Ravenschlag et al., 2001) for subsequent DAPI and FISH analysis. The samples were stored at −20 or 4 °C until processing.

2.2. Cultivation, DNA extractions and PCR amplification

Samples (0.5 g) were taken from each sub-sample and homogenized in 10 ml of 0.85% (wt/v) saline. The aliquots (100 μl) were then spread on R2A medium for heterotrophic organisms (Reasoner and Geldreich, 1985) and then incubated at 25 °C for 3–5 days. Microbial biomass was removed from each plate by adding 1–3 ml of buffer TE (100 mM Tris-HCl–100 mM EDTA [pH 8.0]) and scraping off all colonies. The microbial suspensions (plate-wash samples) were then transferred to micro-centrifuge tubes for subsequent DNA extraction by the CTAB method (Ellis et al., 1999) with modifications (Lugo et al., 2008). Whole DNA was extracted directly from soil samples using an Ultra Clean Soil DNA Purification Kit (Mo Bio Laboratories, USA). The quality and quantity of DNA suspensions were then evaluated by electrophoresis on a 0.8% agarose gel followed by staining with ethidium bromide.

Two approaches were used to amplify 16S rRNA gene fingerprints suitable for denaturing gradient gel electrophoresis (DGGE) analysis, one in which DNA recovered from the plate-wash samples was used (culture dependent approach) and another in which DNA recovered directly from rhizosphere soil samples (culture independent approach, hereafter referred to as soil). DGGE primers 357F-GC (*Escherichia coli* 16S rDNA positions 341–357) and 518R (*E.*

Table 1
Total number of bands obtained by PCR- DGGE of DNA from bacteria culture (cultivable) and those recovered from the rhizospheric soil samples (soil), determined at each altitude and native grasses. References: Code, arbitrary number assigned to each grass species.

Altitude (masl)	Plant	Code	Grass photosynthetic Pathway	Total number of bands	
				Cultivable	Soil
3320	*Eragrostis nigricans* var. *punensis* Nicora	19	C_4	10	11
	Bouteloua simplex Lag.	20	C_4	7	12
	Aristida adscensionis L.	21	C_4	9	15
	Muhlenbergia alopecuroides (Griseb.) P.M. Peterson & Columbus	22	C_3	8	15
	Jarava plumosula (Nees ex Steud.) F. Rojas	23	C_3	12	11
	Eragrostis nigricans var. punensis Nicora	24	C_4	7	7
Total				**53**	**71**
3370	*Eragrostis nigricans var. punensis* Nicora	25	C_4	4	4
	Cynodon incompletus var. *hirsutus* (Stent) de Wet & J.R. Harlan	26	C_4	5	5
	Jarava plumosula (Nees ex Steud.) F. Rojas	27	C_3	5	5
	Muhlenbergia alopecuroides (Griseb.) P.M. Peterson & Columbus	28	C_3	5	6
	Aristida asplundii Henrard	29	C_4	4	6
	Bouteloua barbata Lag.	30	C_4	6	6
Total				**29**	**32**
3520	*Jarava subaristata* (Matthei) Matthei	13	C_3	6	10
	Bouteloua barbata Lag.	14	C_4	5	12
	Cynodon incompletus var. *hirsutus* (Stent) de Wet & J.R. Harlan	15	C_4	4	11
	Eragrostis nigricans (Kunth) Steud.	16	C_4	5	6
	Nassella inconspicua (J. Presl) Barworth	17	C_3	10	7
	Bromus catharticus Vahl	18	C_3	4	8
Total				**34**	**54**
3700	*Bouteloua barbata* Lag.	7	C_4	5	6
	Polypogon interruptus Kunth	8	C_3	6	7
	Cynodon incompletus var. *hirsutus* (Stent) de Wet & J.R. Harlan	9	C_4	8	8
	Bromus catharticus Vahl	10	C_3	7	7
	Aristida asplundii Henrard	11	C_4	6	9
	Calamagrostis rigescens (J. Presl) Scribn.	12	C_3	5	6
Total				**37**	**43**
3870	*Bromus catharticus* Vahl	1	C_3	8	9
	Jarava pungens (Nees & Meyen) Matthei	2	C_3	8	9
	Bouteloua barbata Lag.	3	C_4	9	9
	Bouteloua barbata Lag.	4	C_4	8	9
	Danthonia annableae P.M. Peterson & Rúgolo	5	C_3	9	9
	Jarava subaristata (Matthei) Matthei	6	C_3	7	8
Total				**49**	**53**

coli 16S rDNA positions 518–534) (Muyzer et al., 1993) were used to amplify the V3 region of the 16S rDNA. The PCR reaction mixture consisted of each dNTP at 200 μM, 0.25 μM of each primer, 3 mM $MgCl_2$, 1× PCR buffer and 1 U of Taq DNA polymerase (Inbio Highway, Tandil, Argentina) in a total volume of 50 μl. Approximately 100 ng of target DNA was added to each sample as a template and PCR was conducted by subjecting the samples to the following conditions: initial denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1 min 30 s. A touch down program was performed in which the annealing temperature was decreased following each cycle until 55 °C was reached, after which 15 additional cycles were run. Finally, the samples were subjected to final extension at 72 °C for 5 min. The amplicons were visualized by electrophoresis on a 2% agarose gel after staining with ethidium bromide and then applied to a DGGE gel.

2.3. DGGE and fingerprinting analyses

DGGE was conducted using a D-Code system (Bio-Rad Laboratories, Inc., Hercules, CA). Acrylamide gel (8%) consisting of a 30–70% gradient of urea and formamide was prepared using denaturant that consisted of 40% (v/v) formamide and 7 M urea. About 20 μl (approximately 800 ng) of PCR products were then subjected to electrophoresis at a constant voltage of 100 V at 60 °C for 4.5 h in 0.5× TAE buffer (1× TAE = 0.04 M Tris base, 0.02 M sodium acetate, and 10 mM EDTA; pH adjusted to 7.4). The gel was then stained with SYBR Gold (Molecular Probes, Eugene, OR) and photographed. The DGGE fingerprints were then analyzed using the Quantity One software (version 4.3.1; Bio-Rad Laboratories). The total number of sharp bands per lane was recorded and used to calculate the absolute (number of bands detected per host species) and relative (number of bands per each host species vs. the total number of cultured or soils bands) richness at each altitude.

2.4. Sequencing of DGGE bands

For further analysis of the major resolved DGGE amplicons, selected dominant and sharp bands for each lane were carefully excised with a razor blade while under UV illumination, placed in 30 μl TE buffer, incubated for 15 min at −70 °C or overnight at −20 °C and then centrifuged at room temperature at 10 000×*g* for 3 min. Next, 0.5–2.0 μl of the samples were removed and used for re-amplification with the original primer set (without a GC clamp).

The PCR products were then purified using Wizard PCR Preps (Promega, Madison, WI) and sequenced with primer 357F. Direct sequencing of the amplification products was conducted by Macrogen, Inc. (Korea). The partial sequences were then aligned with the reference 16S rRNA gene sequence using BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997).

2.5. FISH

The fixed samples were suspended and divided into three subsamples, centrifuged at 10 000×*g* and then resuspended with 1× phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.2], 130 mM NaCl). The pellet was then washed twice with 1× PBS and finally stored in 1× PBS-ethanol (1:1) at −20 °C. Each subsample (15 μl) was applied to each well in an 8-well Teflon-coated slide and then dried in an oven at 46 °C. Next, the slides were dehydrated in an ethanol series (3 min each) of 50%, 80% and 98% ethanol, after which they were dried at room temperature. Hybridisation buffer that contained 180 μl 5 M NaCl, 20 μl 1 M Tris [pH 8], 50 μl SDS 10% and 35% formamide (for HGC63a, GAM42a and ARC915) or 50% (for EUB338-II-III and NON338) per millilitre was prepared, and 9 μl of hybridisation buffer and 1 μL of each probe were then applied to each well of the slide. The rest of the hybridisation buffer was then poured onto a paper bed in a 50 ml polypropylene tube. Next, hybridisations were conducted in a prewarmed hybridisation oven at 56 °C (for EUB330-II-III and NON338), 50 °C (for HGC63a), 47 °C (for GAM42a) and 60 °C (for ARC915) for 1–2 h. Thereafter, the slides were washed with a prewarmed washing buffer (100 mM NaCl, 20 mM Tris–HCl [pH: 8]; 0.01% SDS and 50 mM EDTA [pH: 8]) at the same temperature for 20 min. The slides were then rinsed several times with distilled water and air-dried. The following oligonucleotides were used: EUB338-II-III (Amann et al., 1995), complementary to a region of the 16S rRNA conserved in the domain *Bacteria*; GAM42a (Manz et al., 1992), complementary to a region of the 23S rRNAs of the Gamma subclass of *Proteobacteria*; HGC69a for the detection of Gram-positive bacteria with high G + C content: Actinobacteria (Roller et al., 1994); LGC for detection of Gram-positive bacteria with low G + C content: Firmicutes (Meier et al., 1999) and ARC915 for detection of *Archaea* (Manz et al., 1992). The Eub antisense probe NON338 (Manz et al., 1992) was used as a negative control for nonspecific binding. FISH using GAM42a included the labelled probe with unlabelled BET42a probe at the same concentration as a competitor (Manz et al., 1992). The oligonucleotides, which had a 5′Cy3 tag, were purchased from TIB (MOLBIOL Germany). All FISH samples were counterstained with 4′,6′-diamino-2-phenylindole (DAPI, final concentration 2 μg ml^{-1}).

2.6. FISH microscopy and documentation

Fluorescence was detected by epifluorescence microscopy using a Zeiss (Oberkochen, Germany) Axioskop and Zeiss filter set no. 01 for DAPI (excitation, 365 nm; dichroic mirror, 395 nm; suppression, 397 nm), and Zeiss filter set no. 15 for Cy3 (excitation, 546 nm; dichroic mirror, 580 nm; suppression, 590 nm). The means were calculated from 10 randomly chosen fields on each filter section (100 by 100 μm) corresponding to a minimum of 1000 DAPI-stained cells.

2.7. Statistic analyses

The absolute and relative band richness of DGGE gels were analyzed while considering altitude using the Kruskall–Wallis and the *a posteriori* test ($\alpha = 0.05$) with multiple comparisons (Marascuilo and McSweeney, 1977). The absolute and relative band richness determined using the culture and direct extraction approaches were analyzed by the Mann Whitney test considering the altitude from which each sample was collected. Furthermore, these variables were analyzed using the Mann Whitney test to compare samples from plants with different metabolic patterns (C_3, C_4). The mean relative frequencies for DAPI/FISH probes were analyzed by the Kruskal–Wallis test while considering altitude and by the Mann Whitney test when considering the plant metabolic patterns. All tests were conducted at $\alpha = 0.05$ using the Infostat version 2.0 statistical program.

3. Results

3.1. Bacterial community fingerprints

DGGE fingerprints of samples collected at five different altitudes that were obtained using the cultivation-dependent and -independent approaches were compared. The DGGE patterns of grasses sampled at 3370 and 3700 masl are shown in Fig. 1. Although some smears were observed in the individual lanes (independent samples) in addition to the 4–15 distinct bands that were present, only the sharp bands were considered when calculating the band richness. The band absolute richness determined using the culture method differed along the altitudinal gradient ($H = 16.6$, $p = 0.0019$) as did the richness determined using the culture independent method ($H = 18.73$, $p = 0.0007$) (Table 1). However, when plant metabolic patterns were compared no significant differences were observed, regardless of whether the culture method ($W = 239$, $p = 0.35$) or the culture independent method ($W = 216$, $p = 0.97$) was used. Additionally, the relative band richness did not differ significantly between samples analyzed using the culture and culture independent method along the altitudinal gradient ($H = 1.07$, $p = 0.8990$; $H = 0.21$, $p = 0.995$, respectively) or among plants with different metabolic systems ($W = 244$, $p = 0.26$; $W = 200$, $p = 0.48$, respectively) (data not shown). The band absolute richness differed significantly along the gradient when determined using the cultured method and the culture independent method ($W = 750.5$, $p = 0.0142$); however, the relative richness determined using these methods did not differ significantly with altitude ($W = 887$; $p = 0.6786$).

3.2. Diversity estimated by DGGE band sequencing

Of the 455 bands that were detected, 231 prominent bands were excised; however, 23 of these bands could not be re-amplified or sequenced. Nevertheless, most of the re-amplified bands produced legible DNA sequences. Furthermore, DGGE bands that appeared to be identical in the profiles derived using the culture dependent and independent methods also produced identical sequences (Fig. 1 and Table 2). The majority of the DGGE bands showed high levels of similarity to clones of uncultured bacteria recovered from soil environments or sequences that were closely related to organisms that were isolated from soils. The majority of sequences that were related to previously isolated organisms belonged to the defined phylogenetic groups, Gammaproteobacteria and Firmicutes (Table 2). Sequences with uncertain affiliations were obtained by DGGE when both the culture dependent and independent approaches were used, and most of these sequences had high similarities (between 97 and 99%) with database sequences of bacterial clones or DGGE bands recovered from soil environments. These environmental clones represented almost 14% of the total sequences recovered from DGGE gels using the culture approach and nearly 8% of those recovered using the culture independent approach.

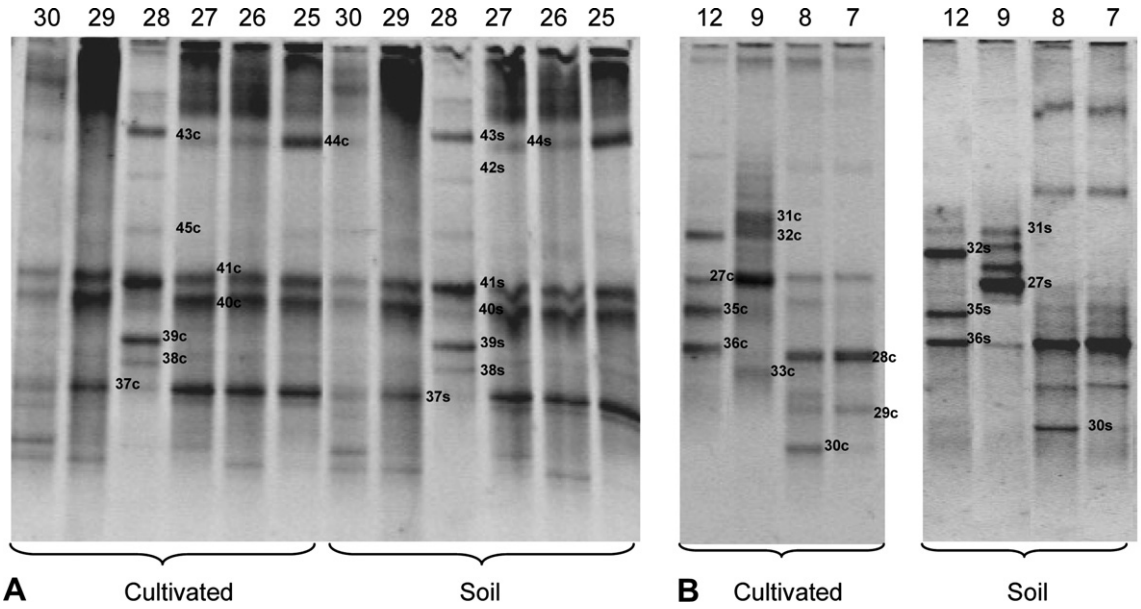

Fig. 1. DGGE profiles obtained at 3370 (A) and 3700 (B) masl. Phylogenetic affiliation based on band sequences as indicated in Table 2. Suffix "s" indicates soil extracted DNA, and "c" indicates culture extracted DNA. Lane numbers indicate plant code (reference Table 1).

In all of the sampled sites, only two previously defined phylogenetic groups (Firmicutes Gram-positive bacteria and Gammaproteobacteria) were identified among the 208 DGGE bands. Based on analysis of the partial 16S rDNA sequences obtained from DGGE bands at each altitude, Gammaproteobacteria accounted for 29–85% of the band sequences, while Firmicutes Gram-positive bacteria accounted for 1–50%. Sequences with no affiliation to a cultivated microorganism accounted for 0–44% of the samples collected along the altitude gradient. These sequences matched those of sequences previously deposited in GenBank during studies conducted using independent culture methods (Table 2) Bands that could not be amplified or sequenced comprised between 7 and 48% between all samples, however, these bands were still considered when calculating the richness (data not shown). In general, *Bacillus* and *Pseudomonas* were the dominant representatives of the Firmicutes Gram-positive bacteria and Gammaproteobacteria groups, respectively. Indeed, these sequences comprised a large proportion of sequences related to database sequences of cultured organisms and showed high homologies with previously identified organisms (97–100%). Both genera were found to be randomly distributed between altitudes and were not associated with any particular grass species. Sequences belonging to Gammaproteobacteria groups were poorly represented among sequences recovered using both approaches. Specifically, one DGGE band recovered from the rhizosphere soil of *Bouteloua barbata* and *Polypogon interruptus* at 3700 masl using both the culture dependent and independent approach was found to be *Luteibacter rhizovicinus* (identity 99%). In addition, two sequences recovered from the rhizospheres of *Cynodon incompletus* var. *hirsutus* (C4) and *Calamagrostis rigescens* (C3) using the culture dependent approach were found to be 99% homologous with *Acinetobacter*. Another sequence of *Acinetobacter* (homology = 96%) was recovered from the rhizosphere soil of *Aristida adscensionis* at 3320 masl. Furthermore, two sequence of distinct *Acinetobacter* species (*Acinetobacter* sp. and *Acinetobacter junii*, homology = 98 and 99%, respectively) were found in the rizhosphere of all grass sampled at 3870 masl using both culture dependent and independent approaches. A single band was found to represent *Xanthomonas campestris* in all samples collected from grasses at 3870 masl, regardless of which approach was used. A lower number of phylotypes than bands were observed in the rhizosphere of the grasses collected from each altitude because one bacterium often produced more than one band on the DGGE gels (Table 2).

3.3. Diversity estimated by FISH analysis

The relative *in situ* abundance of the main subclasses of bacteria in the rhizosphere soil samples was analyzed by FISH. On average (±SD), 77 ± 9% of the DAPI-stained cells were detected using the universal set of probes for bacteria (EUB + cells). Gammaproteobacteria (42 ± 4% of the DAPI count) and Actinobacteria (34 ± 8% of the DAPI count) were the dominant bacterial groups at all altitudes sampled (Fig. 2). Archaea were only detected in a few samples, and appeared to be randomly distributed between each altitude and species of grass (*Bromus catharticus*, *Danthonia annableae*, *Jarava plumosula*, *Jarava subaristata*). Firmicutes Gram-positive cells were not detected in any samples, despite the presence of many sequences belonging to the Firmicutes group being recovered by PCR-DGGE. Several attempts to detect the cells were made using modified versions of the protocol (data not shown) without improved results (Fig. 2).

The mean relative frequencies calculated for DAPI/GAM42a and DAPI/HGC65a did not differ significantly among samples collected from different altitudes ($H = 8.29$, $p = 0.08$; $H = 2.52$, $p = 0.64$, respectively) or the rhizospheres of plants with different metabolic patterns ($W = 237$, $p = 0.4$; $W = 202$, $p = 0.53$) (data not shown).

Because Gammaproteobacteria was the only phylogenetic group detected by all methods, we compared the proportion of sequences recovered by DGGE (using both the culture dependent and independent approach) and Gam42a positive cell counts determined by FISH at each altitude (Fig. 3). At 3520 and 3700 masl, the number of sequences of Gammaproteobacteria was two times greater when the DGGE approach was used when compared to direct detection by FISH; however, biases associated with the application of both techniques could explain these differences (see Discussion).

4. Discussion

In this study, we assessed the rhizospheric bacterial community of native Puna grasses using a combination of cultivation

Table 2
Assignment of taxonomic groups to band sequences extracted from a DGGE gel based on ~180 bp and the closest relatives match of known phylogenetic affiliation. References of plant code at Table 1. DGGE band numbers as indicated on Fig. 1. Suffix "s" indicates soil extraction, and "c" indicates culture extraction. Band sequences were deposited in GenBank under indicated accession numbers.

masl	Plant Code	DGGE band (Accession no.)	Phylogenetic group	Closest relatives	Accession number	Similarity (%)
3320	19, 20, 21, 23	1s, 1c (AM232764)	Gammaproteobacteria	*Pseudomonas* sp. tDp10	AJ971379	98
	19, 20, 23	2c (AM232765)	Gammaproteobacteria	*Pseudomonas* sp. 12M5	AB120337	97
	21	4s, 4c (AM232766)	Proteobacteria	Uncultured bacterium clone AKIW755	DQ129483	93
	21	5c (AM232767)	Gammaproteobacteria	*Acinetobacter* sp. DG880	AY258108	96
	19, 20, 21, 23	6s, 6c (AM232768)	Gammaproteobacteria	*Pseudomonas* sp. DDT-2	DQ241591	98
	19, 20, 23	7c (AM232769)	Gammaproteobacteria	*Pseudomonas* sp. 31	AF388027	99
3370	25, 26, 27, 29, 30	37c (AM232797)	LGC Gram positive	*Bacillus* sp. S-(s)-l-D-3(1)	AB178209	100
	28	38c (AM232798)	Proteobacteria	Uncultured bacterium clone SRS43BBA18	AF389432	98
	28	39c (AM232799)	Gammaproteobacteria	*Pseudomonas marginales* Muzt-F11	AY526699	99
	25, 26, 27, 28, 29, 30	40s, 40c (AM232700)	LGC Gram positive	*Bacillus* sp. 6IX/A01/146	AY576741	99
	25, 26, 27, 28, 29, 30	41s, 41c (AM232801)	Gammaproteobacteria	*Pseudomonas argentinensis* PA01	AY691189	100
	28	42c (AM2327802)	Gammaproteobacteria	*Pseudomonas argentinensis* PAO1	AY691189	100
	28	43s, 43c (AM2327803)	Proteobacteria	Uncultured bacterium DGGE band AHD 11b.2	UBA575898	97
	25, 26, 27	44s, 44c (AM2327804)	LGC Gram positive	*Bacillus cereus* F198–B11	DQ234856	99
	25, 28, 30	45c (AM2327805)	Gammaproteobacteria	*Pseudomonas* sp. B53	AF128872	98
3520	17, 18	10c (AM232771)	Proteobacteria	Uncultured bacterium clone KU71A	AY739681	89
	14, 16, 17	11s, 11c (AM232772)	Proteobacteria	Uncultured *Stenotrophomonas* sp. clone 10J	AY466724	97
	14, 16, 17	12s, 12c (AM232773)	Proteobacteria	Uncultured bacterium clone KU71A	AY739681	90
	15, 16, 17	13s, 13c (AM232774)	LGC Gram positive	*Bacillus mycoides*	AF234860	94
	15, 18	14s, 14c (AM232775)	Gamaproteobacteria	*Pseudomonas* sp. 31	AF388027	100
	14, 17	15c (AM232776)	Proteobacteria	Uncultured sheep mite bacterium	AF289503	100
	15	16c (AM232777)	Gammaproteobacteria	*Pseudomonas argentinensis* strain PA01	AY691189	100
	14, 17	17c (AM232778)	Proteobacteria	Uncultured bacterium DGGE band AAD 8	AF289503	99
	14, 15, 17, 18	18s, 18c (AM232779)	Gammaproteobacteria	*Pseudomonas* sp. WT OTU2	AY965247	98
3700	7, 8, 9, 12	27s, 27c (AM232788)	LGC Gram positive	*Bacillus* sp. 6IX/A01/146	AY576741	100
	7, 8, 9	28s, 28c (AM232789)	LGC Gram positive	*Bacillus* sp. 6IX/A01/146	AY576741	100
	7, 8	29s, 29c (AM232790)	Gammaproteobacteria	*Luteibacter rhizovicinus* OUCZ70	AY785744	99
	8	30s, 30c (AM232791)	Proteobacteria	Uncultured bacterium clone 300I-E04	AY661985	99
	9, 12	31s, 31c (AM232792)	Gammaproteobacteria	*Acinetobacter* sp. AG-LSL 1	DQ152256	99
	9, 12	32s, 32c (AM232793)	Gammaproteobacteria	*Acinetobacter* sp. AG-LSL 1	DQ152256	99
	9	33c (AM232794)	Gammaproteobacteria	*Acinetobacter* sp. YSNZ	DQ234065	99
	9, 12	35s, 35c (AM232795)	LGC Gram positive	*Bacillus* sp. AS-35	AY764132	100
	12	36s, 36c (AM232796)	LGC Gram positive	*Bacillus* sp. AS-35	AY764132	100
3870	1, 2, 3, 4, 5, 6	19s, 19c (AM232780)	LGC Gram positive	*Bacillus* sp. MT04	AY690686	100
	1, 2, 3, 4, 5, 6	20s, 20c (AM232781)	Gammaproteobacteria	*Acinetobacter junii* strain HQ010320B-1	AY787213	99
	1, 2, 3, 4, 5, 6	21s, 21c (AM232782)	Gammaproteobacteria	*Acinetobacter* sp. 18III/A01/072	AY576723	98
	1, 3, 4, 5, 6	22s (AM232783)	Gammaproteobacteria	*Pseudomonas putida* strain S9	AY972464	100
	1, 3, 4, 6	23s, 23c (AM232784)	Gammaproteobacteria	*Pseudomonas* sp. ps4-2	AY303295	96
	1, 2, 3, 5, 6	24s, 24c (AM232785)	Gammaproteobacteria	*Pseudomonas* sp. 31	AF388027	98
	2, 3	25s, 25c (AM232786)	Gammaproteobacteria	*Pseudomonas fluorescens*	DQ207731	99
	1, 2, 3, 4, 5, 6	26s, 26c (AM232787)	Gammaproteobacteria	*Xanthomonas campestris pv. vesicatoria*	AY604178	99

dependent and independent fingerprinting techniques. In this arid and stressed ecosystem, the phylogenetic diversity of rhizospheric bacteria was low when compared to other environments such as salt marshes (Wang et al., 2007), forests (Torsvik et al., 1990) and desert crust soils (Gundlapally and Garcia-Pichel, 2006). In a previous study, we used morphological methodology to describe the arbuscular mycorrhizal fungi and rhizospheric culturable bacterial diversity in the Puna grasses evaluated here. All of the bacteria isolates (27) identified in that study belonged to the genera *Bacillus*, *Pseudomonas* and *Arthrobacter*. Plate counts of aerobic heterotrophic bacteria on R_2A media ranged from 1.31×10^2 to 8.66×10^4 CFU g^{-1} of dry weight of rhizospheric soil (Lugo et al., 2008). In the present study, we attempted to complete the

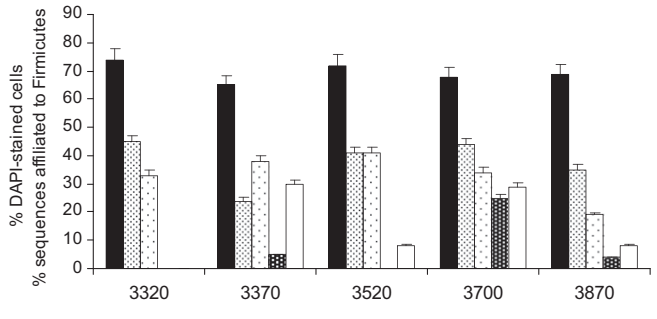

Fig. 2. Proportions of Eubacteria (▮), Gammaproteobacteria (▒), Actinobacteria (▒) and Archaea (▒) phylogenetic groups found by FISH in each altitude sample. Firmicutes (□) was added to the figure, by comparison, as a dominant group identified by cultivation-independent DGGE method (% of sequences affiliated to Firmicutes). The error bars indicate 95% confidence intervals.

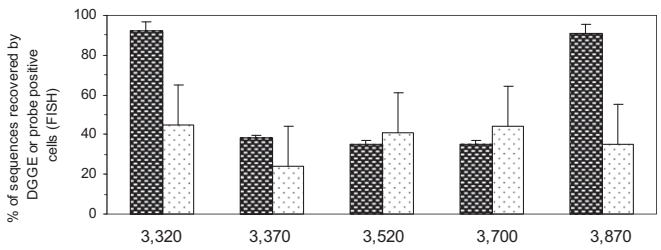

Fig. 3. Proportions of phylotypes of Gammaproteobacteria recovered from DGGE gels (▒) and Gam42a (▒) positive cells detected by FISH at different altitude. The error bars indicate 95% confidence intervals.

characterization of bacterial communities using molecular approaches and by considering the effects of important factors such as the metabolic type of the grass and altitude. Although many more bacterial entities were recorded using these techniques, the phylogenetic data reinforce the low diversity within the Puna system.

The cultivated fraction of rhizosphere soil samples accounted for almost 80% of the bacterial community recovered from soil extracted DNA in the PCR survey. Thus, the use of culture methods for DNA extraction could be useful for the analysis of the bacterial community in the Puna rhizosphere of grasses or other similar systems. In addition, most of the DGGE band sequences with phylogenetic affiliations to sequences already present in GenBank were recovered using both the culture dependent and independent approaches. However, these sequences were most closely related to cultivated organisms and primarily matched divisions containing organisms commonly associated with either soil environments or rhizospheric Gram-positive bacteria and Proteobacteria (Dunbar et al., 1999; Duineveld et al., 2001; Lugo et al., 2008).

Sequencing analysis revealed that the microbial community in the rhizosphere of native grasses was primarily composed of Gammaproteobacteria and Firmicutes, which accounted for nearly 60 and 30% of the total sequences recovered from the DGGE gels, respectively. The selection for Gammaproteobacteria on standard agar plates is a well-known phenomenon that has been observed during analysis by FISH (Wintzingerode et al., 1997). Nevertheless, in this study, the Gammaproteobacteria (especially *Pseudomonas*) represented an important fraction of cultivated bacteria and was one of the main bacterial groups detected using the culture independent method in conjunction with DGGE.

In the rhizosphere of Puna grasses, we observed seven entities of Firmicutes that were all *Bacillus* species. The high levels of *Bacillus* sequences observed in the present study is in agreement with previous descriptions of rhizospheric bacteria communities associated with *Chrysanthemum* sp. (Duineveld et al., 1998), cucumber (Mahafee and Kloepper, 1997) and pinyon pine (Dunbar et al., 1999). Additionally, *Bacillus* was primarily found to be associated with the rhizosphere of *Lasiurus sindicus*, an endemic grass in the Desert of Rajasthan, India (Chowdhury et al., 2009), Similarly, *Bacillus* have frequently been isolated from arid, semi-arid and desert soils, which may be due to its ability to switch to a dormant form under stressful conditions (Driks, 2003). Interestingly, Firmicutes were near the level of detection for FISH in all samples. However, it should be noted that all samples were treated identically to ensure that any biases occurred to the same degree throughout the analysis. In addition to the inherent biases that occurred with FISH (Amann et al., 1995), multiple factors associated with sampling and fixing procedures must be considered. Differential fixation procedures for Gram-negative and positive cells should be conducted, because the fixation schedule is an integral part of *in situ* hybridisation. Moreover, these organisms often form dormant cells or spores to enable them to survive in extreme conditions such as those found in the Puna system. These structures may prevent detection by FISH due to the changes in the cell wall that occur, which is a common phenomenon in spore forming Gram-positive bacteria (Amann et al., 1995). Furthermore, the spores of some *Bacillus* species are enclosed in an additional outer wall that contains one or more glycoproteins and approximately 20 proteins (Bárak et al., 2005; Driks, 2003), which could also interfere with FISH analysis.

Bacillus and *Pseudomonas* genera include nitrate-reducing, free nitrogen-fixing and denitrifying bacteria in soil, and nitrogen is the limited nutrient in arid environments because ammonium and nitrate movement from the soil to the plant can be restricted in dry soil and under low temperatures (Dhillon and Zak, 1993 and reference therein; Philippot and Germon, 2005). Furthermore, it has been suggested that diazotrophs among Enterobacteriaceae were associated with the rhizosphere of salt marshes grasses (Wang et al., 2007). It is well known that *Bacillus* function as mycorrhiza helper bacteria (MHB), which stimulate mycelial growth of mycorrhizal fungi or enhance mycorrhizal formation (Garbaye, 1994). Additionally, AMF interactions with MHB have synergistic beneficial effects on plant growth that occur through either nutrient solubilisation, N fixation by nitrogen-fixing bacteria symbionts or the production of plant hormones (Azcón et al., 1978). Therefore, *Bacillus*, *Pseudomonas* and *Acinetobacter* may play an important role in nutrition and the survival of native grasses in Puna.

In this study, Gram-positive Actinobacteria were only detected by *in situ* hybridisation, suggesting that the PCR-based methods showed an inherent bias against detecting this group of bacteria. In many cultivation-independent studies of the rhizosphere soil bacteria diversity, high proportions of Actinobacteria are often not found because these microorganisms tend to be underrepresented in whole DNA or RNA extractions due to difficulties in their lysis (Liesack et al., 1997; Zhou et al., 1996). However, in our study, almost 6% of the cultured isolates were identified as *Arthrobacter* (identity 99%), indicating that this group was indeed part of the bacterial community in the rhizosphere soil of Puna grasses (Lugo et al., 2008). These findings agree with the results of a study of a desert in India conducted by Chowdhury et al. (2009). Similarly, *Arthrobacter* in their stationary coccal phase were found to be resistant to drought and poor nutrient conditions for extended periods, which indicates that they are well adapted to life in arid soils and other areas subject to extreme conditions such as nuclear waste-contaminated sediments (Fredrickson et al., 2004).

The relative abundance of members of Gammaproteobacteria and Actinobacteria detected by *in situ* hybridisation was high (average 38 and 33% of the DAPI counts, respectively), while Archaea were detected in few samples and were randomly distributed between altitudes. Additionally, the presence of *Archaea* in our soil samples was restricted to few grass species. Specifically, only the rhizosphere of *B. catharticus*, *D. annableae*, *Jarava plumosula* and *J. subaristata* were found to contain Archaea. Surprisingly, samples collected from *J. plumosula* at 3370 masl comprised up to 25% of the DAPI-cell count.

Archaea were originally believed to be associated with extreme environments; however, they have since been shown to be widely distributed among diverse environments including oceans, lakes, sediments, forests and alpine tundra soils, as well as in the rhizosphere of plants (Robertson et al., 2005). Specifically, Archaea have been found in the rhizospheres of *Lycopersicum esculentum*, *Lycopodium*, *Dryopteris*, *Polystichum*, *Pinus*, *Acer*, *Viola*, *Taraxacum*, *Ribes*, *Leonurus*, *Geum*, *Pyrola*, *Carex* and particularly grasses such as *Poa* and *Festuca ovina* (Ochsenreiter et al., 2003; Sliwinski and Goodman, 2004). Archaeal populations were also found in boreal forest rhizospheres of *Alnus glutinosa*, *Betula pendula*, *Picea abies* and *Pinus sylvestris* (Bomberg and Timonen, 2009). Additionally, these populations were dominated by the nonthermophilic *Crenarchaeota* group, which is known to colonize several types of soils and rhizosphere environments (Nicol et al., 2003). Further analysis will be conducted to study this association because several authors revealed that archaeal communities were dependent on the plant rhizosphere when plants were colonized by ectomycorrhizal fungi (Bomberg and Timonen, 2009), but Puna native grasses are associated to arbuscular mycorrhizal fungi (AMF) (Lugo et al., unpublished data). In addition, it has been suggested that colonization of grasses by AMF acts as a selective pressure on the rhizosphere bacterial community (Singh et al., 2008). Therefore, it is possible that AMF may also influence the archaeal community in the

rhizosphere of grasses. Indeed, although the results of a previous morphologically based study revealed no relationships between AMF and cultured heterotrophic bacteria, that study did not consider Archaea (Lugo et al., 2008). Similarly, Puna grasses are known to be associated with arbuscular mycorrhizae (Lugo et al. unpublished data); thus, it is possible that these mycorrhizae also interact with the archaeal bacteria community in their rhizosphere.

DGGE band richness and FISH did not differ among grasses with different metabolic pathways or along the altitudinal gradient, although differences were observed in the cultivated absolute richness of samples collected from droplet sites at 3320 and 3870 m and in the soil absolute richness of samples collected at 3320 and 3370 m. Furthermore, the band absolute richness of samples recovered from 3520 m using culture methods differed from that of samples recovered using culture independent methods. The apparent heterogeneity of the microbial spatial distribution within the Puna grassland could be explained by specific micro-site features such as temperature, pH, organic matter and plant species (Giri et al., 2005; Nunan et al., 2005; Smalla et al., 2001), rather than the general factors evaluated in this study. Several authors have suggested that plant species have a major selective influence on microbial community structure under controlled conditions (Grayston et al., 1998). This is due to differential utilization of C sources taken from the rhizosphere, which may influence the proliferation of particular communities of microorganisms (Berg et al., 2002; Grayston et al., 1998). The functional diversity of microorganisms in different soils may be similar, thereby resulting in a combination of environmental and plant factors influencing which organisms are active, become culturable and proliferate under different conditions (Grayston et al., 1998). The low bacterial diversity and dominance of particular bacterial groups in the native grasses of Puna could be explained by soil poverty, which may select the most efficient bacteria capable of growth under poor nutrient conditions. This low diversity may also mirror the effect of diversity of plant species studied on rhizospheric bacterial community. Our experimental design can identify differences between species because the use of composite sample per grass species but the results involved Archaea and discriminated grass species, could suggest some individual species effect.

The entire bacterial community in the rhizosphere of Puna grasses was dominated by Actinobacteria, Firmicutes and Gammaproteobacteria, and to a lesser extent, Archaea. Among milder conditions in Scottish Borders, *Lolium perenne*, *Anthoxanthum odoratum* and *Agrostis capillaries* rhizospheric bacteria were found to be dominated by Acidobacteria and Proteobacteria, as well as a few unclassified groups (Singh et al., 2007). In arid grasslands of the Colorado Plateau region, the rhizospheres of *Bromus*, *Hilaria* and *Stipa* harboured highly diverse communities of Acidobacteria (Kuske et al., 2002). In a rapidly changing salt marsh system in China, the rhizosphere of *Phragmites australis* and *Spartina alterniflora* contained diverse and abundant Proteobacteria, including Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria, as well as low proportions of other bacterial groups such as Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes and Verrucomicrobia (Wang et al., 2007). In the desert grass, *L. sindicus*, rhizospheric bacterial communities observed after two years of drought were primarily composed of Actinobacteria followed by Firmicutes, with smaller populations of Alphaproteobacteria and Betaproteobacteria (Chowdhury et al., 2009). Thus, the bacterial communities of the rhizosphere of Puna grasses were more similar to that of bacterial communities found in desert soils.

Despite the limitations inherent to PCR-based methods, cultivation-based methods and *in situ* hybridisation biases, the results obtained using these methods appeared to be consistent in all samples collected along the gradient evaluated in this study. As expected, each method revealed partially the diversity of rhizosphere bacteria in the samples; however, some phylogenetic groups were clearly predominant and could be detected by all methods used in this study. Although the roles of the organisms represented by these sequences remain unknown, our results suggest that bacterial communities associated with the rhizosphere of native grasses play an important role in the exchange of nutrients between plants and soil in this stressed, arid and limited-nutrient environment. Accordingly, these associations likely improve the nutrient exchange between plants and soil in the rhizosphere of Puna grasses.

However, it is clear that many questions regarding the bacterial communities of the Puna grassland remain must be addressed in future studies. As a result, further analyses should be conducted to gain a better understanding of the ecology and physiology of the bacterial communities described in this study.

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