Pak. J. Bot., 48(2): 779-791, 2016.

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, Uncultureable 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 osmo-regulatory 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 ± 0.09 - 2.24 ± 0.77 (dSm^{-1}), SAR (Sodium Absorption Ratio) 9.59 ± 1.6-21.8 ± 4.8, pH 8.29 ± 0.21 - 8.48 ± 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 µl 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 µl 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 Porapack 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 rRNAgene: 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 GggatccTACGGCTACCTTGTTACGACTTCACCCC-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 (Fermantas). 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 Pi is the frequency of the ith species. Evenness was calculated as H/Hmax, where Hmax=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 150×10^7, 47×10^7 and130×10^7 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 into17 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.

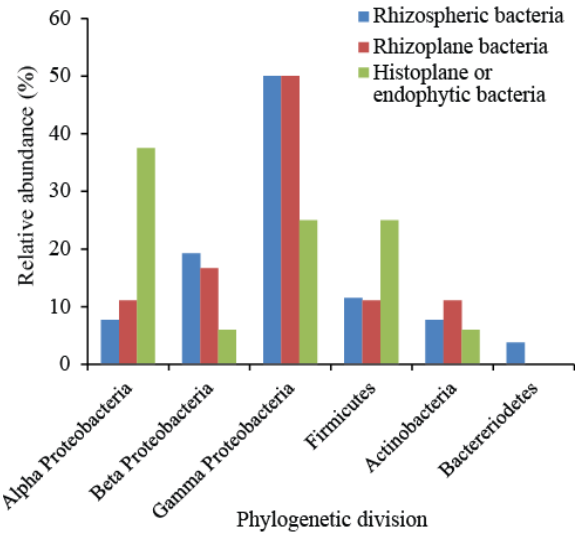

Fig. 1. Relative frequency of bacterial isolates belonging to different phylogenetic groups in the rhizosphere, rhizoplane and histoplane fraction of para grass.

Phylogenetic analysis of culturable bacteria: The rhizospheric bacteria were represented by four phyla, with the majority (51.84%) of the isolates falling within the □-proteobacteria group. Members of the classes, □-proteobacteria and ß-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 □-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 ß-proteobacteria was represented by 4 genera, *Burkholderia* (11.12%), *Comammonas* (3.71%), *Ralstonia* (3.71%) and *Alcaligenes* (3.71%). The □-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 $\times10^7$	Media* used	CFU $\times10^7$	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	*Rhodovibrio*	*salinarum*	1	1	1	
	Agrobacterium	*tumefaciens*			2	
	Azospirillum	*lipoferum*			1	
	Rhizobium	*tropici*			1	
	Paracoccus	*alkenifer*			1	
	Acetobacter	*pasteurianus*		1		
	Uncultured bacteria					1
Betaproteobacteria	*Burkholderia*	*cepacia*	3			
		cenocepacia		1		
	Comamonas	sp.	1			
	Ralstonia	*picketti*	1			
	Alcaligenes	*faccalis*	1			
		sp.		1		
	Sphingomonas	sp.		1		
	Nitrosomonas	sp.			1	
	Massilia	sp.				1
	Duganella	sp.				1
	Uncultured bacteria					2
Deltaproteobacteria	*Chondromyces*	*pediculatus*				1
	Sorangiineae bacteria					1
	Uncultured bacteria					2
Gammaproteobacteria	*Pasteurella*	*multocida*	1			
	Kluyvera	*georgiana*	1			
		ascorbata			1	
	Pseudomonas	*moraviensis*		1		
		putida	2			
		fluorescens	1			
		chlororaphis		1		
		stutzeri		1		
	Xanthomonas	*axonopodis*	2	1		
	Citrobacter	*freundii*	1		1	
	Escherichia	*coli*	1	1		
	Klebsiella	*oxytoca*	1	1		
		pneumoniae	1			
	Vibrio	*proteolyticus*	1			
	Azotobacter	*beijerinckii*	1			
		sp.		1		
	Serratia	*Marcescens*	1			
	Pectobacterium	*carotovorum*		1		
	Aeromonas	*veronii*		1		
	Proteus	*Vulgaris*			1	
	Moraxella	*boevrei*			1	
	Enterobacter	*asburiae*		1	1	
Firmicutes	*Entrococcus*	sp.	1	1	1	
	Streptococcus	*pseudopneumoniae*	1			
	Staphylococcus	*haemolyticus*	1			
		gallinarum			1	
	Veillonella	sp.			1	
	Clostridium	sp.				1
		uncultured sp.				1
	Acetivibrio	*cellulolyticus*				1
	Bacillus	*Megaterium*				1
		subtilis				1
		sp.				1
		uncultured sp.				1
Acidobacteria	Uncultured marine bacteria					1
	Uncultured soil bacteria					5
Actinobacteria	*Micrococcus*	*roseus*	1			
		luteus		2	2	
	Frankia	sp.	1			
	Uncultured soil bacteria					1
Bacteroidetes	*Flavobacterium*	sp.	1			
	Anaerosinus	*Glycerini*				1
Chloroflexi	*Caldilinea*	*tarbellica*				1
		uncultured sp.				1
Cyanobacteria	*Microcoleus*	*steenstrupii*				1
	Microcoleus	sp.				1
Planctomycetes	Uncultured soil bacteria					1
Gemmatonadetes	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

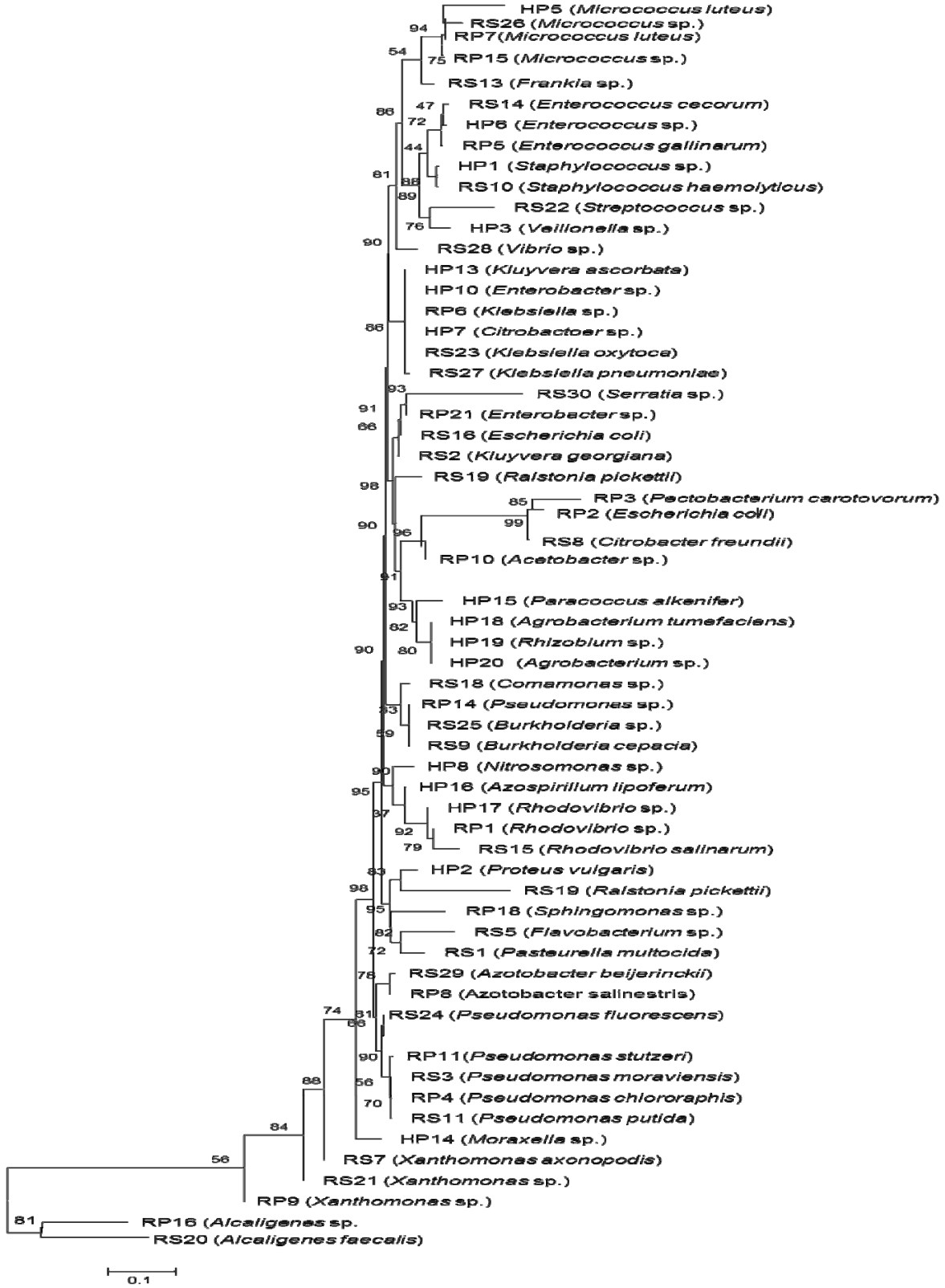

Fig. 2. Phylogenetic tree based on partial 16S rRNA gene sequences of bacterial isolates from the rhizosphere of para grass. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances 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 □-proteobacteria, 16.67% of ß-proteobacteria and 11.11% of □-proteobacteria. All the bacterial isolates from rhizoplane were belonged to 15 genera. The class □-proteobacteriawas represented by eight genera, *Pseudomonas* (16.67%), *Xanthomonas, Escherichia, Klebsiella, Azotobacter, Erwinia, Pectobacterium, Aeromonas* (5.56% each). The ß-proteobacteria was represented by *Burkholderia* (5.56%), *Sphingomonas* (5.56%) and *Alcaligenes* (5.56%). The □-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 □-proteobacteria was represented by five genera, *Agrobacterium* (11.76%), *Rhodovibrio* (5.89%), *Azospirillum* (5.89%), *Rhizobium* (5.89%) and *Paracoccus* (5.89%). The group □-proteobacteria also included five genera *Kluyvera* (5.89%), *Citrobacter* (5.89%), *Proteus* (5.89%), *Moraxella* (5.89%) and *Enterobacter* (5.89%). The ß-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 □-proteobacteria (51.84% and 50%, respectively). The majority of endophytic bacteria were □-proteobacteria (35.29%). Members of □-proteobacteria, ß-proteobacteria, □-proteobacteria, actinobacteria and firmicutes were isolated from all fractions. Bactereriodeteswas 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 *Erwina*, *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 unculturablebacteria 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, bactereriodetes, 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 anaylsis 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, acitnobacteria, cyanobacteria and choroflexi 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, □-proteobacteria, ß-proteobacteria and δ-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 chlorflexi was represented by two groups, uncultured *chloroflexi* bacteria (2.1%) and rarely culturable *Caldilinea* sp. (2.1%). The cyanobacteria and bactereriodetes were represented by *Microcoleus* (4.16%) and *Anaerosinus* (2.1%), respectively. The groups planctomycetes (2.1%), actinobacteria (2.1%) and gemmatonadetes (2.1%) included only one group, uncultured soil bacteria.

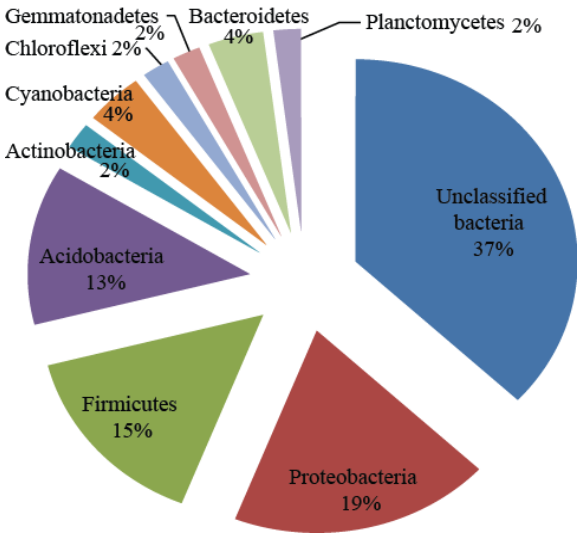

Fig. 3. Percentage distribution of bacterial phyla in soil and roots of para grass, determined by sequencing of 16S rRNA gene.

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)	*Rhodovibrio*	RS15	*Rhodovibrio* sp.	*Rhodovibrio salinarum*	98; HE800441
	Burkholderia	RS9	*Burkholderia capacia*	*Burkholderia capacia*	99; HF678361
		RS4	*Burkholderia* sp.	*Burkholderia capacia*	99; HG328353
		RS25	*Burkholderia* sp.	*Burkholderia cenocepacia*	98; HF678367
	Comamonas	RS18	Unknown	*Comamonas* sp.	98; HF678364
	Ralstonia	RS19	*Ralstonia* sp.	*Ralstonia picketti*	99; HE800442
	Alcaligenes	RS20	Unknown	*Alcaligenes faccalis*	99; HG316109
	Pasteurella	RS1	*Pasteurella multocida*	*Pasteurella multocida*	98; HE800437
	Kluyvera	RS2	*Kluyvera* sp.	*Kluyvera georgiana*	99; HF678358
	Pseudomonas	RS3	*Pseudomonas* sp.	*Pseudomonas putida*	98; HF678359
		RS11	*Pseudomonas putida*	*Pseudomonas putida*	99; HE800440
		RS24	*Pseudomonas florescens*	*Pseudomonas fluorescens*	98; HF678366
	Xanthomonas	RS7	*Xanthomonas* sp.	*Xanthomonas axonopodis*	99; HF678360
		RS21	*Xanthomonas* sp.	*Pseudoxanthomonas japonensis*	99; HE800443
	Citrobacter	RS8	*Citrobacter freundii*	*Citrobacter freundii*	99; HE800438
	Escherichia	RS16	*Escherichia coli*	*Escherichia coli*	99; HF678363
	Klebsiella	RS23	*Klebsiella oxytoca*	*Klebsiella oxytoca*	99; HF678365
		RS27	*Klebsiella* sp.	*Klebsiella pneumonia*	98; HE800445
	Vibrio	RS28	*Vibrio* sp.	*Vibrio proteolyticus*	99; HG316110
	Azotobacter	RS29	Unknown	*Azotobacter beijerinckii*	96; HF678368
	Serratia	RS30	*Serratia* sp.	*Serratia marcescens*	99; HE800446
	Staphylococcus	RS10	*Staphylococcus* sp.	*Staphylococcus haemolyticus*	95; HE800439
	Entrococcus	RS14	*Entrococcus* sp.	*Entrococcus* sp.	99; HG316108
	Streptococcus	RS22	*Streptococcus* sp.	*Streptococcus pseudopneumoniae*	97; HF947006
	Micrococcus	RS26	*Micrococcus roseus*	*Micrococcus roseus*	99; HE800444
	Frankia	RS13	Unknown	*Frankia* sp.	98; HF678362
	Flavobacterium	RS5	Unknown	*Flavobacterium* sp.	97; HF947005
Rhizoplane (RP)	*Rhodovibrio*	RP1	*Rhodovibrio* sp.	*Rhodovibrio salinarum*	99; HF678369
	Acetobacter	RP10	*Acetobacter* sp.	*Acetobacter pasteurianus*	98; HF947008
	Burkholderia	RP14	*Burkholderia* sp.	*Burkholderia cenocepacia*	98; HF678376
	Alcaligenes	RP16	Unknown	*Alcaligenes* sp.	99; HG316114
	Sphingomonas	RP18	Unknown	*Sphingomonas* sp.	99; HG316115
	Escherichia	RP2	*Escherichia coli*	*Escherichia coli*	99; HF678370
	Pectobacterium	RP3	*Erwinia carotovora*	*Pectobacterium carotovorum*	100; HF678371
	Pseudomonas	RP4	*Pseudomonas aurantiaca*	*Pseudomonas chlororaphis*	99; HF678372
		RP11	*Pseudomonas* sp.	*Pseudomonas stutzeri*	100; HF678375
		RP17	*Pseudomonas* sp.	*Pseudomonas moraviensis*	99; HF678377
	Klebsiella	RP6	*Klebsiella* sp.	*Klebsiella oxytoca*	98; HF678373
	Azotobacter	RP8	Unknown	*Azotobacter* sp.	100; HG316112
	Xanthomonas	RP9	*Xanthomonas* sp.	*Xanthomonas* sp.	99; HF678374
	Aeromonas	RP19	*Aeromonas veronii*	*Aeromonas veronii*	99; HG316116
	Entrococcus	RP5	*Entrococcus* sp.	*Entrococcus gallinarum*	98; HG316111
		RP21	*Enterobacter aerogenes*	*Enterobacter aerogenes*	98; HF947009
	Micrococcus	RP7	*Micrococcus luteus*	*Micrococcus luteus*	98; HF947007
		RP15	*Micrococcus* sp.	*Micrococcus luteus*	99; HG316113
Histoplane (HP)	*Paracoccus*	HP15	*Paracoccus* sp.	*Paracoccus alkenifer*	98; HF678381
	Azospirillum	HP16	*Azospirillum* sp.	*Azospirillum lipoferum*	99; HF678382
	Rhodovibrio	HP17	*Rhodovibrio* sp.	*Rhodovibrio salinarum*	98; HF678383
	Agrobacterium	HP18	*Agrobacterium tumefaciens*	*Agrobacterium tumefaciens*	100; HF678384
		HP20	*Argobacterium* sp.	*Agrobacterium tumefaciens*	98; HF678386
	Rhizobium	HP19	*Rhizobium* sp.	*Rhizobium* sp.	99; HF678385
	Nitrosomonas	HP8	Unknown	*Nitrosomonas* sp.	99; HF678378
	Enterobacter	HP10	*Enterobacter* sp.	*Enterobacter asburiae*	100; HF678379
	Kluyvera	HP13	*Kluyvera* sp.	*Kluyvera ascorbata*	99; HF678380
	Moraxella	HP14	*Moraxella* sp.	*Moraxella boevrei*	99; HF947012
	Proteus	HP2	*Proteus vulgaris*	*Proteus vulgaris*	98; HF947011
	Citrobactoer	HP7	*Citrobacter freundii*	*Citrobacter freundii*	99; HG316118
	Stophylococcus	HP1	*Stophylococcus* sp.	*Stophylococcus gallinarum*	99; HF947010
	Entrococcus	HP6	*Entrococcus* sp.	*Entrococcus sp.*	98; HG316117
	Veillonella	HP3	Unknown	*Veillonella* sp.	99; HE800447
	Micrococcus	HP5	*Micrococcus luteus*	*Micrococcus luteus*	95; HE800448
		HP9	*Micrococcus* sp.	*Micrococcus luteus*	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	*Alpha proteobacterium* KC-IT-H1 (FJ711201)	95; HE647639
Rhizobiaceae	S24	Uncultured bacterium (HQ287208)	92; HE800457
Oxalobacteraceae	S23	*Massilia* sp. (JQ014506)	98; HE800456
Oxalobacteraceae	S45	*Duganella* sp. (EF575562)	99; HF560637
Sphingobacteriaceae	S25	Uncultured bacterium (EU445227)	82; HE800458
Comamonadaceae	S29	Uncultured soil bacterium (GU598823)	88; HE800462
Polyangiaceae	S1	*Chondromyces pediculatus* strain Cm p51(GU207875)	85; HE647629
Polyangiaceae	S2	Sorangiineae bacterium SBSr005 (GU249612)	84; HE647630
Proteobacteria	S17	Uncultured proteobacterium (FJ542849)	94; HE800450
Clostridiaceae	S3	*Clostridium* strain FCB90-3 (AJ229251)	95; HE647631
Clostridiaceae	S5	*Acetivibrio cellulolyticus* strain CD2 (NR025917)	91; HE647632
Clostridiaceae	S19	Uncultured bacterium (FN658847)	92; HE800452
Bacillaceae	S9	*Bacillus megaterium* strain MO29 (AY553116)	91; HE647636
Bacillaceae	S16	*Bacillus* sp. (FJ981907)	96; HE800449
Bacillaceae	S26	*Bacillus subtilis* (EF584109)	85; HE800459
Bacillaceae	S28	Uncultured *Bacillus* 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	*Microcoleus* sp. HTT-U-KK5 (EF654070)	92; HE647635
Phormidiaceae	S34	*Microcoleus steenstrupii* (AJ871982)	94; HE800467
Peptococcaceae	S6	*Caldilinea tarbellica* (HM134893)	83; HE647633
Chloroflexaceae	S22	Uncultured *Chloroflexi* 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	*Anaerosinus glycerini* 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

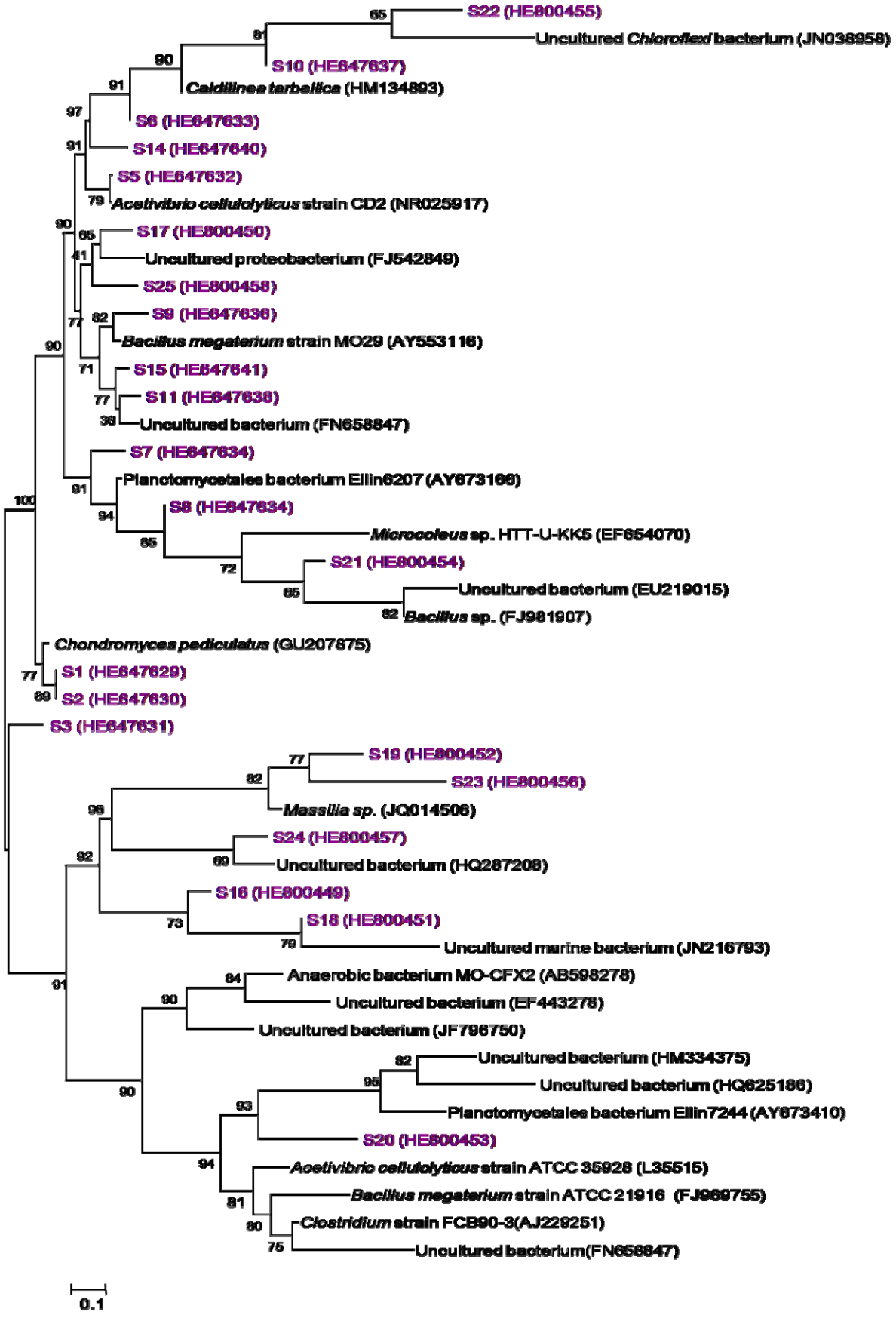
S22 (HE800455)
Uncultured Chloroflexi bacterium (JN038958)
S10 (HE647637)
Caldilinea tarbellica (HM134893)
S6 (HE647633)
S14 (HE647640)
S5 (HE647632)
Acetivibrio cellulolyticus strain CD2 (NR025917)
S17 (HE800450)
Uncultured proteobacterium (FJ542849)
S25 (HE800458)
S9 (HE647636)
Bacillus megaterium strain MO29 (AY553116)
S15 (HE647641)
S11 (HE647638)
Uncultured bacterium (FN658847)
S7 (HE647634)
Planctomycetales bacterium Ellin6207 (AY673166)
S8 (HE647634)
Microcoleus sp. HTT-U-KK5 (EF654070)
S21 (HE800454)
Uncultured bacterium (EU219015)
Bacillus sp. (FJ981907)
Chondromyces pediculatus (GU207875)
S1 (HE647629)
S2 (HE647630)
S3 (HE647631)
S19 (HE800452)
S23 (HE800456)
Massilia sp. (JQ014506)
S24 (HE800457)
Uncultured bacterium (HQ287208)
S16 (HE800449)
S18 (HE800451)
Uncultured marine bacterium (JN216793)
Anaerobic bacterium MO-CFX2 (AB598278)
Uncultured bacterium (EF443278)
Uncultured bacterium (JF796750)
Uncultured bacterium (HM334375)
Uncultured bacterium (HQ625186)
Planctomycetales bacterium Ellin7244 (AY673410)
S20 (HE800453)
Acetivibrio cellulolyticus strain ATCC 35928 (L35515)
Bacillus megaterium strain ATCC 21916 (FJ969755)
Clostridium strain FCB90-3(AJ229251)
Uncultured bacterium(FN658847)
0.1

Fig. 4. Cont'd.

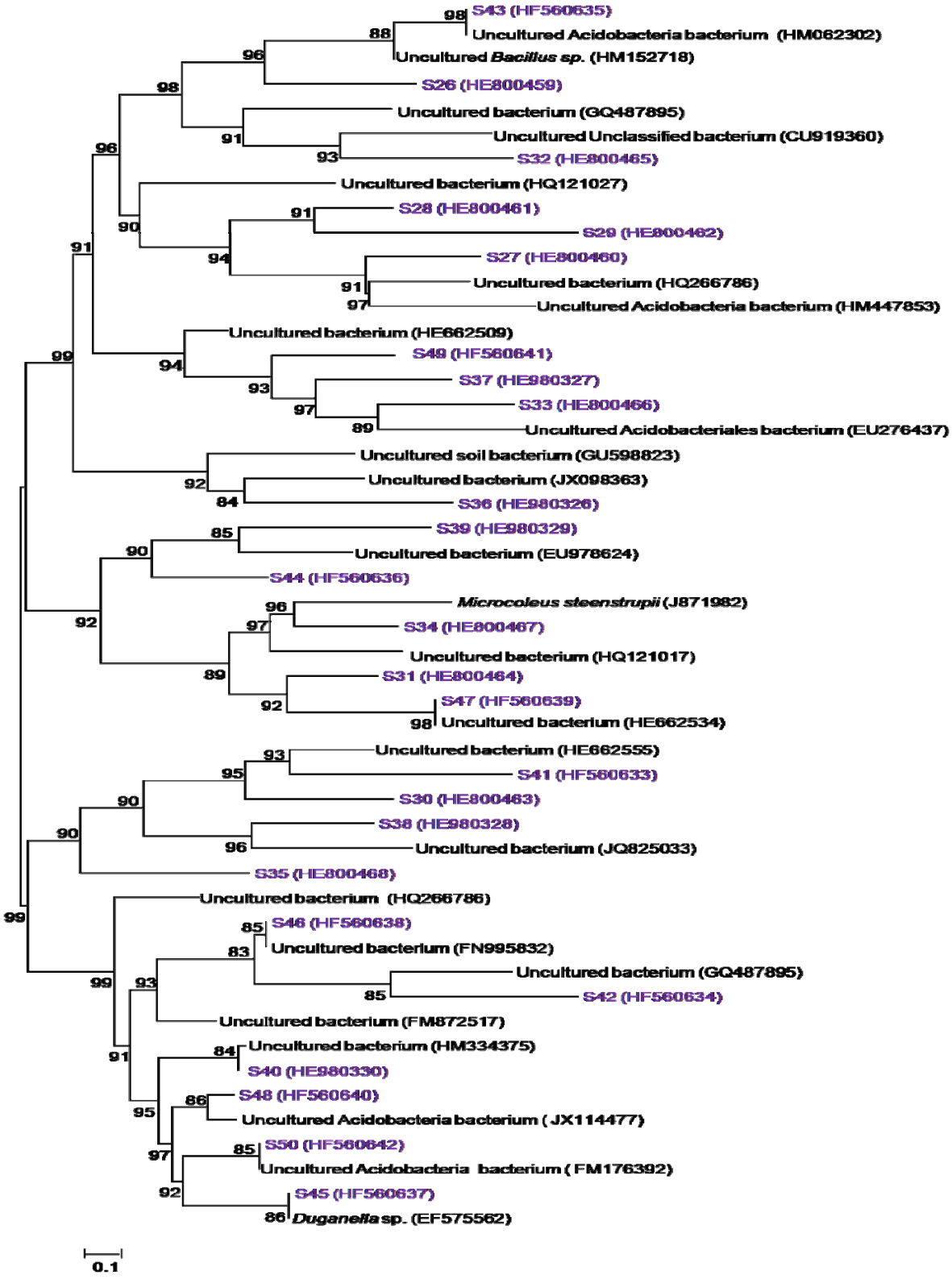

Fig. 4. Phylogenetic tree based on partial 16S rRNA gene clone library sequences directly isolated from the rhizosphere of para grass. Representative sequences (S) were selected to represent each group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap 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 □-proteobacteria, *Pseudomonas*, *Klebsiella, Xanthomonas, Pasteurella, Kluyvera, Escherichia, Azotobacter* and *Serratia* are considered as important constituents in the root-associated microbial community and their ability tocolonize 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 □-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 ß-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 ß-proteobacteria.

Acidobacteria formed the third major group. All the members related to this phylum were unculturable. Aicdobacteria have the ability to degrade cellulose and other compounds found in rhizospheric soil for energy source. Acidobacteriaare generally well-suited to low nutrient environments (Foesel *et al.,* 2014). Bacteroidetes and cyanobacteria, the forth most dominant phyla are the major bacterial groups detected in agricultural and grass land soils (Borneman & Tripplett, 1997). Bacteroidetes may be implicated in degrading of biopolymers and ferment sugars for carbon and energy source (Curtis & Sloan, 2004). Members of thecyanobacteria 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 compare 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).

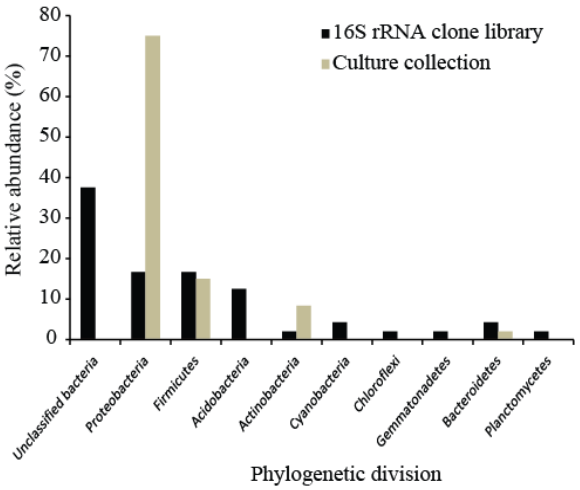

Fig. 5. Comparative analysis of the phylogenetic affiliation of 16S rRNA clones of unculturable bacteria and culturable bacteria from soil and roots of para grass.

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.

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(Received for publication 9 March 2015)