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Diversity of 16S-rRNA and *nifH* genes derived from rhizosphere soil and roots of an endemic drought tolerant grass, *Lasiurus indicus*

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ABSTRACT

Lasiurus indicus is a highly nutritive, drought tolerant, perennial grass, endemic to the Thar Desert of Rajasthan, India. In order to characterize the diversity of bacteria associated with roots of this grass that had survived severe drought stress, 16S-rRNA gene clone libraries were established from RT-PCR amplified products of the total RNA extracted from the washed roots and rhizosphere soil samples. Eight major bacterial taxa were identified in a total of 121 16S-rRNA gene clones. The majority of sequences belonged to Gram-positive bacteria, Actinobacteria being the most predominant ones, closely followed by Firmicutes. Most of the sequences showed similarity with sequences from cultivated bacteria or uncultivated environmental clones associated with arid, semi-arid environments, cold deserts and contaminated soils. PCR amplification of *nifH* genes using total DNA as template produced a total of 48 *nifH* clones from the rhizosphere soil and root samples and revealed a predominance of *nifH* sequences closely affiliated to *Pseudomonas pseudoalcaligenes*, isolated in a previous study from root samples of *Lasiurus indicus*. Some *nifH* sequences showed close similarity to cultivated diazotrophs like *Azospirillum brasilense*, *Rhizobium* sp., and a variety of uncultured nitrogen fixing bacteria. Thus, this study provides us with evidence that *L. indicus* harbors a diversity of bacteria with potential for nitrogen fixation.

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1. Introduction

Lasiurus indicus Henrard (locally known as 'sewan' grass) is one of the most common and endemic perennial grasses of the Thar Desert, India. This agronomically important grass can tolerate prolonged droughts, growing in areas with annual rainfall below 150 mm [16]. *L. indicus* has a high nutritive

value and is preferentially consumed by cattle in the desert. It plays an important role in the development of good rangeland and in stabilizing the blowing sand dunes and expanding desert [7,16]. Severe overgrazing, changes in the land-use patterns and increased aridity pose a serious threat to the survival of this grass in the delicate ecosystem of the Thar.

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There is very little knowledge on rhizosphere microbiology of desert plants, although the rhizosphere effect is qualitatively and quantitatively more pronounced in desert soils as compared to soils in a humid climate [5,27]. Since desert plants survive extreme variations in temperature and moisture, and grow in typically poor soils with low organic content and with limited amounts of bioavailable inorganic nutrients, microorganisms living in association with desert plants should possess adaptive mechanisms to cope with frequent droughts and various other stresses such as starvation, high osmolarity, high temperature and desiccation.

In recent years, many studies have addressed the importance and contribution of biological nitrogen fixation in ecologically unique terrestrial and aquatic habitats by focusing on the diversity of *nifH* sequences [30]. Such studies have provided a rapidly expanding database of *nifH* sequences and revealed a wide diversity of uncultured diazotrophs [23]. The rhizosphere microbiology of endemic grass like *L. indicus* is important in view of the *in situ* conservation of the biodiversity associated with such niches to sustain delicate ecological processes in the oligotrophic desert ecosystem.

The objective of this study was to examine the bacterial community structure present in the rhizosphere soil and roots of *L. indicus*, which had survived a period of drought, by culture-independent methods. Reverse Transcriptase PCR (RT-PCR) technique was applied to directly amplify the 16S-rRNA from the rhizosphere soil and washed roots, followed by cloning and sequence analysis. In addition the diversity of *nifH* gene was examined by PCR amplification of DNA from the rhizosphere soil and washed plant roots.

2. Materials and methods

2.1. Sample collection

Roots from *L. indicus* plants that survived three consecutive years of drought were collected from different spots (at least 8 m apart) from a sample collection site in the Jaisalmer district of Western Rajasthan, India. Root samples (15–20 cm) with adhering rhizosphere soil from each plant were transferred to separate sterile screw-capped plastic tubes and transported in ice to the laboratory and further analysis was done within 24 h.

2.2. Nucleic acid extraction

For isolation of nucleic acids a modified protocol for co-extraction of RNA and DNA [13] was used. Briefly, root samples were washed with PBS (pH 7.0, 0.1 M) to remove adhering rhizosphere soil and particles. Washed root tissues were cut into segments, frozen in liquid nitrogen and ground to a fine powder in a mortar with pestle. A total of 0.5 g of this powder and rhizosphere soil (wet weight) were separately used for nucleic acid extraction using Bio-101 Multimix 2 Matrix tubes in combination with the FastPrep FP120 bead beating system (Bio-101, Vista, CA). Extractions were performed by the addition of 0.5 ml of hexadecyltrimethylammonium bromide (CTAB) extraction buffer and 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8.0). Samples were lysed for 30 s

at a machine speed setting of 5.5 m/s (FastPrep® Instrument, BIO101, Carlsbad, CA) and the aqueous phase containing nucleic acids were separated by centrifugation (16,000 × *g*) for 5 min at 4 °C. Phenol was removed by chloroform-isoamyl alcohol (24:1). Total nucleic acids were subsequently precipitated from the extracted aqueous layer with two volumes of 30% (wt/vol) polyethylene glycol 6000 (Fluka BioChemika)-1.6 M NaCl for 2 h on ice, followed by centrifugation (18,000 × *g*) at 4 °C for 10 min. Pelleted nucleic acids were then washed in ice cold 70% (vol/vol) ethanol and air dried prior to resuspension in 50 µl of RNase free water. For extraction of RNA, nucleic acid samples were treated with RNasin® Ribonuclease Inhibitor (Promega Corporation, Madison, WI) according to manufacturer's instructions.

2.3. 16S-rRNA RT-PCR analysis

Prior to RT-PCR, RNA samples were treated with RQ1 RNase-Free DNase (Promega Corporation, Madison, WI) for removal of DNA according to the manufacturer's instructions. RT-PCR was performed using the AccessQuick™ RT-PCR System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. A pair of universal primers 616-Forward (5'-AGAGTTTGATYMTGGCTCAG-3') and 606-R-Reverse-II (5'-TRACGGSCRGTTGTA-3'), corresponding to *E. coli* 16S-rDNA binding position 8–27 and 1393–1408 respectively [4], were used for amplification of a 1.4 kb region of the 16S-rRNA coding genes. The reactions were performed on a programmable Primus 25 PCR-System Thermal Cycler (MWG-Biotech AG, Ebersberg, Germany). The one-tube PCR cycle consisted of a reverse transcription step of 48 °C for 45 min, followed by initial denaturation of cDNA for 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C with a final extension of 5 min at 72 °C. The amplified product was visualized by UV excitation in 0.8% agarose gel after staining with ethidium bromide (0.5 mg l⁻¹).

2.4. *nifH* gene amplification

PCR amplification of *nifH* gene fragments from the rhizosphere soil and root DNA was performed. To obtain pure DNA, nucleic acid mixture was incubated at 37 °C with RNase A (Promega Corporation, Madison, WI) at a final concentration of 100 µg ml⁻¹ for 10 min. A pair of universal degenerate primers [29] Zehr-*nifHf* (5'-TGYGAYCCNAARGCNGA-3') and Zehr-*nifHr* (5'-ADNGCCATCATYTCNCC-3'), were used to amplify a 360 bp fragment of the *nifH* gene. These primers have been widely and successfully used to amplify *nifH* gene from distantly related diazotrophs [23]. The samples were amplified in a PCR mixture containing 4 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate, 100 picomoles of each primer, 2.5 U of *Taq* DNA polymerase with 1× buffer with (NH₄)₂SO₄ (MBI Fermentas, Vilnius, Lithuania) in 50 µl volumes for 40 cycles at 94 °C for 1 min, 57 °C for 2 min and 72 °C for 2 min on Primus 25 PCR-System Thermal Cycler (MWG-Biotech AG, Ebersberg, Germany).

2.5. Cloning and sequencing reactions

PCR products were cloned using the TOPO TA Cloning kit (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's

instructions. Clone libraries were constructed in vector pCR2.1-TOPO and positive clones were selected. Plasmids were prepared using Nucleospin Plasmid kit (Macherey Nagel GmbH, Düren, Germany) according to manufacturer's instructions. Clones were screened for the correct size of the insert by EcoRI digestion. A 2 µl aliquot of the purified plasmid was used for restriction digestion with EcoRI (New England Biolabs Inc., USA) at 37 °C for 3 h. Enzymes were inactivated by heating the reaction mixture to 65 °C for 10 min. The reaction products were analyzed on a 1.5% agarose gel at 5 V cm⁻¹ for 4 h and visualized by UV excitation after staining with ethidium bromide (0.5 mg l⁻¹). The plasmids were quantified using ND-1000 a Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Sequencing was performed from both strands using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Rotkreuz, Switzerland) using M13 reverse and forward primers. Sequencing was done on ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

2.6. Phylogenetic analysis

The 16S-rRNA coding gene sequences obtained from the plasmid sequencing were added to an existing database of small-subunit rRNA gene sequences by using the fast alignment tool implemented in the ARB software package [19] (<http://www.arb-home.de>). Alignments were manually examined. Phylogenetic analyses were performed by applying maximum likelihood, maximum parsimony, and neighbor joining methods by use of respective tools in the ARB software package. The *nifH* sequences obtained from the clones were compared with the sequences available in the GenBank database to using the Basic Local Alignment Search Tool, BLAST [1], at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST>). The program ClustalW [8]; (<http://www.ebi.ac.uk/Tools/clustalw>) was used for multiple alignments of translated NifH protein sequences. The basic information they provide is identification of conserved sequence regions.

2.7. Statistical analysis of 16S-rRNA gene clone libraries

Clones sequences with >97% sequence similarity to previously published data available at NCBI (<http://ncbi.nlm.nih.gov>) were clustered into operational taxonomic units (OTUs) and a number of diversity indices were calculated. These indices included: (i) library coverage calculated by the equation $C = [1 - (n/N)] * 100$, where n is the number of unique clones and N is the total number of clones examined [11]; (ii) species richness (S) or the total number of OTUs [3]; (iii) the Shannon–Weaver diversity index, calculated by the equation $H = - \sum p_i * \ln(p_i)$, where p_i is the relative contribution of an individual OTU i , to the whole library [21]; (iv) the Simpson's index, calculated by the equation $D = 1 - \sum (p_i)^2$ [22]; and (v) evenness, calculated from the Shannon diversity function by using the equation $J = H/H_{max}$, where $H_{max} = \ln S$.

2.8. Nucleotide sequence accession numbers

All sequences determined in this study are available at GenBank under the accession no. DQ298259 to DQ298321 (16S-rRNA sequences from rhizosphere soil clones),

DQ298322 to DQ298379 (16S-rRNA sequences from plant root clones), DQ304814 to DQ304861 (*nifH* clones).

3. Results

3.1. 16S-cDNA clone libraries

16S-rRNA RT-PCR products (1.4 kb), amplified from the total RNA extracted from rhizosphere soil and washed root samples, were used to establish 16S-rDNA gene clone libraries. Products from three separate amplification preparations were pooled prior to cloning in order to minimize PCR drift [24]. A total of 63 clones obtained from the rhizosphere soil (SR) and 58 clones from root samples (PR) were analyzed by comparative sequence analysis. The number of clone sequences and phylotypes for each bacterial taxon is presented in Table 1. Selected clone sequences representing dominant phylotypes are listed in Table 2 with affiliation to higher taxa, number of clones per phylotype and percentage identity to the closest related sequence.

3.2. Identification and distribution of 16S-cDNA clones

A total of eight major bacterial taxa were identified by analyzing the 16S-cDNA sequences of 121 clones. Altogether, the phylotypes belonging to Actinobacteria were the predominant ones (43.1% of the total rhizosphere soil phylotypes and 44.8% of the total root associated phylotypes), followed by Firmicutes (31.8 and 14.2%), β -Proteobacteria (9.0 and 10.5%) and α -Proteobacteria (6.1 and 13.15%). Phylotypes affiliated to γ -Proteobacteria, δ -Proteobacteria, Bacteroidetes and Acidobacteria were only observed in the library of root inhabiting community (Table 1). Phylotypes of unclassified bacteria were observed only in the clone library from rhizosphere soil.

Table 1 – Number of phylotypes and 16S-cDNA clones from rhizosphere soil and washed roots of *L. sidicus* for each of the bacterial taxa observed in the two clone libraries

Bacterial division/subdivision	Total	Rhizosphere soil	Washed roots	Common in both rhizosphere and plant root
Total	85 ^a /121 ^b	44/63	49/58	8/22
Actinobacteria	37/47	19/31	22/26	4/10
Firmicutes	19/22	14/21	7/9	2/8
α -Proteobacteria	6/7	2/2	5/5	½
β -Proteobacteria	7/7	3/3	4/4	–
γ -Proteobacteria	1/1	–	1/1	–
δ -Proteobacteria	1/1	–	1/1	–
Bacteroidetes/ Chlorobi	2/2	–	2/2	–
Acidobacteria	1/2	–	½	–
Environmental samples	11/11	6/6	6/7	½

a Phylotypes: clones with sequence identities higher than 97%.

b Clones.

Table 2 – 16S-cDNA clones and their closest affiliation in the database

Closest GenBank sequence (accession number)	Clones from rhizosphere soil (percentage of similarity)	Clones from washed roots (percentage of similarity)	Source of closest sequence
Gram-positive Actinobacteria; Actinomycetales; Micrococcaceae			
<i>Arthrobacter</i> sp. LMG 20239 (AJ316305)	SR11 (97.4), SR16 (97.2), SR50 (97.9)		Bio deteriorated painting
<i>Arthrobacter atrocyaneus</i> strain SMW-1 (AY605543)	SR15 (95.9)	PR46 (92.5)	Metacrate-degrading, contaminated soil
<i>Arthrobacter</i> sp. R-23173 (AJ786821)	SR26 (99.4)		Commercial nitrifying inoculum
<i>Arthrobacter nitroguajacolicus</i> (AJ512504)	SR27 (95.7)		4-nitroguaiacol-degrading, contaminated soil
<i>Arthrobacter</i> sp. AG1 (AY651317)	SR30 (92.9)		Atrazine-degrading, contaminated soil
<i>Arthrobacter</i> sp. M4 (AY177360)	SR44 (99.10)		Phenanthrene-degrading, contaminated soil
<i>Arthrobacter</i> sp. NSA34 (AB177880)	SR53 (99.9), SR59 (99.9)		Dibenzofuran-degrading, contaminated soil
<i>Arthrobacter</i> sp. 83b (AJ879123)	SR68 (94.8)		2-methylpyridine degrading, contaminated soil
Actinomycetales; Actinomycetaceae			
Actinomycetaceae bacterium (X87310)		PR67 (97.6)	Soil
Actinomycetales; Microbacteriaceae			
<i>Microbacterium chocolateum</i> (AM181503)	SR69 (98.1)	PR17 (95.0)	Culture contamination
<i>Microbacterium</i> sp. PHD-5 (DQ227343)		PR65 (92.8)	Phenol-degrading, contaminated soil
Actinomycetales; Streptomycetaceae			
<i>Streptomyces steffisburgensis</i> (AB045889)		PR30 (94.4)	Soil
<i>Streptomyces misionensis</i> (AB184285)		PR33 (94.7)	Soil
Actinomycetales; Nocardioideae			
<i>Nocardioides simplex</i> (AF005013)	SR47 (95.4)		Soil
<i>Nocardioides albus</i> (AF004997)	SR67 (98.8)	PR58 (96.5), PR64 (98.8)	Soil
<i>Nocardioides</i> sp. V4.BE.17 (AJ244657)	SR46 (97.0), SR52 (98.2), SR60 (97.2)		Marine
<i>Nocardioides fulvus</i> (AF005016)		PR66 (95.1)	Soil
<i>Nocardioides aromaticivorans</i> (AB087721)	SR51 (95.4), SR62 (95.1), SR70 (95.5)		Dibenzofuran-degrading, polluted soil
<i>Aeromicrobium alkaliterrae</i> (AY822044)		PR56 (97.7)	Alkaline soil
<i>Kribbella</i> sp. HD9 (AY253866)		PR52 (95.6)	Soil
<i>Kribbella alba</i> (AY082062)	SR17 (92.6),		Soil
<i>Marmoricola</i> sp. CNJ780 PL04 (DQ448720)	SR38 (93.8)	PR16 (97.3), PR62 (97.3)	Marine sediment
Actinomycetales; Micromonosporaceae			
<i>Catellatospora citrea</i> (AF152106)	SR42 (94.9)		Soil
Actinomycetales; Promicromonosporaceae			
<i>Isoptericola variabilis</i> (AJ298873)	SR71 (98.6)		Cellulolytic bacterium, Termite gut
Actinomycetales; Pseudonocardiaceae			
<i>Pseudonocardia saturna</i> IMSNU (AJ252829)	SR24 (92.1)		Soil
<i>Pseudonocardia benzenivorans</i> (AJ556156)		PR40 (96.5)	Tetrachlorobenzene contaminated soil
<i>Pseudonocardia</i> sp. CNS139 PL04 (DQ448726)		PR31 (96.0)	Marine sediment
<i>Kibdelosporangium aridum</i> (AJ311174)		PR11 (97.3)	Arid soil
<i>Amycolatopsis fastidiosa</i> AS 4.1172 (AY389140)		PR37 (93.2)	Soil
Actinomycetales; Actinosynnemataceae			
<i>Lechevalieria aerocolonigenes</i> (AB020030)		PR10 (98.8), PR49 (92.9)	Soil
<i>Lechevalieria flava</i> (AF114808)		PR7 (98.6)	Soil
Actinomycetales; Corynebacteriaceae			
<i>Corynebacterium cyclohexanicum</i> (AB210282)	SR20 (95.7), SR32 (98.4), SR35 (98.6), SR45 (98.5), SR48 (98.5), SR56 (98.2)		Cyclohexanecarboxylate-degrading, contaminated soil

(continued on next page)

Table 2 (continued)

Closest GenBank sequence (accession number)	Clones from rhizosphere soil (percentage of similarity)	Clones from washed roots (percentage of similarity)	Source of closest sequence
Actinomycetales; Geodermatophilaceae <i>Geodermatophilus obscurus</i> (L40620)	–	PR26 (97.5)	Plant
Actinomycetales; Mycobacteriaceae <i>Mycobacterium goodii</i> (AY457079)	–	PR48 (98.8)	Human wound
Actinobacteria; environmental samples Uncultured Actinobacteria bacterium clone AKYG500 (AY921685)		PR25 (95.2), PR63 (95.7)	Farm soil
Uncultured bacterium clone MB-A2-100 (AY093455)		PR35 (93.3)	Methane hydrate-containing marine sediment
Uncultured bacterium clone C-F-4 (AF443580)		PR36 (99.12)	Semi-arid soil
Firmicutes; Bacillales; Bacillaceae <i>Bacillus pumilus</i> (AY030327)	SR1 (99.9)		Spacecraft assembly facilities
<i>Bacillus</i> sp. LMG 20241 (AJ316313)	SR7 (98.1)		Biodeteriorated painting
<i>Bacillus maegaterium</i> (DQ267829)	SR8(99.5)		Soil
<i>Bacillus</i> sp. No.49 (AB066347)	SR36 (99.4)		Compost
<i>Bacillus endophyticus</i> (AF295302)	SR25 (97.3)	PR47 (99.2)	Cotton endophyte
<i>Bacillus</i> sp. LMG 19490 (AJ315058)	SR10 (99.1), SR13 (99.1), SR33 (98.3)		Biodeteriorated painting
<i>Bacillus</i> sp. LMG 19415 (AJ276809)	SR18 (97.0)		Biodeteriorated painting
<i>Bacillus firmus</i> (AY833571)	SR12 (96.3), SR21 (98.0), SR55 (99.5)		Epilithic biofilms
<i>Bacillus niacini</i> strain SAFN-019 (AY167811)	SR37 (99.1), SR39 (99.6), SR40 (99.5), SR61 (99.3)	PR18 (97.3), PR39 (99.2)	Spacecraft assembly facilities
<i>Bacillus pichinoty</i> strain RS2 (AF519464)	SR43 (99.7)		Soil
<i>Bacillus</i> sp. R3 (AY939830)	SR9 (99.4)		Glyphosate resistant, contaminated soil
<i>Bacillus soli</i> (AJ542513)	SR66 (98.2)		Soil
<i>Bacillus</i> sp. KL-152 (AY030333)		PR1 (99.6)	Spacecraft assembly facility
<i>Bacillus humi</i> (AJ627209)		PR4 (99.1)	Soil
<i>Bacillus</i> sp. Str 86348 (AF227865)	–	PR19 (95.7)	Environmental isolate
<i>Bacillus sphaericus</i> (AB116123)		PR32 (95.3)	Compost
<i>Bacillus cereus</i> isolate PN24 (DQ423485)		PR42 (98.7), PR44 (98.9)	Polyethylene degrading, contaminated soil
<i>Bacillus barbaricus</i> (AJ422145)	SR6 (98.1)		Wall painting
Firmicutes; environmental samples Uncultured firmicute clone SM1E07 (AF445685)	SR65 (96.4)		Thermal spring
Bacteria; environmental samples Uncultured soil bacterium clone 528-1 (AF423273)	SR29 (96.7)		Soil
Uncultured soil bacterium clone 432-1 (AF423263)		PR8 (96.4)	Soil
Uncultured soil bacterium clone 1200-1 (AF423214)		PR9 (98.1)	Soil
Uncultured bacterium clone X20 (DQ083105)		PR14 (96.3)	Soil
Uncultured bacterium (AJ604545)		PR27 (97.6)	Fungus (<i>Folsomia candida</i>)
Uncultured bacterium clone AKIW574 (DQ129514)	SR14 (93.4)	PR43 (98.9)	Urban aerosol
Uncultured bacterium clone AKIW1098 (DQ129555)	SR22 (99.1)		Urban aerosol
Uncultured organism clone ctg_CGOGA47 (DQ395956)	SR57 (96.1)		Marine
Uncultured bacterium clone AKIW421 (DQ129533)	SR63 (98.3)		Urban aerosol
Unclassified bacteria Bacterium 7A1 (DQ298783)		PR55 (99.1) PR61 (99.0)	Community exposed to nutrient flux
Uncultured candidate division SPAM bacterium (AY921949)	SR31 (94.1)		Farm soil
Gram-negative Proteobacteria: α-Proteobacteria Uncultured soil bacterium clone TD3 (DQ248308)	SR2 (98.2)	PR22 (98.4)	Carbon tetrachloride contaminated soil
<i>Methylobacterium</i> sp. AC72a (AY776209)	SR34 (96.0)		Legume species, arid soil
Uncultured alpha proteobacterium clone (AY922042)		PR12 (97.6)	Soil
Uncultured alpha proteobacterium clone (AY921875)		PR38 (93.0)	Soil
Uncultured bacterium clone (DQ125843)		PR41 (97.4)	Uranium contaminated soil
<i>Rhizobium galegae</i> strain NBIMTC 2246 (Z79620)		PR50 (93.0)	Plant

Table 2 (continued)

Closest GenBank sequence (accession number)	Clones from rhizosphere soil (percentage of similarity)	Clones from washed roots (percentage of similarity)	Source of closest sequence
β-Proteobacteria			
Uncultured bacterium clone (DQ129594)	SR3 (98.5)		Urban aerosol
Uncultured <i>Burkholderia</i> sp. clone (DQ279344)	SR5 (99.1)		Plant tuber
<i>Schlegelella thermodepolymerans</i> strain SA8 (AY538708)	SR58 (96.8)		Thermotolerant, hot compost
<i>Ralstonia</i> sp. A-471 (AF525456)		PR6 (98.1)	Moderately thermophilic, chitin-degrading bacterium, crab waste compost
<i>Ramlibacter henchirensis</i> (AF439400)		PR24 (98.8)	Subdesert soil in Tunisia
<i>Rastonia</i> sp. PHS1 (AF239160)		PR34 (99.7)	Thermotolerant bacterium, hydrocarbon contaminated soil
<i>Ramlibacter tataouinensis</i> TTB310 (AF144383)		PR59 (98.7)	Tataouine desert, Tunisia
γ-Proteobacteria			
Uncultured γ -Proteobacterium clone (AY922146)		PR23 (97.4)	Soil
δ-Proteobacteria			
<i>Thaxtera salina</i> / <i>Enhygromyxa salina</i> (AB097591)		PR5 (98.6)	Halophilic, coastal soil
Bacteroidetes/Chlorobium group Bacteroidetes; Sphingobacteria; environmental samples			
Uncultured <i>Cytophagales</i> bacterium clone (AY250875)		PR21 (96.2)	Antarctic cryptoendolithic community
<i>Pontibacter actiniarum</i> strain KMM 6156 (AY989908)		PR29 (95.1)	Marine coelenterate
Fibrobacteres/Acidobacteria group Acidobacteria; environmental samples			
Uncultured <i>Acidobacteria</i> bacterium clone (AY921811)		PR3 (97.6), PR15 (97.4)	Soil

Only eight phylotypes (9.4% of the total number of phylotypes analyzed) were found in both rhizosphere soil and root RNA clone libraries (Table 1). These phylotypes accounted for 18% of the total sequences and did not represent the most abundant ones within the respective phylogenetic groups. A number of sequences showed similarity to those from uncultured environmental clones (12.9% of the total number of phylotypes). 16S-cDNA of *Firmicutes* were more abundantly retrieved from rhizosphere soil (21 clones) as compared to washed roots (nine clones). In contrast, 16S-cDNA of proteobacteria was more frequently obtained from washed roots (15 clones) as compared to rhizosphere soil (five clones).

3.3. Statistical analysis and diversity indices

Statistical analyses showed that expected number of phylotypes was higher in the root associated clone library than the rhizosphere soil clone library. Shannon–Weaver diversity indices (3.848 for the root associated library and 3.601 for rhizosphere soil clone library) were consistent with the Simpson diversity indices (0.005 for the root associated library and 0.019 for rhizosphere soil clone library). The Shannon evenness index was 0.989 and 0.952, respectively. The species richness calculated as the number of phylotypes represented by the libraries was found to be higher in case of the root associated clone library than the rhizosphere soil clone library (49 and 44 respectively). The coverage of libraries was calculated to assess the completeness of the clone libraries. Coverage was higher in case of rhizosphere soil library (42.8%) compared to the root associated library (29.3%).

3.4. Phylogenetic assignment of 16S-rRNA sequences

High and low G + C Gram-positive bacteria were represented quite frequently in the clone libraries (Tables 1 and 2). Low G + C Gram-positive bacteria belonged mostly to different species of the genus *Bacillus* (18 sequences). High G + C Gram-positive bacteria (37 sequences) were mainly affiliated to different families of *Actinomycetales* isolated from various environments. The most abundant genus was *Arthrobacter* (eight sequences). Only six sequences retrieved from the rhizosphere soil library and 15 sequences from the root associated library were related to Gram-negative bacteria. Several clones clustered with sequences from uncultured bacterial clone sequences obtained in other studies. Gram-negative bacteria formed distinct lineages and were represented by α -, β -, γ -, δ -Proteobacteria. Two clones affiliated to the phyla *Acidobacteria* and *Bacteroidetes* (Table 2) were present in the rhizosphere soil library.

3.5. Phylogenetic analysis of *nifH*-DNA clone library

PCR products with the correct size were amplified from the total DNA extracted from rhizosphere soil and washed root samples and used to establish *nifH* clone libraries. A total of 48 clones obtained from rhizosphere soils (25 clones, named as S1 and S2 series respectively) and root samples (23 clones, named as Pn and P2 series respectively) were analyzed by sequencing. The sequences and their closest affiliations with known diazotrophs on the basis of sequence similarity are listed in Table 3.

Table 3 – List of *nifH*-DNA clones^a obtained and their relatedness to closely affiliated known *nifH* nucleotide sequences and their translated amino acid sequences from databases

Clone (number of sequences)	Nearest relative	Accession number	% Identity nucleotide	% Identity amino acids
S2n (1)	<i>Pelomonas saccharophila</i> strain IAM 14368	AB188120	98	98
S2b, S2c, S2k, S2l, S2m, S2s, S1a, S1b, S1c, S1d, S1h, S1i, P2c, P2i, Pn1, Pn12 (16)	Uncultured microorganism clone H01_RNA_A10	EF568537	96–97	100
S2e, S2o, P2d, Pn3, Pn5, Pn6, Pn 7, Pn13 (8)	Uncultured bacterium clone MINH-43A	AJ716399	96–98	100
S2i, S2p, Pn4, P2g (4)	Uncultured bacterium clone MINH-10B	AJ716391	96–99	100
P2f (1)	<i>Methylosinus trichosporium</i>	AF378724	98	98
P2a (1)	<i>Sinorhizobium</i> sp. TJ170	AJ505315	98	100
S1e (1)	<i>Azohydromonas australica</i> IAM 12664	AB188121	97	100
P2b (1)	<i>Azorhizobium caulinodans</i>	AB303393	99	99
Pn2 (1)	<i>Methylococcus capsulatus</i>	AJ563958	96	98
Pn10, P2j (2)	Uncultured bacterium clone IPA64	EU048006	94–95	97–99
S2a, S2d, S2g, S2j (4)	<i>Azospirillum brasilense</i>	X51500	98–100	99–100
Pn9 (1)	<i>Methylobacterium nodulans</i>	AJ512205	99	100
S2h, Pn8, Pn11 (3)	Uncultured soil bacterium clone 1GA04-26	DQ776514	84–87	94–97
S2f (1)	Uncultured bacterium clone M5b-39	AY819566	96	99
P2e (1)	Uncultured nitrogen-fixing bacterium clone g1-HW4	AY196408	98	98
P2h (1)	Uncultured nitrogen-fixing bacterium clone A101	DQ098179	91	98
S1g (1)	Gamma Proteobacterium BAL286	AY972875	98	98

a Rhizosphere soil clones are designated as series S1 and S2 and root associated clones as series Pn and P2 respectively.

Most of the sequences from our study (35/48; 72.9%) clustered in the subcluster 1K of the *nifH* cluster I [30]. About 58.3% (28 out of 48) of the obtained sequences were highly similar and shared a 99–100% amino acid sequence identity with each other. They were closely affiliated to the *nifH* sequences from uncultured bacterium clones in the database (Table 3). Another 22.9% (11/48) sequences from both the rhizosphere and roots were related with *nifH* sequences of known cultivated diazotrophs. The others showed phylogenetic affiliations with *nifH*-clones of yet uncultivated nitrogen fixing bacteria.

4. Discussion

In the present study we have used a culture-independent approach to analyze the bacterial community that was present in the rhizosphere soil and roots of *L. indicus* grass which suffered a prolonged period of drought. Even though the grass was dry, the underground roots harbored diverse bacteria representing a broad phylogenetic spectrum. Whereas the ribosomal RNA clone library was dominated by Actinobacteria and Firmicutes and had only little representation from proteobacteria, the *nifH* clone library showed a predominance of proteobacteria. While we could derive 16S-rRNA gene libraries from roots and rhizosphere soil successfully, it was not possible for *nifH* RNA. The copy number of 16S-rRNA is usually very high in metabolically active cells, whereas the *nifH* mRNA is

more labile and exists in much lower copy number. Since the samples could not be frozen in dry ice, immediately after sampling in the desert, most of the *nifH* mRNA was presumably degraded during transportation of the samples to the laboratory. As DNA is much more stable, *nifH*-DNA clone libraries could be constructed successfully by direct amplification using total DNA as template.

The majority of sequences obtained from the 16S-rRNA clone libraries belonged to Gram-positive bacteria (Table 1). Gram-positive spore-formers have been found to be dominant in the Thar Desert, with significant populations even during the harsh summers (up to 50 °C at the soil surface), of which Actinomycetes may constitute about 50% of the total bacterial population [20]. The Firmicutes were mainly dominated by different species of *Bacillus*, which have been frequently isolated from arid, semi-arid and desert soils and switch to dormant forms as a strategy for surviving under arid conditions [2]. The Actinobacteria were mostly represented by genera commonly found in soils. Most of the clone sequences showed close similarities with sequences obtained from contaminated soils and belonging to nutritionally versatile bacteria using a variety of substrates in their oxidative metabolism including, nicotine, nucleic acids, and various herbicides and pesticides (Table 2). The unusually high abundance of the sequences of Actinobacteria in the clone libraries indicates that high-G + C Gram-positive bacteria might play a more dominant role in the rhizosphere than was previously believed [12].

Most of the obtained 16S-rRNA gene sequences representing Gram-negative bacteria (proteobacteria) showed similarity with sequences from uncultured bacterial clones obtained from various contaminated soils. Only few cultivable genera were represented, which are either commonly associated with rhizosphere of grasses or are halophilic/thermophilic/thermotolerant (Table 2). Interestingly, within the Gram-negative bacteria, a single sequence each showed similarity with *Methylobacterium* sp. (isolated from the root nodules of *Phaseolus vulgaris* from Ethiopia) [26], and with a *Rhizobium galegae* strain (isolated from wild legumes in Tunisia) [28], both of which are arid regions. We also obtained two sequences, which showed similarity with *Ramlibacter tataouinensis* and *Ramlibacter henchirensis*, isolated from the sand samples of Tataouine Desert [6,15]. Our results corroborate the fact that such bacteria possibly have the ability to colonize arid and stressful environments. Only a few of the 16S-rRNA sequences indicate the presence and actual activity of potential nitrogen fixing bacteria. This reflects that under the conditions of sampling during a severe drought period, only very minor fractions of the active bacterial diversity are diazotrophs.

Most of the sequences obtained in this study show similarities with sequences from cultivated or uncultivated environmental samples associated with arid, semi-arid environments, cold deserts and contaminated soils. A correlation exists between all these types of environments with respect to the adaptability of microbes to water stress. It is evident from our results that the plant root associated clones showed a greater diversity and represented sequences from α -, β -, γ -, δ -proteobacteria, *Bacteroidetes* and *Acidobacteria* (Table 1). Thus, it appears that the roots in the desert plants might offer a milder and more hospitable microenvironment for the survival of bacteria than the rhizosphere soil, where e.g. *Actinobacteria* are most frequent (Table 1).

Measures of the diversity among the clone libraries revealed that the levels of diversity in the rhizosphere soil and the plant roots were significant and comparable. Relatively similar values for phylotype richness and evenness were obtained. However, the taxa present in the rhizosphere soil library were absent from the root library and vice versa. The coverage of the two libraries represented an approximate estimate of the diversity, which was calculated relative to total richness. High phylogenetic diversity reported in our study is in agreement with other studies of such soils [6,10,17].

A comparison of the sequences obtained from the *nifH* clones described in this study with that obtained from culturable diazotrophs associated with *L. indicus* [9] revealed common genera like *Azospirillum* and *Rhizobium*. The predominance of *nifH* clones related to *nifH* sequences from *Proteobacteria* and the absence of *nifH* clones related to sequences from *Cyanobacteria*, *Firmicutes* and *Archaea* has also been reported from a study of Nickel mine soils revegetated with *Gymnostoma webbianum* and *Serianthes calycina* [14], from dead aboveground biomass of *Spartina alterniflora* [18] and Douglas fir forest site [25]. Most interestingly, the *nifH* sequence from the bacterial isolate 3R1 obtained from root samples of *Lasiurus indicus* of the Thar desert [9] (identified as *Pseudomonas pseudoalcaligenes*) showed approximately 99% nucleotide similarity and 100% amino acid sequence similarity with 28 other *nifH* DNA sequences, retrieved from root and rhizosphere soil samples. A ClustalW

alignment of the translated products of these 28 *nifH* sequences with that from isolate 3R1 (*Pseudomonas pseudoalcaligenes*) showed that they are highly conserved. Thus, it is possible, that *Pseudomonas pseudoalcaligenes* is one of the culturable, more abundant diazotrophs associated with *L. indicus*. However, reinoculation and colonization studies are needed to demonstrate that *Pseudomonas pseudoalcaligenes* is the most dominant endophyte associated with this grass.

The 'sewan' rangelands are marked by the absence or scanty presence of wild legumes and by natural regeneration of grasses in the absence of external input of nitrogen. These unique features of the Thar Desert ecosystem could possibly have restricted the diversity of diazotrophs and allowed the selection of some unique ones. The diversity of *nifH* sequences present in the *nifH* clone libraries from roots and the rhizosphere indicated that *Pseudomonas pseudoalcaligenes*-related *nifH* genes may be very important, together with the other characterized diazotrophs like *Azospirillum brasilense*, and *Rhizobium* sp. This study provides us with a glimpse of the probable nitrogen fixers associated with *Lasiurus indicus*, endemic to the Thar Desert ecosystem. Further studies targeting the metabolically active diazotrophs could provide us with evidences for *in planta* nitrogen fixation in this agronomically important grass.

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