

## MICROBIAL DIVERSITY AND METAGENOMIC ANALYSIS OF THE RHIZOSPHERE OF PARA GRASS (*UROCHLOAMUTICA*) GROWING UNDER SALINE CONDITIONS

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### Abstract

Para grass is a salt tolerant plant, grown on salt affected soils of Punjab, Pakistan. The aim of this study was to investigate the distribution of culturable and non-culturable bacteria in the rhizosphere, rhizoplane and histoplane of para grass, growing under saline conditions. A total of seventy four, bacterial strains were isolated and characterized. Among these, thirty two from rhizosphere, twenty two from rhizoplane and twenty were from the histoplane. Cultureable bacteria were characterized by biochemical tests and 16S rRNA gene sequence analysis. Non-culturable bacteria were identified by PCR amplification of 16S rRNA gene, using metagenomic approach. Seventy seven percent bacterial isolates from rhizosphere and rhizoplane fractions were identified as member of Proteobacteria. Twenty five percent isolates of histoplane fraction were members of firmicutes while 68.75% were of Proteobacteria. Of total isolates, 50% could grow in nitrogen free medium and 21.67% on halophilic medium. Nitrogen fixers and halophilic bacteria were more abundant in the rhizosphere as compared to roots. 16S rRNA gene clone library analysis showed that out of 48 clones, 14 were uncultured, classified; eighteen un-cultured un-classified, while others related to 16 different known cultured groups of bacteria. Results for cultured and uncultured bacteria revealed a wide diversity of bacterial population present in the rhizosphere of para grass.

**Key words:** Halophilic bacteria, 16S rRNA gene, Unculturable bacteria, Metagenomics, Para grass.

### Introduction

Salinity is a worldwide problem with nearly 600 million hectares throughout the world being salt affected which results in poor soil fertility and thus adversely affects crop productivity (Qureshi *et al.*, 1993). In Pakistan, approximately 6.3 million hectares is affected by salinity, of which nearly half is used for irrigated agriculture (Qureshi *et al.*, 2008). Salt tolerant plants like kallar grass (*Leptochloa fusca*) and para grass (*Urochloa mutica*) grow well in saline soil and with brackish water. Such plants can be used for economic utilization of salt affected lands by raising biomass for bio-energy or being used as fodder or forage (Khan, 2009; Chaoyan *et al.*, 2015; Karakas *et al.*, 2016). In this context, Para grass is a suitable species for forage production on moderately saline-sodic soils with brackish underground water and can be grown in both summer and winter seasons. All such soils are low in soil fertility. Plants growing in these environments have to meet their nutritional requirements.

Rhizosphere is a site of intense microbial activity and responsible for nutrient cycling. These microorganisms can have a neutral, pathogenic or beneficial interaction with their host plant (Sharma *et al.*, 2011; Huang *et al.*, 2013). It is also important to study the organisms from saline rhizosphere habitats because these organisms have adapted to osmoregulatory mechanisms which are still not well known. Studying diversity of such soil will contribute towards long term goal of improving plant-microbe interactions for salinity affected fields and crop productivity (Miransari, 2011; Wu *et al.*, 2015).

Traditional methods of bacterial identification relied heavily upon morphological, biochemical and physiological characteristics but recently the 16S rRNA gene sequence analysis has also become important as a mean to identify an unknown bacterium up to the genus or species level (Fierer *et al.*, 2007). In extreme environments, most microorganisms are reluctant to cultivation-based approaches (Amann *et al.*, 1995; Bastida *et al.*, 2013). Most of the scientists estimated that only 1% of the existing bacteria on earth are culturable (Cardenas & Tiedje, 2008). Metagenomics exploits the fact that while some microorganisms are culturable and others are not, all of them (i.e., 100%) are life-forms based on DNA as a carrier of genetic information. Therefore, culture-independent metagenomic strategies are promising approaches to assess the phylogenetic composition and functional potential of microbial communities living in extreme environments (Rincon *et al.*, 2013; Sheng *et al.*, 2014). Unprecedented analysis of microbial communities of various environments has become possible due to development of bioinformatics tools (Chu *et al.*, 2010). The biosphere is dominated by microorganisms that have much practical significance in medicine, engineering and agriculture. Due to their significance, genetic and biological diversity of microorganisms is an important area of scientific research (Ghazanfar & Azim, 2009).

Our goal was to compare a culture-based technique with culture independent metagenomic technique to evaluate their respective effectiveness at capturing the complete range of bacterial species in the rhizosphere of para grass growing under saline environment. Previous culture based techniques in the rhizosphere of halophytes suggested that we would identify bacteria related to alpha-

proteobacteria, Firmicutes and Actinobacteria. We expected to identify a greater diversity of species using culture independent metagenomic technique, giving us a more complete understanding of the entire community. The identification of bacterial species through culture independent technique in the rhizosphere of halophytes is a first step toward understanding the genetic potential and the interaction between all community members which may lead to the discovery of specialized enzymes or metabolic pathways.

## Material and Methods

**Sampling:** Rhizosphere soil and roots of para grass plants, growing under field conditions at the BioSaline Research Station (BSRS), Faisalabad, were used for this study. The soil was saline-sodic with medium to light texture. The rhizosphere soil profile depth of para grass was 0 - 60 cm,  $EC_{1:1}$  (Electrical Conductivity)  $1.14 \pm 0.09 - 2.24 \pm 0.77$  ( $dSm^{-1}$ ), SAR (Sodium Absorption Ratio)  $9.59 \pm 1.6-21.8 \pm 4.8$ , pH  $8.29 \pm 0.21 - 8.48 \pm 0.31$ . For rhizoplane and histoplane fractions, samples were processed as described by Seeley and Van Demark, 1981.

**Isolation of culturable bacteria:** For the isolation of culturable bacteria, four media, i.e., Luria-Bertani (LB), alkaliphilic, halophilic (HaP) and nitrogen free malate medium (NFM) (Dobereiner & Day, 1976; Akhtar *et al.*, 2008) were used. For rhizosphere fraction (RS), the soil was mixed thoroughly, sieved and one gram representative soil sample was taken. Bacterial fraction from rhizoplane (RP) was isolated by shaking one gram of washed roots with 9 ml saline along pebbles for 30 minutes (Bilal & Malik, 1988). For the isolation from histoplane (HP), roots were sealed at both ends with wax after washing with water and surface sterilized by using 3%  $HgCl_2$  for 3 minutes. After sterilization, waxed ends of roots were removed. Roots were macerated by using FastPrep® instrument (MP Biomedicals, USA) for 30 seconds at speed 4 meter/second. Serial dilutions ( $10^{-1} - 10^{-10}$ ) were made for all samples (RS, RP and HP).

One hundred  $\mu l$  of each serial dilution, ranging from  $10^{-3}$  to  $10^{-6}$  were spread on LB, AP and HaP agar plates with three replicates, to calculate the total bacterial population. For MPN counts (Alexander, 1982), 100  $\mu l$  of serial dilutions  $10^{-5}$  to  $10^{-10}$  were inoculated in NFM vials, each with five replicates. Plates and vials were incubated at 28°C until the appearance of bacterial colonies and pellicles, respectively. The bacterial cultures from plates were further purified by repeated sub-culturing on LB agar plates. MPN counts and nitrogen fixing ability of all bacterial isolates were assessed by acetylene reduction assay. Single bacterial colonies of each isolate was inoculated in vials containing NFM semisolid medium (5 ml/vial) and incubated at 28°C for 48 h. After 48 h, acetylene [10% (v/v)] was injected into all vials and re-incubated at 28°C. After 24 h, the samples were analyzed for acetylene reduction by Gas chromatography (Buck Scientific; Model 910/310 Gas Chromatograph; Column Porapak N).

**Morphological and biochemical characterization of bacterial isolates:** Bacterial colonies were characterized on the basis of color, shape, size, margin and elevation. The cell size, shape and motility of bacterial strains were observed under light microscope (Model, Nikon LABOPHOTO-2, Japan). Biochemical tests of all bacterial isolates were performed using QTS-24 miniaturized identification system (DESTO Laboratories, Karachi, Pakistan).

**Isolation of DNA from bacterial isolates and rhizosphere sample:** Modified CTAB method (Winnepenninckx *et al.*, 1993) was used for genomic DNA isolation from bacterial isolates. Metagenomic DNA from rhizosphere soil sample was extracted with Fast DNA Spin kit for soil using FastPrep® instrument (MP Biomedicals, USA).

**Amplification, cloning and sequencing of 16S rRNA gene:** The genomic and metagenomic DNA samples were used as templates for PCR. 16S rRNA gene was amplified using universal forward primer P1 (5'-GggatccAGAGTTTGATCCTGGTCAGAACGAACGCT-3' and universal reverse primer P6 (5'-C GggatccTACGGCTACCTGTTACGACTTCACCCC-3') for prokaryotes (Tan *et al.*, 1997) PCR products were purified by using QIA quick PCR purification kit (QIAGEN, USA) and inserted into pTZ57R/T vector using TA cloning kit (Fermentas). Positive clones were confirmed through double digestion of plasmids DNA with restriction enzymes *Hind* III and *Xba* I. Plasmid DNA samples were sequenced by M13 forward primer.

**Sequence alignment and construction of phylogenetic tree:** The sequence data were assembled and analyzed with the help of Chromus Lite 2.01 sequence analysis software. The gene sequences were compared to those deposited in the GenBank nucleotide database using the BLAST program. Sequences were aligned using Clustal X 2.1 program and phylogenetic trees were constructed using neighbor-joining method (Saitou & Nei, 1987). Bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance tree topologies obtained (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA5 (Tamura *et al.*, 2011). The sequences from metagenomic and cultured isolates were deposited in the GenBank database under the accession numbers HE647629-HE647641, HE800449-HE800468, HE980326-HE980330, HF560633-HF560642 and HG316108-HG316118, HG328353-HG328354, HF678358-HF678386, HF947005-HF947012, HE800437-HE800448, respectively.

**Calculation of diversity indices:** An operational taxonomic unit (OTU) was defined as a 16S ribosomal DNA (rDNA) sequence group in which sequences

differed by less than 3% (Martin, 2002). Phylotype richness (S) was calculated as the total number of OTUs. The Shannon–Wiener index was calculated as follows:

$$H = - \sum_{i=1}^S P_i \ln P_i$$

where  $P_i$  is the frequency of the  $i$ th species. Evenness was calculated as  $H/H_{max}$ , where  $H_{max}=\ln(S)$ .

**Results**

**Quantification of bacterial populations:** Culturable nitrogen fixing bacteria were in abundance in the rhizosphere and roots of para grass. MPN for the nitrogen fixing bacteria were  $150 \times 10^7$ ,  $47 \times 10^7$  and  $130 \times 10^7$  per gram dry weight, from the rhizosphere, rhizoplane and histoplane, respectively (Table 1). For rhizosphere fraction, the values of CFU were the highest on LB medium and lowest on AP. The total number of bacterial isolates obtained from the rhizosphere were 32, 22 from rhizoplane 22 and 20 from histoplane.

**Diversity of culturable bacteria from the rhizosphere and roots of paragrass:** Out of 74, twelve isolates were found to be similar on the basis of morphological and biochemical results and these were not used for 16S rRNA gene amplification. On the basis of biochemical tests and 16S rRNA gene sequence analysis, 27 isolates from rhizosphere were grouped into 26 OTUs; 18 isolates from rhizoplane were grouped into 17 OTUs, and 17 isolates from histoplane were grouped into 16 OTUs.

All isolates were related to four phyla within the domain bacteria, namely proteobacteria, firmicutes, bactereriodetes and actinobacteria (Figs. 1 and 2, Table 2). Phylotype richness (S), Shannon–Wiener index (H), and evenness (E) of the rhizospheric, rhizoplane, and histoplane bacterial communities were calculated as 24, 15, 15; 3.12, 2.65, 2.67 and 0.93, 0.91, 0.94, respectively. Diversity analysis by the Shannon-Wiener test, suggested that the rhizosphere, rhizoplane and histoplane of para grass plants have highly diverse bacterial communities.

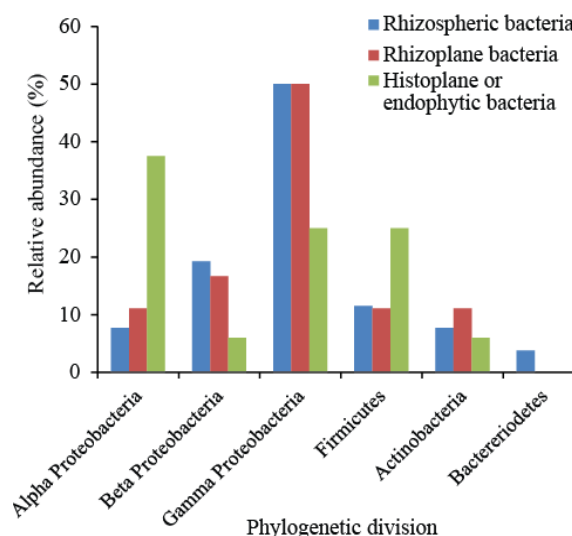


Fig. 1. Relative frequency of bacterial isolates belonging to different phylogenetic groups in the rhizosphere, rhizoplane and histoplane fraction of para grass.

**Phylogenetic analysis of culturable bacteria:** The rhizospheric bacteria were represented by four phyla, with the majority (51.84%) of the isolates falling within the  $\alpha$ -proteobacteria group. Members of the classes,  $\alpha$ -proteobacteria and  $\beta$ -proteobacteria were 3.71%, and 22.22% respectively. Members of the phyla, firmicutes, actinobacteria and bactereriodetes were 11.14%, 7.41% and 3.8%, respectively. All the bacterial isolates from rhizosphere were assigned to 20 genera. The  $\alpha$ -proteobacteria represented by 10 genera, *Pseudomonas* (11.12%), *Klebsiella* (7.40%), *Xanthomonas* (7.40%), *Pasteurella*, *Kluyvera*, *Citrobacter*, *Escherichia*, *Vibrio*, *Azotobacter* and *Serratia* (3.71% each). The  $\beta$ -proteobacteria was represented by 4 genera, *Burkholderia* (11.12%), *Comamonas* (3.71%), *Ralstonia* (3.71%) and *Alcaligenes* (3.71%). The  $\alpha$ -proteobacteria was represented by a single genus, *Rhodovibrio*, which accounted for 3.7% of the isolates (Figs. 1 and 2, Table 2).

**Table 1. Isolation and quantification of bacteria from the rhizospheric soil, rhizoplane and histoplane.**

| Zone             | MPN $\times 10^7$ | Media* used | CFU $\times 10^7$ | Total isolates obtained |
|------------------|-------------------|-------------|-------------------|-------------------------|
| Rhizosphere (RS) | 150               | LB          | 54                | 30                      |
|                  |                   | AP          | 31                |                         |
|                  |                   | HaP         | 34                |                         |
| Rhizoplane (RP)  | 47                | LB          | 47                | 22                      |
|                  |                   | AP          | 7.6               |                         |
|                  |                   | HaP         | 15                |                         |
| Histoplane (HP)  | 130               | LB          | 4.9               | 20                      |
|                  |                   | AP          | 2.5               |                         |
|                  |                   | HaP         | 4.7               |                         |

\*LB-Luria Bertani, HaP-Halophilic Medium, AP-Alkaliphilic Medium

Table 2. Distribution of representative bacterial taxa in the rhizosphere, rhizoplane, and root of para grass.

| Phylogenetic group    | Genus                      | Species                 | Culturable bacteria from soil and roots |                 |                | Unculturable bacteria from rhizosphere (48) |
|-----------------------|----------------------------|-------------------------|---|-----------------|----------------|---|
|                       |                            |                         | Rhizosphere (27)                        | Rhizoplane (18) | Endophyte (17) |   |
| Alphaproteobacteria   | <i>Rhodovibrio</i>         | <i>salinarum</i>        | 1                                       | 1               | 1              |   |
|                       | <i>Agrobacterium</i>       | <i>tumefaciens</i>      |   |                 | 2              |   |
|                       | <i>Azospirillum</i>        | <i>lipoferum</i>        |   |                 | 1              |   |
|                       | <i>Rhizobium</i>           | <i>tropici</i>          |   |                 | 1              |   |
|                       | <i>Paracoccus</i>          | <i>alkenifer</i>        |   |                 | 1              |   |
|                       | <i>Acetobacter</i>         | <i>pasteurianus</i>     |   | 1               |                |   |
| Betaproteobacteria    | Uncultured bacteria        |                         |   |                 |                | 1   |
|                       | <i>Burkholderia</i>        | <i>cepacia</i>          | 3                                       |                 |                |   |
|                       |                            | <i>cenocepacia</i>      |   | 1               |                |   |
|                       | <i>Comamonas</i>           | sp.                     | 1                                       |                 |                |   |
|                       | <i>Ralstonia</i>           | <i>picketti</i>         | 1                                       |                 |                |   |
|                       | <i>Alcaligenes</i>         | <i>faccalis</i>         | 1                                       |                 |                |   |
|                       |                            | sp.                     |   | 1               |                |   |
|                       | <i>Sphingomonas</i>        | sp.                     |   | 1               |                |   |
|                       | <i>Nitrosomonas</i>        | sp.                     |   |                 | 1              |   |
|                       | <i>Massilia</i>            | sp.                     |   |                 |                | 1   |
| Deltaproteobacteria   | <i>Duganella</i>           | sp.                     |   |                 |                | 1   |
|                       | Uncultured bacteria        |                         |   |                 |                | 2   |
|                       | <i>Chondromyces</i>        | <i>pediculatus</i>      |   |                 |                | 1   |
|                       | Sorangiiineae bacteria     |                         |   |                 |                | 1   |
| Gammaproteobacteria   | Uncultured bacteria        |                         |   |                 |                | 2   |
|                       | <i>Pasteurella</i>         | <i>multocida</i>        | 1                                       |                 |                |   |
| Firmicutes            | <i>Kluyvera</i>            | <i>georgiana</i>        | 1                                       |                 |                |   |
|                       |                            | <i>ascorbata</i>        |   |                 | 1              |   |
|                       | <i>Pseudomonas</i>         | <i>moraviensis</i>      |   | 1               |                |   |
|                       |                            | <i>putida</i>           | 2                                       |                 |                |   |
|                       |                            | <i>fluorescens</i>      | 1                                       |                 |                |   |
|                       |                            | <i>chlororaphis</i>     |   | 1               |                |   |
|                       |                            | <i>stutzeri</i>         |   | 1               |                |   |
|                       | <i>Xanthomonas</i>         | <i>axonopodis</i>       | 2                                       | 1               |                |   |
|                       | <i>Citrobacter</i>         | <i>freundii</i>         | 1                                       |                 | 1              |   |
|                       | <i>Escherichia</i>         | <i>coli</i>             | 1                                       | 1               |                |   |
|                       | <i>Klebsiella</i>          | <i>oxytoca</i>          | 1                                       | 1               |                |   |
|                       |                            | <i>pneumoniae</i>       | 1                                       |                 |                |   |
|                       | <i>Vibrio</i>              | <i>proteolyticus</i>    | 1                                       |                 |                |   |
|                       | <i>Azotobacter</i>         | <i>beijerinckii</i>     | 1                                       |                 |                |   |
|                       |                            | sp.                     |   | 1               |                |   |
|                       | <i>Serratia</i>            | <i>Marcescens</i>       | 1                                       |                 |                |   |
|                       | <i>Pectobacterium</i>      | <i>carotovorum</i>      |   | 1               |                |   |
|                       | <i>Aeromonas</i>           | <i>veronii</i>          |   | 1               |                |   |
|                       | <i>Proteus</i>             | <i>Vulgaris</i>         |   |                 | 1              |   |
|                       | <i>Moraxella</i>           | <i>boevrei</i>          |   |                 | 1              |   |
| <i>Enterobacter</i>   | <i>asburiae</i>            |                         | 1                                       | 1               |                |   |
| Actinobacteria        | <i>Entrococcus</i>         | sp.                     | 1                                       | 1               | 1              |   |
|                       | <i>Streptococcus</i>       | <i>pseudopneumoniae</i> | 1                                       |                 |                |   |
|                       | <i>Staphylococcus</i>      | <i>haemolyticus</i>     | 1                                       |                 |                |   |
|                       |                            | <i>gallinarum</i>       |   |                 | 1              |   |
|                       | <i>Veillonella</i>         | sp.                     |   |                 | 1              |   |
|                       | <i>Clostridium</i>         | sp.                     |   |                 |                | 1   |
|                       |                            | uncultured sp.          |   |                 |                | 1   |
|                       | <i>Acetivibrio</i>         | <i>cellulolyticus</i>   |   |                 |                | 1   |
|                       | <i>Bacillus</i>            | <i>Megaterium</i>       |   |                 |                | 1   |
|                       |                            | <i>subtilis</i>         |   |                 |                | 1   |
|                       | sp.                        |                         |   |                 | 1              |   |
|                       | uncultured sp.             |                         |   |                 | 1              |   |
| Acidobacteria         | Uncultured marine bacteria |                         |   |                 | 1              |   |
|                       | Uncultured soil bacteria   |                         |   |                 | 5              |   |
| Bacteroidetes         | <i>Micrococcus</i>         | <i>roseus</i>           | 1                                       |                 |                |   |
|                       |                            | <i>luteus</i>           |   | 2               | 2              |   |
|                       | <i>Frankia</i>             | sp.                     | 1                                       |                 |                |   |
| Chloroflexi           | Uncultured soil bacteria   |                         |   |                 |                | 1   |
|                       | <i>Flavobacterium</i>      | sp.                     | 1                                       |                 |                |   |
| Cyanobacteria         | <i>Anaerosinus</i>         | <i>Glycerini</i>        |   |                 |                | 1   |
|                       | <i>Caldilinea</i>          | <i>tarbellica</i>       |   |                 |                | 1   |
| Planctomycetes        | uncultured sp.             |                         |   |                 |                | 1   |
|                       | <i>Microcoleus</i>         | <i>steenstrupii</i>     |   |                 |                | 1   |
| Gemmatonadetes        | <i>Microcoleus</i>         | sp.                     |   |                 |                | 1   |
|                       | Uncultured soil bacteria   |                         |   |                 |                | 1   |
| unclassified bacteria | Uncultured bacteria        |                         |   |                 |                | 18  |

\*Numbers indicate strains assigned to each species, and those in parentheses are the total numbers of isolates

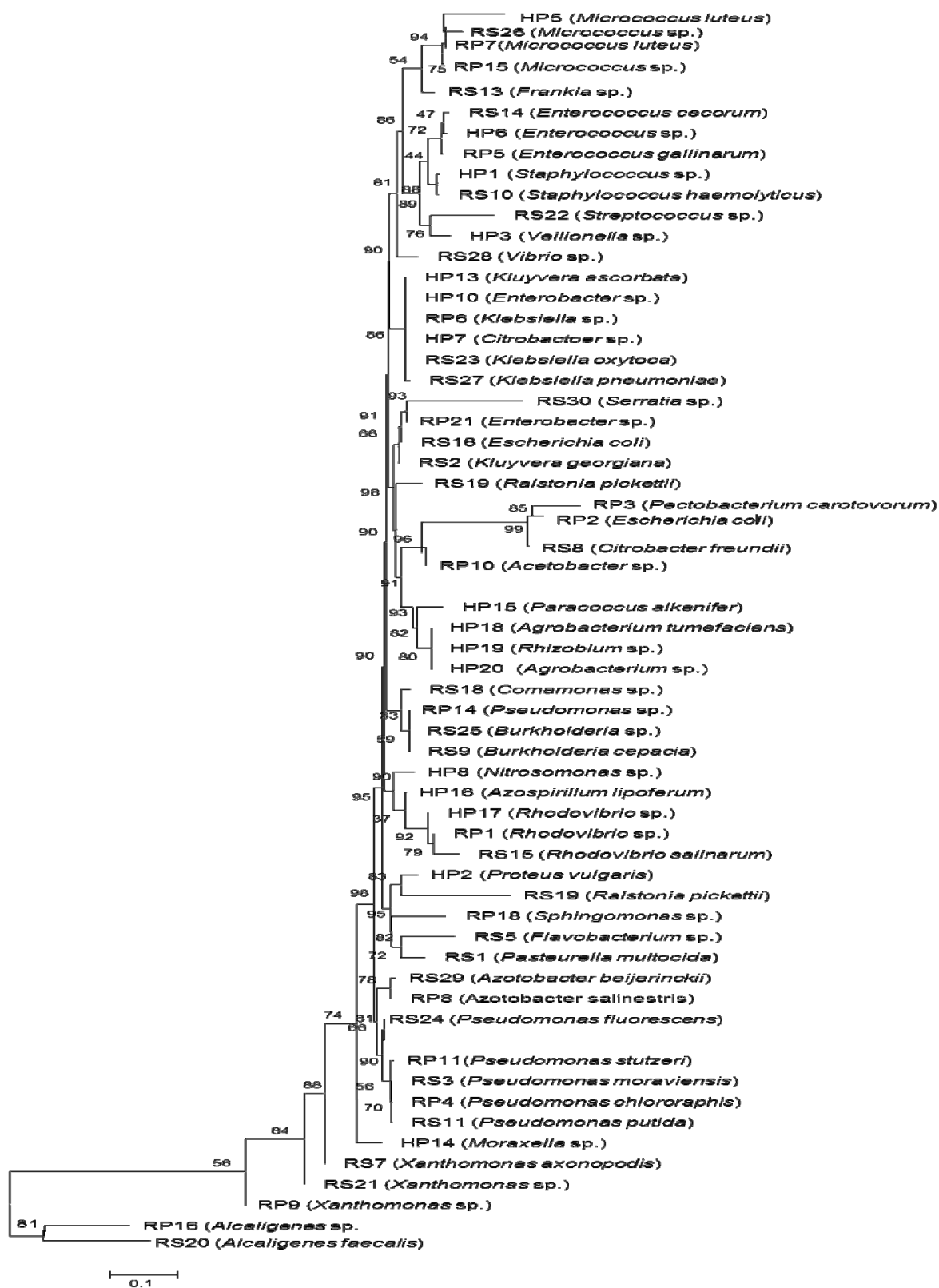


Fig. 2. Phylogenetic tree based on partial 16S rRNA gene sequences of bacterial isolates from the rhizosphere of para grass. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of number of base substitutions per site.

The rhizoplane isolates were represented by three phyla: proteobacteria (77.78%), firmicutes (11.11%) and actinobacteria (11.11%). Among the isolates from the proteobacteria, 50% were members of  $\alpha$ -proteobacteria, 16.67% of  $\beta$ -proteobacteria and 11.11% of  $\gamma$ -proteobacteria. All the bacterial isolates from rhizoplane were belonged to 15 genera. The class  $\alpha$ -proteobacteria was represented by eight genera, *Pseudomonas* (16.67%), *Xanthomonas*, *Escherichia*, *Klebsiella*, *Azotobacter*, *Erwinia*, *Pectobacterium*, *Aeromonas* (5.56% each). The  $\beta$ -proteobacteria was represented by *Burkholderia* (5.56%), *Sphingomonas* (5.56%) and *Alcaligenes* (5.56%). The  $\gamma$ -proteobacteria was represented by *Rhodovibrio* (5.56%) and *Acetobacter* (5.56%). The groups firmicutes and actinobacteria included *Enterococcus* (11.11%) and *Micrococcus* (11.11%), respectively (Figs. 1 and 2, Table 2). The endophytic (HP) bacteria were grouped into three phyla: proteobacteria (70.59%), firmicutes (17.65%) and actinobacteria (11.76%). All endophytic bacteria (HP) belonged to fifteen genera. Among the isolates from the histoplane, the group  $\alpha$ -proteobacteria was represented by five genera, *Agrobacterium* (11.76%), *Rhodovibrio* (5.89%), *Azospirillum* (5.89%), *Rhizobium* (5.89%) and *Paracoccus* (5.89%). The group  $\alpha$ -proteobacteria also included five genera *Kluyvera* (5.89%), *Citrobacter* (5.89%), *Proteus* (5.89%), *Moraxella* (5.89%) and *Enterobacter* (5.89%). The  $\beta$ -proteobacteria was represented by *Nitrosomonas* (5.89%). The group firmicutes was represented by *Enterococcus* (5.89%), *Staphylococcus* (5.89%) and *Veillonella* (5.89%). Actinobacteria was represented by one genus, *Micrococcus* (11.76%) (Figs. 1 and 2, Table 2).

Among the bacterial strains isolated from the rhizosphere, rhizoplane, and histoplane, there were clear differences. More than fifty percent isolates from rhizosphere and rhizoplane fractions were members of  $\alpha$ -proteobacteria (51.84% and 50%, respectively). The majority of endophytic bacteria were  $\alpha$ -proteobacteria (35.29%). Members of  $\alpha$ -proteobacteria,  $\beta$ -proteobacteria,  $\gamma$ -proteobacteria, actinobacteria and firmicutes were isolated from all fractions. Bacteroidetes was represented by one genus, isolated from the rhizosphere (Figs. 1 and 2, Table 2).

A total of 34 bacterial genera were present in the rhizosphere and the roots of para grass among which 20 genera were present in the rhizosphere, 15 in the rhizoplane and 15 in the interior of the roots. Isolates of genera *Micrococcus*, *Enterococcus* and *Rhodovibrio* were common in all three fractions, however some were detected in one or other fraction. Strains of *Pasteurella*, *Flavobacterium*, *Frankia*, *Comamonas* and *Serratia* were isolated from rhizosphere; isolates of *Erwinia*, *Acetobacter*, *Sphingomonas* and *Aeromonas* were found in the rhizoplane; *Azospirillum*, *Rhizobium*, *Moraxella*, *Veillonella* and *Proteus* were detected in the histoplane (Figs. 1 and 2, Table 2). Culturable bacteria were identified by biochemical and molecular methods and comparison showed that results of biochemical methods were in agreement with molecular method for the identification of fifty strains up to genus level and 16 of them were correctly identified upto species level (Table 3). Biochemical tests could not identify 10 strains that were identified by molecular method.

**Diversity of unculturable bacteria from the rhizosphere of paragrass:** A total of forty eight 16S rRNA clones from rhizosphere were grouped into 25 OTUs. All the clones were related to 9 phyla within the domain bacteria, namely proteobacteria, firmicutes, acidobacteria, cyanobacteria, chloroflexi, bacteroidetes, gemmatonadetes, planctomycetes and actinobacteria (Table 2). Phylotype richness (S), Shannon–Wiener index (H), and evenness (E) of the unculturable bacterial communities were calculated as 25; 2.48 and 0.7, respectively.

**Phylogenetic analysis of unculturable bacteria:** Nucleotide BLAST search of different clones of the 16S rRNA gene showed that 62% of the clones were uncultured bacteria. Of total 16S rRNA clones identified, 66.67% had more than 90% identity with other clones and were rarely culturable or unculturable. About 37.5% clones were related to uncultured and unclassified bacteria. Members of the phyla, proteobacteria and firmicutes were 16.67% each. Uncultured bacteria related to phylum, acidobacteria formed 12.5% of the total bacterial population. Bacteria related to phyla, actinobacteria, cyanobacteria and chloroflexi formed 2.1%, 4.2% and 2.1%, respectively. About 4.2% uncultured bacteria related to bacteroidetes, 2.1% related to gemmatonadetes and 2.1% related to planctomycetes (Fig. 3, Table 2).

Members of the classes,  $\alpha$ -proteobacteria,  $\beta$ -proteobacteria and  $\delta$ -proteobacteria were 2.1%, 8.33% and 8.33%, respectively (Fig. 4, Table 4). The group firmicutes was represented by *Bacillus* (8.33%), *Clostridium* (4.16%) and *Acetovibrio* (2.1%). The acidobacteria formed two groups represented by uncultured marine bacteria (2.1%) and uncultured soil bacteria (10.41%). The chloroflexi was represented by two groups, uncultured *chloroflexi* bacteria (2.1%) and rarely culturable *Caldilinea* sp. (2.1%). The cyanobacteria and bacteroidetes were represented by *Microcoleus* (4.16%) and *Anaerostinus* (2.1%), respectively. The groups planctomycetes (2.1%), actinobacteria (2.1%) and gemmatonadetes (2.1%) included only one group, uncultured soil bacteria.

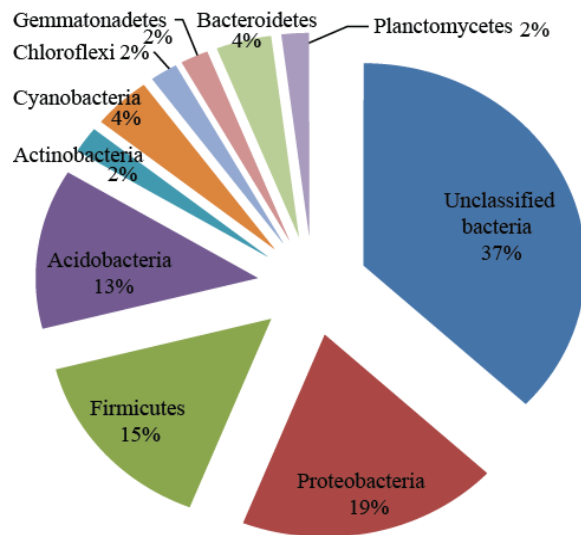


Fig. 3. Percentage distribution of bacterial phyla in soil and roots of para grass, determined by sequencing of 16S rRNA gene.

**Table 3. Identification of bacterial isolates from soil and roots of para grass on the basis of QTS 24 bacterial identification kit and 16S rRNA gene sequence analyses.**

| Root domains       | Phylogenetic group (genus) | Isolates                      | Identification Methods           |                                       |                                      |
|--------------------|----------------------------|-------------------------------|----------------------------------|---------------------------------------|--------------------------------------|
|                    |                            |                               | QTS24 Identification kit         | 16S rDNA                              | Sequence identity (%); Accession No. |
| Rhizosphere (RS)   | <i>Rhodovibrio</i>         | RS15                          | <i>Rhodovibrio</i> sp.           | <i>Rhodovibrio salinarum</i>          | 98; HE800441                         |
|                    | <i>Burkholderia</i>        | RS9                           | <i>Burkholderia capacia</i>      | <i>Burkholderia capacia</i>           | 99; HF678361                         |
|                    |                            | RS4                           | <i>Burkholderia</i> sp.          | <i>Burkholderia capacia</i>           | 99; HG328353                         |
|                    |                            | RS25                          | <i>Burkholderia</i> sp.          | <i>Burkholderia cenocepacia</i>       | 98; HF678367                         |
|                    | <i>Comamonas</i>           | RS18                          | Unknown                          | <i>Comamonas</i> sp.                  | 98; HF678364                         |
|                    | <i>Ralstonia</i>           | RS19                          | <i>Ralstonia</i> sp.             | <i>Ralstonia picketti</i>             | 99; HE800442                         |
|                    | <i>Alcaligenes</i>         | RS20                          | Unknown                          | <i>Alcaligenes faccalis</i>           | 99; HG316109                         |
|                    | <i>Pasteurella</i>         | RS1                           | <i>Pasteurella multocida</i>     | <i>Pasteurella multocida</i>          | 98; HE800437                         |
|                    | <i>Kluyvera</i>            | RS2                           | <i>Kluyvera</i> sp.              | <i>Kluyvera georgiana</i>             | 99; HF678358                         |
|                    | <i>Pseudomonas</i>         | RS3                           | <i>Pseudomonas</i> sp.           | <i>Pseudomonas putida</i>             | 98; HF678359                         |
|                    |                            | RS11                          | <i>Pseudomonas putida</i>        | <i>Pseudomonas putida</i>             | 99; HE800440                         |
|                    |                            | RS24                          | <i>Pseudomonas fluorescens</i>   | <i>Pseudomonas fluorescens</i>        | 98; HF678366                         |
|                    | <i>Xanthomonas</i>         | RS7                           | <i>Xanthomonas</i> sp.           | <i>Xanthomonas axonopodis</i>         | 99; HF678360                         |
|                    |                            | RS21                          | <i>Xanthomonas</i> sp.           | <i>Pseudoxanthomonas japonensis</i>   | 99; HE800443                         |
|                    | <i>Citrobacter</i>         | RS8                           | <i>Citrobacter freundii</i>      | <i>Citrobacter freundii</i>           | 99; HE800438                         |
|                    | <i>Escherichia</i>         | RS16                          | <i>Escherichia coli</i>          | <i>Escherichia coli</i>               | 99; HF678363                         |
|                    | <i>Klebsiella</i>          | RS23                          | <i>Klebsiella oxytoca</i>        | <i>Klebsiella oxytoca</i>             | 99; HF678365                         |
|                    |                            | RS27                          | <i>Klebsiella</i> sp.            | <i>Klebsiella pneumonia</i>           | 98; HE800445                         |
|                    | <i>Vibrio</i>              | RS28                          | <i>Vibrio</i> sp.                | <i>Vibrio proteolyticus</i>           | 99; HG316110                         |
|                    | <i>Azotobacter</i>         | RS29                          | Unknown                          | <i>Azotobacter beijerinckii</i>       | 96; HF678368                         |
|                    | <i>Serratia</i>            | RS30                          | <i>Serratia</i> sp.              | <i>Serratia marcescens</i>            | 99; HE800446                         |
|                    | <i>Staphylococcus</i>      | RS10                          | <i>Staphylococcus</i> sp.        | <i>Staphylococcus haemolyticus</i>    | 95; HE800439                         |
|                    | <i>Enterococcus</i>        | RS14                          | <i>Enterococcus</i> sp.          | <i>Enterococcus</i> sp.               | 99; HG316108                         |
|                    | <i>Streptococcus</i>       | RS22                          | <i>Streptococcus</i> sp.         | <i>Streptococcus pseudopneumoniae</i> | 97; HF947006                         |
|                    | <i>Micrococcus</i>         | RS26                          | <i>Micrococcus roseus</i>        | <i>Micrococcus roseus</i>             | 99; HE800444                         |
|                    | <i>Frankia</i>             | RS13                          | Unknown                          | <i>Frankia</i> sp.                    | 98; HF678362                         |
|                    | <i>Flavobacterium</i>      | RS5                           | Unknown                          | <i>Flavobacterium</i> sp.             | 97; HF947005                         |
| Rhizoplane (RP)    | <i>Rhodovibrio</i>         | RP1                           | <i>Rhodovibrio</i> sp.           | <i>Rhodovibrio salinarum</i>          | 99; HF678369                         |
|                    | <i>Acetobacter</i>         | RP10                          | <i>Acetobacter</i> sp.           | <i>Acetobacter pasteurianus</i>       | 98; HF947008                         |
|                    | <i>Burkholderia</i>        | RP14                          | <i>Burkholderia</i> sp.          | <i>Burkholderia cenocepacia</i>       | 98; HF678376                         |
|                    | <i>Alcaligenes</i>         | RP16                          | Unknown                          | <i>Alcaligenes</i> sp.                | 99; HG316114                         |
|                    | <i>Sphingomonas</i>        | RP18                          | Unknown                          | <i>Sphingomonas</i> sp.               | 99; HG316115                         |
|                    | <i>Escherichia</i>         | RP2                           | <i>Escherichia coli</i>          | <i>Escherichia coli</i>               | 99; HF678370                         |
|                    | <i>Pectobacterium</i>      | RP3                           | <i>Erwinia carotovora</i>        | <i>Pectobacterium carotovorum</i>     | 100; HF678371                        |
|                    | <i>Pseudomonas</i>         | RP4                           | <i>Pseudomonas aurantiaca</i>    | <i>Pseudomonas chlororaphis</i>       | 99; HF678372                         |
|                    |                            | RP11                          | <i>Pseudomonas</i> sp.           | <i>Pseudomonas stutzeri</i>           | 100; HF678375                        |
|                    |                            | RP17                          | <i>Pseudomonas</i> sp.           | <i>Pseudomonas moraviensis</i>        | 99; HF678377                         |
|                    | <i>Klebsiella</i>          | RP6                           | <i>Klebsiella</i> sp.            | <i>Klebsiella oxytoca</i>             | 98; HF678373                         |
|                    | <i>Azotobacter</i>         | RP8                           | Unknown                          | <i>Azotobacter</i> sp.                | 100; HG316112                        |
|                    | <i>Xanthomonas</i>         | RP9                           | <i>Xanthomonas</i> sp.           | <i>Xanthomonas</i> sp.                | 99; HF678374                         |
|                    | <i>Aeromonas</i>           | RP19                          | <i>Aeromonas veronii</i>         | <i>Aeromonas veronii</i>              | 99; HG316116                         |
|                    | <i>Enterococcus</i>        | RP5                           | <i>Enterococcus</i> sp.          | <i>Enterococcus gallinarum</i>        | 98; HG316111                         |
|                    | RP21                       | <i>Enterobacter aerogenes</i> | <i>Enterobacter aerogenes</i>    | 98; HF947009                          |                                      |
| <i>Micrococcus</i> | RP7                        | <i>Micrococcus luteus</i>     | <i>Micrococcus luteus</i>        | 98; HF947007                          |                                      |
|                    | RP15                       | <i>Micrococcus</i> sp.        | <i>Micrococcus luteus</i>        | 99; HG316113                          |                                      |
| Histoplane (HP)    | <i>Paracoccus</i>          | HP15                          | <i>Paracoccus</i> sp.            | <i>Paracoccus alkenifer</i>           | 98; HF678381                         |
|                    | <i>Azospirillum</i>        | HP16                          | <i>Azospirillum</i> sp.          | <i>Azospirillum lipoferum</i>         | 99; HF678382                         |
|                    | <i>Rhodovibrio</i>         | HP17                          | <i>Rhodovibrio</i> sp.           | <i>Rhodovibrio salinarum</i>          | 98; HF678383                         |
|                    | <i>Agrobacterium</i>       | HP18                          | <i>Agrobacterium tumefaciens</i> | <i>Agrobacterium tumefaciens</i>      | 100; HF678384                        |
|                    |                            | HP20                          | <i>Agrobacterium</i> sp.         | <i>Agrobacterium tumefaciens</i>      | 98; HF678386                         |
|                    | <i>Rhizobium</i>           | HP19                          | <i>Rhizobium</i> sp.             | <i>Rhizobium</i> sp.                  | 99; HF678385                         |
|                    | <i>Nitrosomonas</i>        | HP8                           | Unknown                          | <i>Nitrosomonas</i> sp.               | 99; HF678378                         |
|                    | <i>Enterobacter</i>        | HP10                          | <i>Enterobacter</i> sp.          | <i>Enterobacter asburiae</i>          | 100; HF678379                        |
|                    | <i>Kluyvera</i>            | HP13                          | <i>Kluyvera</i> sp.              | <i>Kluyvera ascorbata</i>             | 99; HF678380                         |
|                    | <i>Moraxella</i>           | HP14                          | <i>Moraxella</i> sp.             | <i>Moraxella boevrei</i>              | 99; HF947012                         |
|                    | <i>Proteus</i>             | HP2                           | <i>Proteus vulgaris</i>          | <i>Proteus vulgaris</i>               | 98; HF947011                         |
|                    | <i>Citrobacter</i>         | HP7                           | <i>Citrobacter freundii</i>      | <i>Citrobacter freundii</i>           | 99; HG316118                         |
|                    | <i>Staphylococcus</i>      | HP1                           | <i>Staphylococcus</i> sp.        | <i>Staphylococcus gallinarum</i>      | 99; HF947010                         |
|                    | <i>Enterococcus</i>        | HP6                           | <i>Enterococcus</i> sp.          | <i>Enterococcus</i> sp.               | 98; HG316117                         |
|                    | <i>Veillonella</i>         | HP3                           | Unknown                          | <i>Veillonella</i> sp.                | 99; HE800447                         |
| <i>Micrococcus</i> | HP5                        | <i>Micrococcus luteus</i>     | <i>Micrococcus luteus</i>        | 95; HE800448                          |                                      |
|                    | HP9                        | <i>Micrococcus</i> sp.        | <i>Micrococcus luteus</i>        | 95; HG328354                          |                                      |

Table 4. Sequence analysis of 16S rRNA cloned from the rhizospheric soil and roots of para grass.

| Phylogenetic group    | Clones from rhizosphere soil | Closest relative (Accession No.)                          | Sequence identity (%); Accession No. |
|-----------------------|------------------------------|---|--------------------------------------|
| Rhizobiaceae          | S13                          | <i>Alpha proteobacterium</i> KC-IT-H1 (FJ711201)          | 95; HE647639                         |
| Rhizobiaceae          | S24                          | Uncultured bacterium (HQ287208)                           | 92; HE800457                         |
| Oxalobacteraceae      | S23                          | <i>Massilia</i> sp. (JQ014506)                            | 98; HE800456                         |
| Oxalobacteraceae      | S45                          | <i>Duganella</i> sp. (EF575562)                           | 99; HF560637                         |
| Sphingobacteriaceae   | S25                          | Uncultured bacterium (EU445227)                           | 82; HE800458                         |
| Comamonadaceae        | S29                          | Uncultured soil bacterium (GU598823)                      | 88; HE800462                         |
| Polyangiaceae         | S1                           | <i>Chondromyces pediculatus</i> strain Cm p51(GU207875)   | 85; HE647629                         |
| Polyangiaceae         | S2                           | Sorangiiineae bacterium SBSr005 (GU249612)                | 84; HE647630                         |
| Proteobacteria        | S17                          | Uncultured proteobacterium (FJ542849)                     | 94; HE800450                         |
| Clostridiaceae        | S3                           | <i>Clostridium</i> strain FCB90-3 (AJ229251)              | 95; HE647631                         |
| Clostridiaceae        | S5                           | <i>Acetivibrio cellulolyticus</i> strain CD2 (NR025917)   | 91; HE647632                         |
| Clostridiaceae        | S19                          | Uncultured bacterium (FN658847)                           | 92; HE800452                         |
| Bacillaceae           | S9                           | <i>Bacillus megaterium</i> strain MO29 (AY553116)         | 91; HE647636                         |
| Bacillaceae           | S16                          | <i>Bacillus</i> sp. (FJ981907)                            | 96; HE800449                         |
| Bacillaceae           | S26                          | <i>Bacillus subtilis</i> (EF584109)                       | 85; HE800459                         |
| Bacillaceae           | S28                          | Uncultured <i>Bacillus</i> sp. (HM152718)                 | 89; HE800461                         |
| Acidobacteriaceae     | S18                          | Uncultured marine bacterium (JN216793)                    | 93; HE800451                         |
| Acidobacteriaceae     | S27                          | Uncultured bacterium (HQ266786)                           | 88; HE800460                         |
| Acidobacteriaceae     | S33                          | Uncultured Acidobacteriales bacterium (EU276437)          | 94; HE800466                         |
| Acidobacteriaceae     | S43                          | Uncultured Acidobacteria bacterium (HM062302)             | 98; HF560635                         |
| Acidobacteriaceae     | S48                          | Uncultured Acidobacteria bacterium ( JX114477)            | 95; HF560640                         |
| Acidobacteriaceae     | S50                          | Uncultured Acidobacteria bacterium ( FM176392)            | 93; HF560642                         |
| Phormidiaceae         | S8                           | <i>Microcoleus</i> sp. HTT-U-KK5 (EF654070)               | 92; HE647635                         |
| Phormidiaceae         | S34                          | <i>Microcoleus steenstrupii</i> (AJ871982)                | 94; HE800467                         |
| Peptococcaceae        | S6                           | <i>Caldilinea tarbellica</i> (HM134893)                   | 83; HE647633                         |
| Chloroflexaceae       | S22                          | Uncultured <i>Chloroflexi</i> bacterium (JN038958)        | 95; HE800455                         |
| Planctomycetaceae     | S7                           | Planctomycetales bacterium Ellin6207 (AY673166)           | 88; HE647634                         |
| Acidimicrobiaceae     | S11                          | Uncultured bacterium (AB517669)                           | 87; HE647638                         |
| Gemmatimonaceae       | S14                          | Gemmatimonadetes bacterium KBS708 (HM154525)              | 81; HE647640                         |
| Bacteroidaceae        | S15                          | <i>Anaerobaculum glycerini</i> strain DSM 5192 (NR025297) | 93; HE647641                         |
| Unclassified Bacteria | S10                          | Anaerobic bacterium MO-CFX2 (AB598278)                    | 84; HE647637                         |
| Unclassified Bacteria | S20                          | Uncultured bacterium (JF910629)                           | 92; HE800453                         |
| Unclassified Bacteria | S21                          | Uncultured bacterium (EU219015)                           | 94; HE800454                         |
| Unclassified Bacteria | S30                          | Uncultured bacterium (HQ011588)                           | 83; HE800463                         |
| Unclassified Bacteria | S31                          | Uncultured bacterium (HQ121017)                           | 91; HE800464                         |
| Unclassified Bacteria | S32                          | Uncultured Unclassified bacterium (CU919360)              | 87; HE800465                         |
| Unclassified Bacteria | S35                          | Uncultured bacterium (HQ121027)                           | 95; HE800468                         |
| Unclassified Bacteria | S36                          | Uncultured bacterium (JX098363)                           | 96; HE980326                         |
| Unclassified Bacteria | S37                          | Uncultured bacterium (HE662509)                           | 98; HE980327                         |
| Unclassified Bacteria | S38                          | Uncultured bacterium (JQ825033)                           | 99; HE980328                         |
| Unclassified Bacteria | S39                          | Uncultured bacterium (EU978624)                           | 97; HE980329                         |
| Unclassified Bacteria | S40                          | Uncultured bacterium (HM334375)                           | 94; HE980330                         |
| Unclassified Bacteria | S41                          | Uncultured bacterium (HE662555)                           | 93; HF560633                         |
| Unclassified Bacteria | S42                          | Uncultured bacterium (GQ487895)                           | 94; HF560634                         |
| Unclassified Bacteria | S44                          | Uncultured bacterium (FM872517)                           | 95; HF560636                         |
| Unclassified Bacteria | S46                          | Uncultured bacterium (FN995832)                           | 86; HF560638                         |
| Unclassified Bacteria | S47                          | Uncultured bacterium (HE662534)                           | 89; HF560639                         |
| Unclassified Bacteria | S49                          | Uncultured bacterium (EU803523)                           | 93; HF560641                         |



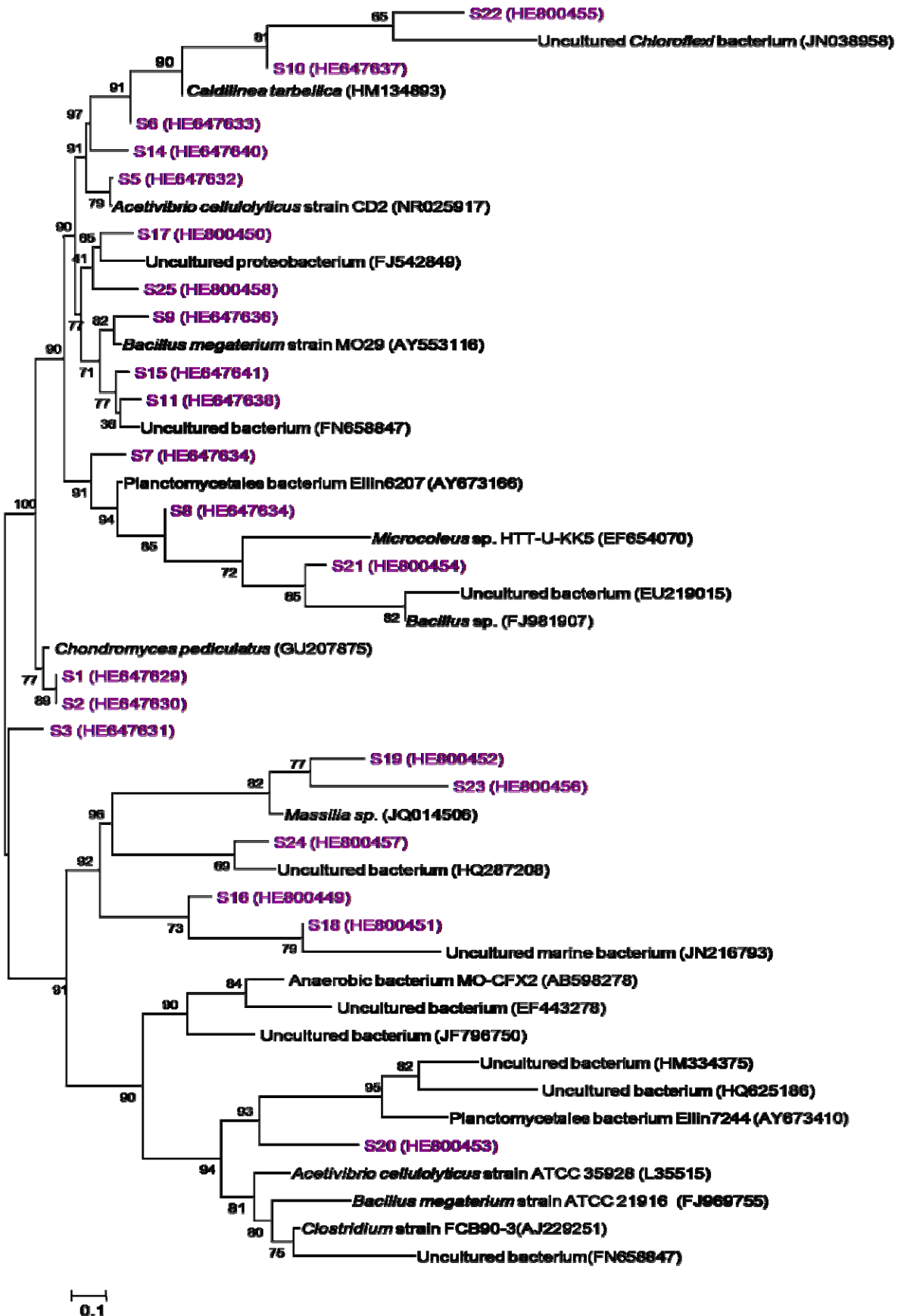


Fig. 4. Cont'd.

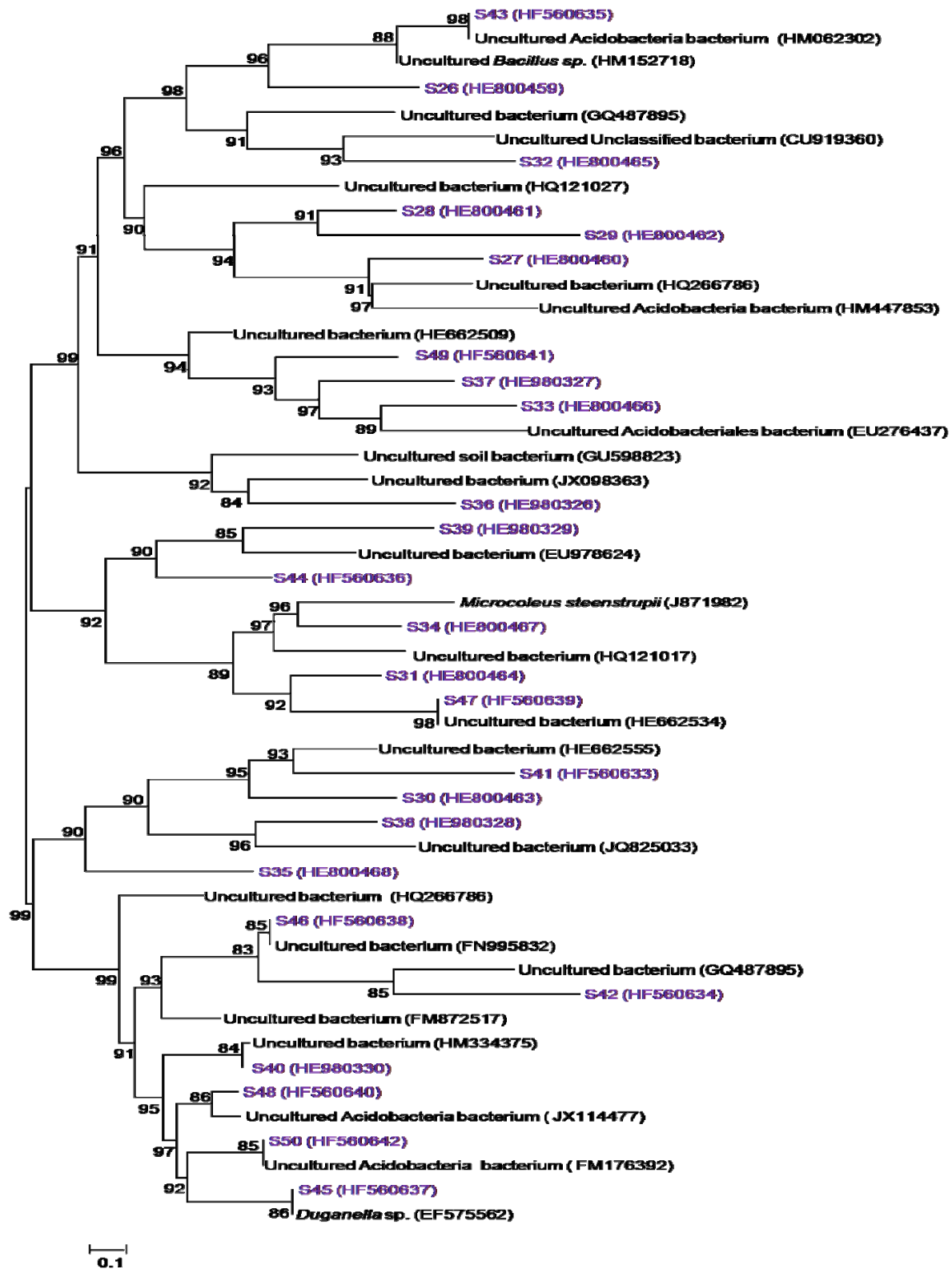


Fig. 4. Phylogenetic tree based on partial 16S rRNA gene clone library sequences directly isolated from the rhizosphere of para grass. Representative sequences (S) were selected to represent each group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches.

## Discussion

In the present work microbial as well as metagenomic analysis of RS, RP and HP fractions of para grass were studied. The population of diazotrophic bacteria was the most abundant in the rhizosphere and the least abundant in the rhizoplane which is similar to previous reports (Malik *et al.*, 1997; Itoh *et al.*, 2010). The values of CFU per gram dry weight were highest for LB medium as compared to HaP and AP media, from all fractions (Table 1). The rhizosphere attracts a great diversity and population density of microorganisms as it contains important sources of nutrients (proteins, carbohydrates, alcohols, vitamins and hormones (Compant *et al.*, 2005; Ahemad & Kibret, 2014).

The identification of culturable bacteria was based on the biochemical tests, following Bergey's manual and 16S rRNA gene sequence analysis. So far, the 16S rRNA gene sequence analysis is the most reliable technique for bacterial identification (Sacchi *et al.*, 2002; Jayachandra *et al.*, 2013) due to a very large data bank as compared to any other data bank.

*Flavobacterium*, the only genus of bacteroidetes was detected in the rhizosphere of para grass. Many studies have revealed that effective colonization of flavobacteria (antagonistic bacteria) on roots through competition for limited nutrients and/or niches against plant pathogens leads to successful disease suppression by protecting infection courts from plant pathogens (Haggag & Timmusk, 2008). Members of  $\alpha$ -proteobacteria, *Pseudomonas*, *Klebsiella*, *Xanthomonas*, *Pasteurella*, *Kluyvera*, *Escherichia*, *Azotobacter* and *Serratia* are considered as important constituents in the root-associated microbial community and their ability to colonize the root surface, preventing the development of plant pathogens and improving plant growth, is well known (James & Olivares, 1997; Vacheron *et al.*, 2013). Members of  $\alpha$ -proteobacteria; *Agrobacterium*, *Azospirillum*, *Rhizobium* and *Paracoccus* were only found in the root interior while *Rhodovibrio* and *Acetobacter* were found in the rhizosphere and the rhizoplane of para grass. Members of  $\beta$ -proteobacteria; *Burkholderia*, *Comamonas*, *Alcaligenes*, *Sphingomonas*, *Nitrosomonas* and *Ralstonia* were found in all three fractions (rhizosphere, rhizoplane and histoplane). These isolates can potentially be used as bioinoculants through production of phytohormones, biological nitrogen fixation, phosphorous release, increased nutrient uptake, enhanced stress resistance, biocontrol of both major and minor plant pathogens and improved water status (Beneduzi *et al.*, 2012; Glick, 2012). Strains of *Enterococcus*, *Streptococcus* and *Staphylococcus* were present in the rhizosphere, while the genus, *Veillonella* was present only in the root interior (Han *et al.*, 2009; Nakade, 2013).

The phylogenetic analysis of unculturable bacteria revealed that the largest proportion of bacterial population in the rhizosphere of para grass related to uncultured than cultured bacteria. Proteobacteria and firmicutes, the second most dominant phyla based on the metagenomic studies are the two most important phyla in grass land and agricultural soils (Thurmer *et al.*, 2010; Ma & Gong, 2013). Among the members of the proteobacteria, 50% were the members of  $\beta$ -proteobacteria.

Acidobacteria formed the third major group. All the members related to this phylum were unculturable. Acidobacteria have the ability to degrade cellulose and other compounds found in rhizospheric soil for energy source. Acidobacteria are generally well-suited to low nutrient environments (Foesel *et al.*, 2014). Bacteroidetes and cyanobacteria, the fourth most dominant phyla are the major bacterial groups detected in agricultural and grass land soils (Borneman & Triplett, 1997). Bacteroidetes may be implicated in degrading of biopolymers and ferment sugars for carbon and energy source (Curtis & Sloan, 2004). Members of the cyanobacteria are typically found in surface soil and laminated ecosystems. Cyanobacteria due to their physiological flexibility are considered to play a fundamental role, together with diatoms, in soil stability and nutrient cycling in the extreme environments (Tseng & Tang, 2014).

Actinobacteria, chloroflexi, gemmatonadetes and planctomycetes were detected as minor components in the rhizosphere of para grass. These groups are in abundance in soils (Daniel, 2004; Poisot *et al.*, 2013). Actinobacteria identified in this work are related to iron-reducing, moderately thermophilic group of actinobacteria isolated from a solfataric field and can grow aerobically and heterotrophically. Chloroflexi are typically plant symbionts and are chemo-organotrophs or phototrophs. They are filamentous bacteria and mostly consuming the organic products of the autotrophic cyanobacteria. They are found in surface soils and some strains can use hydrogen or sulphide as an electron donor and grow autotrophically (Macrae *et al.*, 2000; Bjornsson *et al.*, 2002). Members of the gemmatonadetes were identified from activated sludge in a sewage treatment system. These bacteria are rarely isolated in cultivation studies. These bacteria can be used for biological phosphorus removal for wastewater treatment. Environmental sequence data indicate that this phylum is widespread in nature (Zhang *et al.*, 2003). Planctomycetales are abundant in oxic and anoxic soils, marine sediments and water habitats (Neef *et al.*, 1998). They play an important role in nutrient cycling and determinants of plant nitrate bioavailability as they are responsible for the anaerobic oxidation of ammonia (Wyman *et al.*, 2013).

The phylogenetic analysis of 16S rRNA clone library and bacterial isolates of the culture collection yielded different descriptions of the composition of the microbial community in the rhizosphere of para grass. The bacterial population identified through metagenomic analysis showed greater diversity as compared to culturable bacterial population. The 16S rRNA clone library was dominated by bacteria belonging to unclassified uncultured bacteria whereas in case of culturable bacteria, 75% bacterial population belonged to the phylum Proteobacteria. The members of Firmicutes isolated from pure cultures formed 15% and identified through 16S rRNA analysis 16.67%. Actinobacteria and Bacteroidetes identified through 16S rRNA clone library were both 2% and from the pure cultures were 8.33% and 2%, respectively. The members of Acidobacteria, Cyanobacteria, Chloroflexi, Gemmatonadetes and Planctomycetes were identified only through 16S rRNA gene sequence analyses (Fig. 5).

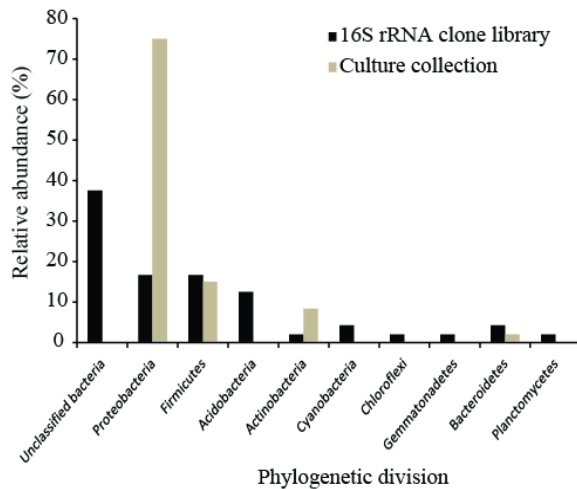


Fig. 5. Comparative analysis of the phylogenetic affiliation of 16S rRNA clones of unculturable bacteria and culturable bacteria from soil and roots of para grass.

This study provides information about the bacterial community associated with para grass, a moderate salt tolerant plant growing in Punjab, Pakistan. This study based culture dependent as well as culture-independent approaches. Studies on extreme environments such as saline conditions have revealed the presence of a considerable diversity of microorganisms. Though, the microbial study is important from such environments because it delineates biodiversity increases the prospect of having microbial resource in hand which can be further used for other purposes at a later stage but metagenomic studies enhances the extent of biodiversity contained in these environments.

## References

- Ahemad, M. and M. Kibret. 2014. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *J. King. Saud. Univ. Sci.*, 26: 1-20.
- Akhtar, N., M.A. Ghauri, A. Iqbal, M.A. Anwar and K. Akhtar. 2008. Biodiversity and phylogenetic analysis of culturable bacteria indigenous to Khewra salt mine of Pakistan and their industrial importance. *Braz. J. Microbiol.*, 39: 143-150.
- Alexander, M. 1982. Most probable number method for microbial populations, In: (Ed.): Page A.L., R.H. Miller, D.R. Keeney. *Methods of Soil Analysis, part 2: Chemical and microbiological properties*, 2<sup>nd</sup> edn. American Society of Agronomy, Madison, WI, pp. 815-820.
- Amann, R.L., W. Ludwig and K.H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.*, 59(1): 143-169.
- Bastida, F., T. Hernández, J. Albaladejo and C. García. 2013. Phylogenetic and functional changes in the microbial community of long-term restored soils under semiarid climate. *Soil. Biol. Biochem.*, 65: 12-21.
- Beneduzi, A., A. Ambrosini and M.P. Luciane. 2012. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. *Genet. Mol. Biol.*, 35 (4): 1044 -1051.
- Bilal, R. and K.A. Malik. 1988. Characterization of *Azospirillum* and related diazotrophs associated with roots of plants growing in saline soils. *World. J. Microbiol. Biotech.*, 6: 46-52.

- Bjornsson, L., P. Hugenholtz, G.W. Tyson and L.L. Blackall. 2002. Filamentous Chloroflexi (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. *Microbiology*, 148: 2309-2318.
- Borneman, J. and E.W. Triplett. 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.*, 63: 2647-2653.
- Cardenas, E. and J.M. Tiedje. 2008. New tools for discovering and characterizing microbial diversity. *Curr. Opin. Biotech.*, 19(6): 544-549.
- Chaoyan, L.V., X. Zhang and G. Liu. 2015. Variability of *Haloxylon ammodendron* (C.A. MEY) Bunge populations from different habitats. *Pak. J. Bot.*, 47(6): 2135-2141.
- Chu, H., N. Fierer, C.L. Lauber, J.G. Caporaso, R. Knight and P. Grogan. 2010. Soil bacterial diversity in the Arctic is not fundamentally different from that found in other biomes. *Environ. Microbiol.*, 12(11): 2998-3006.
- Compant, S., B. Duffy, J. Nowak, C. Clement and E.A. Barka. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.*, 71: 4951-4959.
- Curtis, T.P. and W.T. Sloan. 2004. Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. *Curr. Opin. Microbiol.*, 7(3): 221-226.
- Daniel, R. 2004. The soil metagenome - a rich resource for the discovery of novel natural products. *Curr. Opin. Biotech.*, 15 (3):199-204.
- Dobereiner, J. and J.M. Day. 1976. Associative symbiosis and free-living systems. *Plant. Soi.*, 174: 195-209.
- Felsenstein, J. 1985. Phylogenies and the comparative method. *Amer. Nat.*, 125: 1-15.
- Fierer, N., M.A. Bradford and R.B. Jackson. 2007. Toward an ecological classification of soil bacteria. *Ecology*, 88: 1354-1364.
- Foesel, B.U., V. Nägele and A. Naether. 2014. Determinants of acidobacteria activity inferred from the relative abundances of 16S rRNA transcripts in German grassland and forest soils. *Environ. Microbiol.*, 16(3): 658-675.
- Ghazanfar, S. and A. Azim. 2009. Metagenomics and its application in rumen ecosystem: potential biotechnological prospects. *Pak. J. Nut.*, 8(8): 1309-1315.
- Glick, B.R. 2012. Plant growth promoting bacteria: mechanisms and applications. *Scientifica*, 2012: 1-15.
- Haggag, W.M. and S. Timmusk. 2008. Colonization of peanut roots by biofilm-forming *Paenibacillus polymyxa* initiates biocontrol against crown rot disease. *J. Appl. Microbiol.*, 104: 961-969.
- Han, J., D. Xia, L. Li, L. Sun, K. Yang and L. Zhang. 2009. Diversity of culturable bacteria isolated from root domains of mosobamboo (*Phyllostachysedulis*). *Microb. Ecol.*, 58: 363-373.
- Huang, Q., H. Jiang, B.R. Briggs, S. Wang, W. Hou, G. Li, G. Wu, R. Solis, C.A. Arcilla, T. Abrajano and H. Dong. 2013. Archaeal and bacterial diversity in acidic to circumneutral hot springs in the Philippines. *FEMS. Microbiol. Ecol.*, 85(3): 452-64.
- Itoh, T., K. Yamanoi, T. Kudo, M. Ohkuma and T. Takashina. 2010. *Aciditerrimonas ferrireducens* gen. nov., sp. nov., a novel iron-reducing *Thermoacidophilic actinobacterium* isolated from a solfataric field in Japan. *Int. J. Syst. Evol. Microbiol.*, 12: 423-428.
- James, E.K. and F.L. Olivares. 1997. Infection and colonization of sugarcane and other graminaceous plants by endophytic bacteria. *Cri. Rev. Plant. Sci.*, 17: 77-119.

- Jayachandra, S.Y., K.S. Anil, Y.S. Shouche and M.B. Sulochana. 2013. Culturable diversity of extremely halophilic bacteria from West coast of Karnataka, India. *IJBPAS*, 2(2): 391-405.
- Karakas, S., M.A., Cullu, C. Kaya and M. Dikilitas, 2016. Halophytic companion plants improve growth and physiological parameters of tomato plants grown under salinity. *Pak. J. Bot.*, 48(1): 21-28.
- Khan, A.G. 2009. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. *J. Trace. Elem. Med. Biol.*, 18: 355-364.
- Ma, B. and J. Gong. 2013. A meta-analysis of the publicly available bacterial and archeal sequence diversity in saline soils. *World. J. Microbiol. Biotechnol.*, 29(12): 2325-2334.
- Macrae, A., D.L. Rimmer and A.G. O'Donnell. 2000. Novel bacterial diversity recovered from the rhizosphere of oilseed rape (*Brassica napus*) determined by the analysis of 16S ribosomal DNA. *Anton. Leu. Int. J.*, 78:13-21.
- Malik, K.A., R. Bilal, S. Mehnaz, G. Rasool, M.S. Mirza and S. Ali. 1997. Association of nitrogen-fixing, plant growth promoting rhizobacteria (PGPR) with kallar grass and rice. *Plant Soi.*, 194: 37-44.
- Martin, A.P. 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. *Appl. Environ. Microbiol.*, 68: 3673-3682.
- Miransari, M. 2011. Soil microbes and plant fertilization. *Appl. Microbiol. Biotechnol.*, 92: 875-885.
- Nakade, D.B. 2013. Bacterial diversity in sugarcane (*Saccharum officinarum*) rhizosphere of saline soil. *Int. Res. J. Biological. Sci.*, 2(2): 60-64.
- Neef, A., R. Amann, H. Schlesner and K.H. Schleifer. 1998. Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology*, 144: 3257-3266.
- Poisot, T., B. Pequin and D. Gravel. 2013. High-throughput sequencing: a roadmap toward community ecology. *Ecol. Evol.*, 3: 1125-1139.
- Qureshi, A.S., P.G. McCornick, M. Qadir and Z. Aslam. 2008. Managing salinity and water logging in the Indus Basin of Pakistan. *Agric. Water. Manag.*, 95: 1-10.
- Qureshi, R.H., M. Aslam and M. Rafiq. 1993. Expansion in the use of forage halophyte in Pakistan. In: (Eds.): Davidson, N. & R. Galloway. Productive Use of Saline Land. *ACIAR. Proc.*, 42: 12-6.
- Rincon-Florez, V.A., L.C. Carvalhais and P.M. Schenk. 2013. Culture-independent molecular tools for soil and rhizosphere microbiology. *Diversity*, 5: 581-612.
- Sacchi, C.T., A.M. Whitney, M.W. Reeves, L.W. Mayer and T. Popovic. 2002. Sequence diversity of *Neisseria meningitidis* 16S rRNA genes and use of 16S rRNA gene sequencing as a molecular subtyping tool. *J. Clin. Microbiol.*, 40(12): 4520-4527.
- Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4: 406-425.
- Seeley, H.W. and P.J. Van Demark. 1981. Microbes in action. A laboratory manual of Microbiology. 3<sup>rd</sup>edn. W.H Freeman and Company USA, pp. 350.
- Sharma, S.K., B.N. Johri, A. Ramesh, O.P. Joshi and S.V. Prasad. 2011. Selection of plant growth-promoting *Pseudomonas* spp. that enhanced productivity of soybean-wheat cropping system in central India. *J. Microbiol. Biotech.*, 21: 1127-1142.
- Sheng, J.L., S.H. Zheng, N.H. Li, L. Jie, H.S. Su, X.C. Lin, L.K. Jia, L. Jun, H. Min and S.S. We. 2014. Microbial communities evolve faster in extreme environments. *Scient. Rep.*, 4(6205): 1-9.
- Tamura, K., J. Dudley, M. Nei and S. Kumar. 2011. MEGA 5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, 28: 2731-2739.
- Tamura, K., M. Nei and S. Kumar. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA.*, 101: 11030-11035.
- Tan, Z.Y., X.D. Xu, E.T. Wan, J.L. Gao, E.M. Romer and W.X. Chen. 1997. Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related *Rhizobia*. *Int. J. Sys. Bacteriol.*, 47(3): 874-879.
- Thurmer, W.C., A. Wollherr, H. Nacke, N. Herold. 2010. Horizon specific bacterial community composition of German grassland soils, as revealed shotgun forest soil metagenomics by pyro-sequencing-based analysis of 16S rRNA genes. *Appl. Environ. Microbiol.*, 76: 6751-6759.
- Tseng, C.H. and S.L. Tang. 2014. Marine microbial metagenomics: from Individual to the environment. *Int. J. Mol. Sci.*, 15: 8878-8892.
- Vacheron, J., G. Desbrosses, M.L. Bouffaud, B. Touraine, Y. Moëgne-Loccoz and D. Muller. 2013. Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant. Sci.*, 4:356.
- Winnepenninckx, B., T. Backeljau and R. de Wachter. 1993. Extraction of high molecular weight DNA from molluscs. *Trends. Genet.*, 9: 407-412.
- Wu F.Z., C., Shao-Can, C., Ling, A., Mei-Jun, Z. Xin-gang and X.U. Wei-hui. 2015. Rhizosphere microbial communities from resistant and susceptible watermelon cultivars showed different response to *Fusarium oxysporum* F. sp. *niveum* inoculation. *Pak. J. Bot.*, 47(4): 1535-1546.
- Wyman, M., S. Hodgson and C. Bird. 2013. Denitrifying alpha-proteobacteria from the Arabian Sea that express the gene (*nosZ*) encoding nitrous oxide reductase in oxic and sub-oxic waters. *Appl. Environ. Microbiol.*, 79: 2670-2681.
- Zhang, H., Y. Sekiguchi, S. Hanada, P. Hugenholtz, H. Kim, Y. Kamagata and Nakamura 2003. *Gemmatimonas aurantiaca* gen. nov., sp. nov., a gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. *Int. J. Syst. Evol. Microbiol.*, 53(4): 1155-1163.

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