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Original article

A comparative study of bacterial diversity based on culturable and culture-independent techniques in the rhizosphere of maize (*Zea mays* L.)

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ABSTRACT

Objective: Maize is an important crop for fodder, food and feed industry. The present study explores the plant-microbe interactions as alternative eco-friendly sustainable strategies to enhance the crop yield.
Methodology: Bacterial diversity was studied in the rhizosphere of maize by culture-dependent and culture-independent techniques by soil sampling, extraction of DNA, amplification of gene of interest, cloning of desired fragment and library construction.
Results: Culturable bacteria were identified as *Achromobacter, Agrobacterium, Azospirillum, Bacillus, Brevibacillus, Bosea, Enterobacter, Microbacterium, Pseudomonas, Rhodococcus, Stenotrophomonas* and *Xanthomonas* genera. For culture-independent approach, clone library of 16S ribosomal RNA gene was assembled and 100 randomly selected clones were sequenced. Majority of the sequences were related to Firmicutes (17%), Acidobacteria (16%), Actinobacteria (17%), Alpha-Proteobacteria (7%), Delta-proteobacteria (4.2%) and Gemmatimonadetes (4.2%) However, some of the sequences (30%) were novel that showed no homologies to phyla of cultured bacteria in the database. Diversity of diazotrophic bacteria in the rhizosphere investigated by analysis of PCR-amplified *nif*H gene sequence that revealed abundance of sequences belonging to genera *Azoarcus* (25%), *Aeromonas* (10%), *Pseudomonas* (10%). The diazotrophic genera *Azotobacter, Agrobacterium* and *Zoogloea* related *nif*H sequences were also detected but no sequence related to *Azospirillum* was found showing biasness of the growth medium rather than relative abundance of diazotrophs in the rhizosphere.
Conclusion: The study provides a foundation for future research on focussed isolation of the *Azoarcus* and other diazotrophs found in higher abundance in the rhizosphere.
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1. Introduction

Maize (*Zea mays* L.) is one of the most widely grown crop for fodder, food and feed industry. Maize used as raw material for energy generation in world (Byrt et al., 2011). Recent introduction of high yielding hybrid varieties coupled with adoption of cropping system with two crops per year instead of a single maize crop have contributed to popularity of this crop among the farming

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community in Pakistan. Securing sustainable yields requires a detailed knowledge of genetic and environmental factors that influence crop. Developed nations have used extensive breeding and management strategies for maximizing yields with higher inputs of fertilizers and insecticides. As the environmental and economic concerns of using these chemical increased. Alternative strategies are being employed to enhance the cropping system sustainability using eco-friendly approaches while retaining the competitive crop yields. The interaction between rhizosphere microbiota colonizing the plant root plays an important role in crop yield. The plant-microbe interactions taking place between "plant growth promoting rhizobacteria (PGPR) and plant root" mediate plant's nutrient acquisition and disease tolerance. These rhizobacteria are a diverse group of microbes like *Azospirillum, Azotobacter, Bacillus, Burkholderia, Herbaspirillum and Pseudomonas* (Bhattacharyya and Jha, 2012; Qaisrani et al., 2014; Ayyaz et al., 2016) and involved in plant stimulation by atmospheric N_2-fixation, phytohormone production, antagonism against pathogens, phosphate solubilization, siderophore production and biofilm formation. The beneficial effects of PGPR inoculation have been studied on various crops including maize (Bhattacharyya and Jha, 2012; Sheng et al., 2012; Zaheer et al., 2016).

Traditionally, analysis of bacterial communities and diversity has been dependent upon the cultivation of the microbes from the environment. However, culture-based studies provide limited information of community structure because majority of bacteria cannot be cultured in laboratory due to lack of information on specific growth requirements. As a result a large proportion of microbial population remained un-explored. Advancement in culture-independent techniques like sequence analysis of amplicons of 16S rRNA and *nif*H genes from soil DNA, has facilitated microbial diversity studies by comparing composition, richness, and structure of the prokaryotic communities in soil and other environments (Mirza et al., 2014; Hakim et al., 2018). These studies have even facilitated soil microbiologists to make more focused attempts to isolate useful microbes.

Previously, analysis of bacterial taxa associated with maize was based on culturable fraction or culture-independent fraction (Sanguin et al., 2006) separately. No data is available on the comparative analysis or the *nif*H based analysis of culture-dependent and culture-independent fractions of rhizosphere communities from the rhizosphere of maize. In present study maize rhizosphere's soil samples were collected and investigated the culturable fraction of bacterial community from maize by isolations on growth media, followed by *16S rRNA* based identification of isolates. Bacterial diversity studies were extended to non-culturable fraction by extracting DNA directly from that soil for PCR amplification of 16S rRNA and *nif*H genes, followed by sequence and phylogenetic analysis.

2. Methodology

2.1. Analysis of bacterial diversity through culture-dependent technique

Rhizospheric soil and roots samples of maize plants (variety FSH 810) were collected from experimental fields of NIBGE (National Institute for Biotechnology and Genetic Engineering). The field soil was a sandy loam and bacteria were isolated on LB (Luria-Bertani) agar and NFM (Nitrogen-Free Medium) medium (Okon et al., 1977) using serial dilution plating technique. Colonies with different shape, size and color purified separately through subculturing on the same medium. Nitrogen fixers were obtained by enrichment technique, root pieces (5–10 mm length) were inoculated along with rhizospheric soil to NFM medium and after 5–6 enrichments, single colonies were purified on LB plates. Colony morphology was studied after 24 h of incubation at 28 ± 2 °C.

2.2. PCR amplification and cloning

CTAB (Cetyl Trimethyl Ammonium Bromide) method was used to extract total genomic DNA from pure bacterial strains. 16S ribosomal RNA gene was amplified by primers PH: 5′-AAGGAGGTGATC CAGCCGCA-3′ and PA: 5′-AGAGTTTGATCCTGGCTCAG-3′ using conditions reported by Qaisrani et al. (Qaisrani et al., 2014). The PCR products were cloned in pTZ57/R vector (Fermentas, Germany), confirmed by restriction analysis and sequenced commercially from Macrogen, Korea. Molecular phylogenetic analysis of strains were done as per Zaheer et al. (2016) study.

2.3. Analysis of bacterial diversity by culture-independent technique

2.3.1. Soil sampling and DNA extraction

Rhizospheric soil samples were collected from three maize plants and pooled to prepare a composite sample. From this composite sample two sub-samples (0.5 g each) were used for extraction of soil DNA. Soil DNA was extracted using Fast DNA Spin Kit (MP Biomedicals Inc, France).

2.3.2. PCR amplification, cloning and library construction

To amplify 16S rRNA gene from soil DNA, primers and reaction conditions were same as reported earlier by Qaisrani et al. (2014). For amplification of *nif*H; PolF and PolR primers was used. PCR conditions were the same as Qaisrani et al. (2014); except annealing temperature of 48 °C. The PCR products were cloned in pTZ57/R vector (Fermentas, Germany), confirmed by restriction analysis. 100 clones were randomly selected and sequenced commercially from Macrogen, Korea. For *nif*H, PCR products of *nif*H from six independent reactions were combined and cloned. Forty clones were selected randomly and sequenced.

3. Results

3.1. Bacterial diversity using culture-dependent method

Ten isolates were identified as *Bacillus,* four as *Azospirillum brasilense,* two *Pseudomonas stutzeri,* three *Stenotrophomonas* spp., two *Enterobacter* spp. and one each of *Brevibacillus, Agrobacterium, Bosea* and *Microbacterium* sp., based on 16S rRNA gene analysis (Table 1). *Bacillus* genera came as dominant genera in culturable population followed by *Azospirillum* sp. and *Stenotrophomonas* sp. (Fig. 1).

3.2. Bacterial diversity revealed by 16S rRNA gene sequence analysis

Out of 100 clones sequenced randomly from *16S rRNA* clone library, 70 clones provided the good read-length and sequence information (Table 2). Most of the clones (30%) were related to the uncultured bacterial sequences, which did not show any similarity with the known phyla or taxa (Fig. 2). Firmicutes, acidobacteria, actinobacteria, alpha-proteobacteria, delta-proteobacteria, and gemmatimonadetes were the major phyla found.

3.3. Diversity of diazotrophs revealed by nifH sequence analysis

Twenty clones provided the sequence information out of 40 clones sequenced from *nif*H gene clone library. Among the *nif*H sequences obtained in the present study, 65% sequences showed similarity with those of culturable diazotrophs and the remaining 35% showed sequence similarity with non-culturable bacteria

Table 1
Identification of pure strains on the basis of 16S rRNA gene.

Isolate ID	Accession # of the isolates	Description	Maximum similarity (%) in the databank and accession number
M1	HE646771	*Azospirillum brasilense*	FR745918 (98)
M7	HE646772	*Azospirillum brasilense*	FR745918 (99)
M9	HE646773	*Pseudomonas stutzeri*	GQ402828 (97)
M14	HE646774	*Pseudomonas stutzeri*	GQ402828 (99)
M18	HE984300	*Enterobacter* sp.	AB641897 (96)
M25	HE646775	*Achromobacter* sp.	EU220009 (99)
M27	HE646776	*Stenotrophomonas*sp.	GQ360071 (99)
M28	HE646777	*Rhodococcus*sp.	FJ752527 (99)
M32	HE646779	*Bacillus niabensis*	JQ946067 (98)
M34	HE646785	*Bacillus* sp.	KF596683 (99)
M35	HE984301	*Azospirillum brasilense*	HE977616 (97)
M36	HE646783	*Agrobacterium* sp.	FJ719340 (86)
M37	HE646782	*Stenotrophomonas* sp.	HM461149 (89)
M38	HE646793	*Bosea* sp.	JQ689184 (77)
M39	HE646781	*Stenotrophomonas* sp.	AB508855 (85)
MA	HE646786	*B. megaterium*	KC692200 (99)
MB	HE646787	*B. tequilensis*	KC172005 (99)
MC	HE646788	*B. licheniformis*	JN852814 (99)
ME	HE646789	*B. thuringensis*	KF317874 (99)
MF	HE646790	*B. nanhaiensis*	KC992295 (99)
MG	HE646791	*B. niabensis*	JQ946066 (98)
MH	HE646792	*Brevibacillusbervis*	JF772474 (98)
MP4	HE984302	*Microbacterium* sp.	JQ660077 (98)
MP5	HE646780	*Bacillus* sp.	FJ784129 (98)
MP7	HE984303	*Enterobacter* sp.	FJ868807 (98)
MP8	HE984304	*Bacillus lichneformis*	HQ266667 (99)
ZN1	HE646778	*Azospirillum brasilense*	DQ288686 (99)

(Table 3). The *nif*H sequences similar to culturable diazotrophs belonged to genera *Aeromonas*, *Agrobacterium*, *Azoarcus*, *Azotobacter*, *Bacillus*, *Pseudomonas* and *Zoogloea*, which showed culturing with their respective member in phylogenetic analysis (Fig. 3).

4. Discussion

Investigation of bacterial communities and diversity in the plant's rhizosphere is very important as these microbes exert direct beneficial or pathogenic effect on plants. Despite the abundance of bacterial species in the rhizosphere, more than 99% of these species cannot be cultured that include 31 bacterial phyla. Metagenomic analysis provided detailed information of microbial diversity, composition, richness, structure and function (Mirza et al., 2014). Comparison of culturable and non-culturable community will help to determine the structurally abundant, functionally viable and potentially valuable bacteria that can ultimately be used as inoculum to influence the plant health in a positive manner.

Limited studies are available regarding the bacterial diversity in maize rhizosphere. In the present study, bacterial diversity was compared by using culture-dependent technique and culture-independent technique. Among the culturable population obtained dominant (37%) were members from *Bacillus* spp. which have been widely stated in the rhizosphere of different plants (Hakim et al., 2018). Bacterial isolates showing plump rods with vibroid motility in N-free semi solid NFM medium, showed high sequence similarity with *Azospirillum brasilense* strains. Azospirilla have been isolated from many crops including cereals, legumes and grasses (Qaisrani et al., 2014; Ayyaz et al., 2016).

Among others, following genera *Pseudomonas stutzeri, Stenotrophomonas maltiphilia Enterobacter*, *Agrobacterium, Microbacterium, Bosea,* and *Brevibacillus* spp. were obtained. From maize rhizosphere, the isolation of *Pseudomonas, Enterobacter*, *Microbacterium*

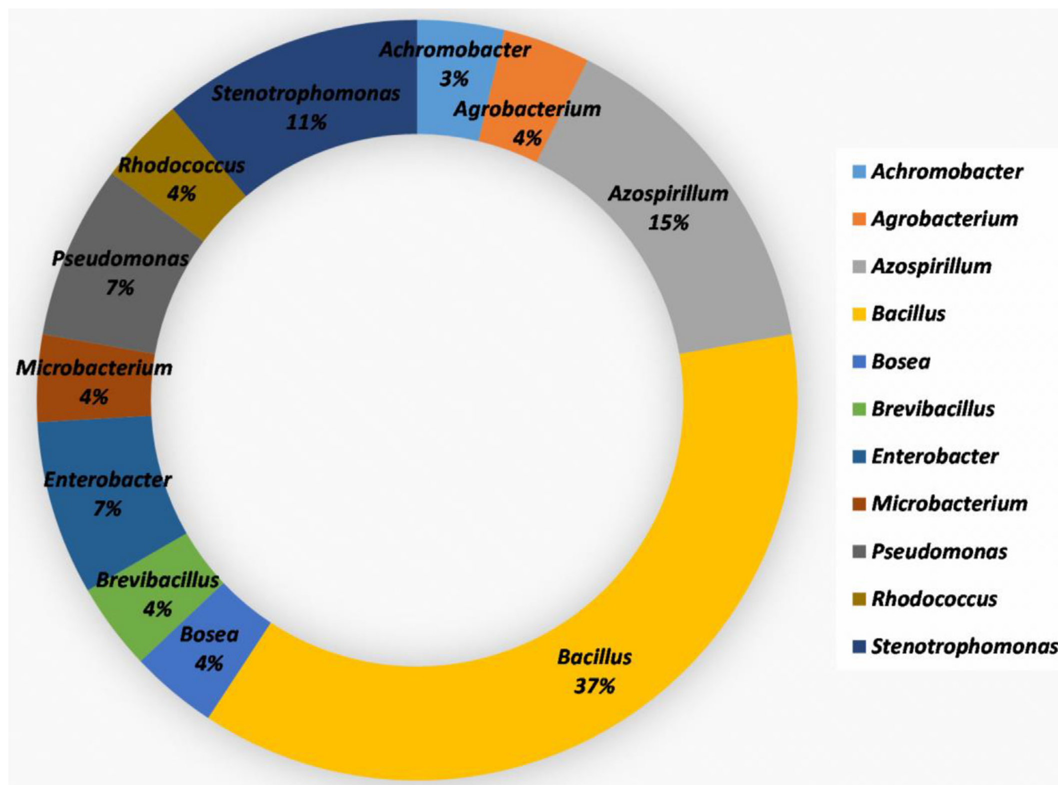

Fig. 1. On the base of *16S rRNA*, detection of culturable bacteria from the rhizoshperic soil of maize.

Table 2
Clone identification on the basis of 16S rRNA gene rhizospheric DNA of maize.

Clone ID	Accession # of the clones	Description	Maximum similarity (%) in the databank and accession number
MRS1	HE585109	Uncultured (U) bacterium	EU160410 (98)
MRS2	HE585110	*Bacillus* sp.	AJ315064 (95)
MRS3	HE585111	U. Myxococcales bacterium	EU445232 (95)
MRS4	HE585112	U. bacterium	EU676444 (94)
MRS5	HE585113	U. *Acitivibrio* sp.	JX505257 (98)
MRS6	HE585114	*Actinomadura* sp.	AF131317 (95)
MRS8	HE585115	U. bacterium	FJ893527 (84)
MRS9	HE585116	*Paenibacillus validus*	GU191921 (95)
MRS11	HE585117	U. *Acidobacterium*	DQ514045 (99)
MRS12	HE585118	U. *Acidimicrobium*	FJ551475 (94)
S1	HE599540	U. *Bacillus* sp.	HE646746 (99)
S2	HE599541	U. *Gemmatimonas* sp.	HM447783 (96)
S3	HE599542	U. *Bacillus* sp.	JQ793577 (97)
S4	HE599543	U. bacterium	JN177890 (98)
S5	HE599544	U. acetobacteraceae	EU193084 (98)
S6	HE599545	U. Gemmatimonodetes	AY921704 (100)
S7	HE599546	U. *Acidobacteria*	HM447891 (98)
S8	HE599547	U. Nocardiodes	HE662540 (98)
S9	HE599548	U. *Acidobacteria*	HE646770 (76)
S10	HE599549	U. Acidobacteriaceae	FJ550882 (92)
S11	HE599550	U. *Acidobacteria*	EU979108 (93)
S12	HE599551	*Syntrophobacter fumaroxidans*	EU266858 (92)
S13	HE599552	U. acidobacterium	JN409041 (93)
S14	HE599553	U. Acidobacteriaceae	HM438249 (99)
S15	HE599554	U. *Conexibacter* sp.	FJ551841 (97)
S16	HE599555	U. *Kaistobacter*sp.	FJ889320 (98)
S17	HE599556	U. Acidobacteriaceae	HM438240 (99)
S18	HE599557	U. bacterium	JN030403 (98)
S19	HE599558	U. Sphingomonadales	FJ889322 (98)
S20	HE599559	*Bacillus*sp.	AB082678 (85)
S21	HE646745	*Streptomyces prasinosporus*	JX192604 (99)
S22	HE646746	*Bacillus subterraneus*	NR104749 (99)
S23	HE646747	U. bacterium	JF910325 (95)
S24	HE646748	*Terribacillus* sp.	EU435359 (98)
S25	HE646749	U. bacterium	GQ306031 (93)
S26	HE646750	U. bacterium	JN417563 (99)
S27	HE646751	U. *Bacillussp.*	JN082282 (99)
S28	HE646752	U. bacterium	HM437987 (98)
S29	HE646753	*Catenulis poraacidiphila*	CP001700 (97)
S30	HE646754	U. bacterium	HM37969 (99)
S31	HE646755	*Agrobacterium tumefaciens*	JF513176 (97)
S32	HE646756	U. Rubrobacteraceae	FJ552011 (98)
S33	HE646757	U. *Bacillus* sp.	AY082367 (99)
S34	HE646758	U. Actinobacterium	JN037890 (97)
S35	HE646759	U. Chloroflexi	HQ397103 (96)
S36	HE646760	U. Bacillaceae bacterium	JQ793415 (99)
S37	HE646761	U. delta-Proteobacterium	KF247583 (95)
S38	HE646762	U. bacterium	JQ428756 (96)
S39	HE646763	U. bacterium	JN038819 (93)
S40	HE646764	*Nonomuraea* sp.	KC417349 (99)
S41	HE646765	U. Rhizobaiales	HM447746 (94)
S42	HE646766	Acidobacteriaceae	HM438224 (90)
S43	HE646767	U. Bacteroidetes bacterium	KC449976 (98)
S44	HE646768	U. *Gemmatimonas* sp.	HM438475 (96)
S45	HE646769	U. bacterium	JN178474 (98)
S46	HE646770	U. Acidobacteria bacterium	JQ957800 (97)
58	HE798162	U. bacterium	FJ152787 (98)
59	HE798163	U. bacterium	JQ769654 (98)
60	HE798164	U. bacterium	KF037819 (98)
61	HE798165	U. bacterium	JN037990 (84)
62	HE798166	U. bacterium	JN869202 (94)
63	HE798167	U. *Sphingomonas* sp.	JN628042 (99)
64	HE798168	U. bacterium	KC554081 (97)
65	HE798169	U. alpha-Proteobacterium	KF437571 (97)
66	HE798170	U. *Actinobacterium*	HQ183925 (98)
67	HE798171	*Bacillus* sp.	AB062678 (99)
69	HE798173	Alpha-Proteobacterium bacterium	KF43757 (96)
70	HE798174	*Nocardioides* sp.	NR_044185 (99)
71	HE798175	U. bacterium	FJ755754 (97)
72	HE798176	*Janibacter* sp.	JN644568 (90)

neimengense and *Agrobacterium* spp. have been described. Isolation of *Stenotrophomonas* from the rhizospheric soil of *Astragalus bisulcatus* and similarly from the rhizospheric soil of sugarcane *Brevibacillus* has been reported. The genus *Bosea* has been isolated from the agriculture soil (Qaisrani et al., 2014; Hakim et al., 2018). The low sequence homologies (<90%) obtained for *Agrobac-*

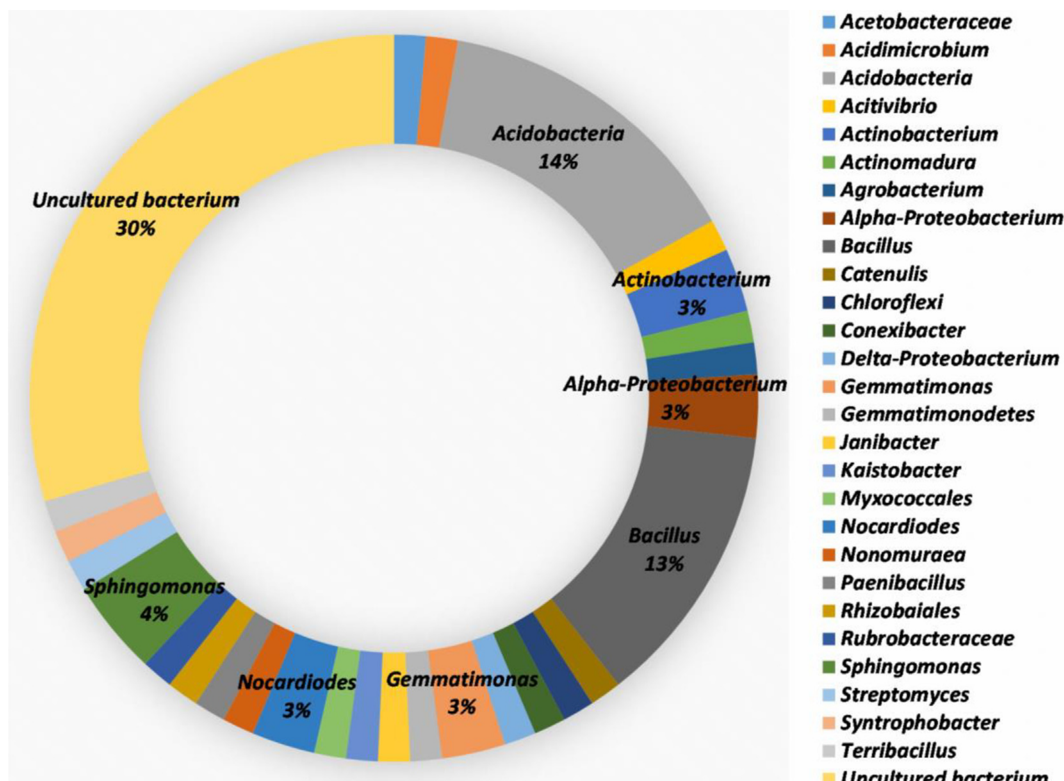

Fig. 2. On the base of *16S rRNA*, detection of non-culturable bacteria from the rhizoshperic soil of maize.

Table 3
Clone sequences of partial *nif*H gene detected in the rhizosphere of maize.

Clone ID	Accession No. of the clones	Description	Maximum similarity (%) in the databank and Accession number
C-1	LN624092	Uncultured *Azoarcus* sp. partial sequence of nifH gene	EF158389 (96)
C-2	LN612752	Uncultured bacterium partial sequence of nifH gene	HQ335683 (94)
C-3	LN624093	Uncultured *Azotobacter chroococcum* partial sequence of nifH gene	M73020 (97)
C-4	LN624094	Uncultured *Agrobacterium tumefaciens* partial sequence of nifH gene	FJ822995 (98)
C-5	LN624095	Uncultured *Aeromonas* sp. partial sequence of nifH gene	FJ687522 (1 0 0)
C- 6	LN624096	Uncultured bacterium partial sequence of nifH gene	EF494089 (98)
C-7	LN624097	Uncultured *Azoarcus* sp. partial sequence of nifH gene	AF200742 (96)
C-8	LN624098	Uncultured *Aeromonas* sp. partial sequence of nifH gene	FJ687522 (98)
C-9	LN624099	Uncultured *Pseudomonas stutzeri* partial sequence of nifH gene	FR669139 (93)
C-10	LN624100	Uncultured bacterium partial sequence of nifH gene	AY196413 (97)
C-11	LN624101	Uncultured *Azoarcus* sp. partial sequence of nifH gene	EF158389 (95)
C-12	LN624102	Uncultured *Zoogloea oryzae* partial sequence of nifH gene	AB201046 (96)
C-13.	LN624103	Uncultured *Azoarcus* sp. partial sequence of nifH gene	Y12545 (94)
C-14	LN624104	Uncultured *Bacillus* sp. partial sequence of nifH gene	EU693342 (94)
C-15	LN624105	Uncultured *Pseudomonas stutzeri* partial sequence of nifH gene	DQ776415 (91)
C-16	LN624106	Uncultured bacterium partial sequence of nifH gene	HM210352 (87)
C-17.	LN624107	Uncultured bacterium partial sequence of nifH gene	GU121497 (96)
C-18	LN612756	Uncultured *Azoarcus* sp. partial sequence of nifH gene	EF158389 (95)
C-19	LN612757	Uncultured bacterium partial sequence of nifH gene	AY196413 (96)
C-20	LN612755	Uncultured bacterium partial sequence of nifH gene	GU193145 (84)

terium, *Bosea* and *Stenotrophomonas* spp. might be due to the partial 16S ribosomal RNA gene sequences gained in the present study for sequence comparison.

The cultivation-independent analysis demonstrated that majority of the sequences (78.6%) obtained from the soil DNA derived *16S rRNA* clone library were related to the uncultured bacteria. About 30% of the total cloned sequences showed no similarity with the known phyla or taxa and were considered as novel sequences. Other genera detected were firmicutes (17%), acidobacteria (16%), actinobacteria (17%), alpha-proteobacteria (7%), delta-proteobacteria (4.2%) and gemmatamonedales (4.2%). Abundance of proteobacteria was earlier reported in canola (Kaiser et al., 2001) and two pasture soils followed by actinomycetes (McCaig et al., 1999). Moreover, abundance of α-proteobacteria was reported in rice clone libraries along with acidobacteria, firmicutes, bacteriodetes groups (Arjun and Harikrishnan, 2011).

Similarities of clone sequences showing relatedness to culturable bacteria were further computed to find the PGPRs among

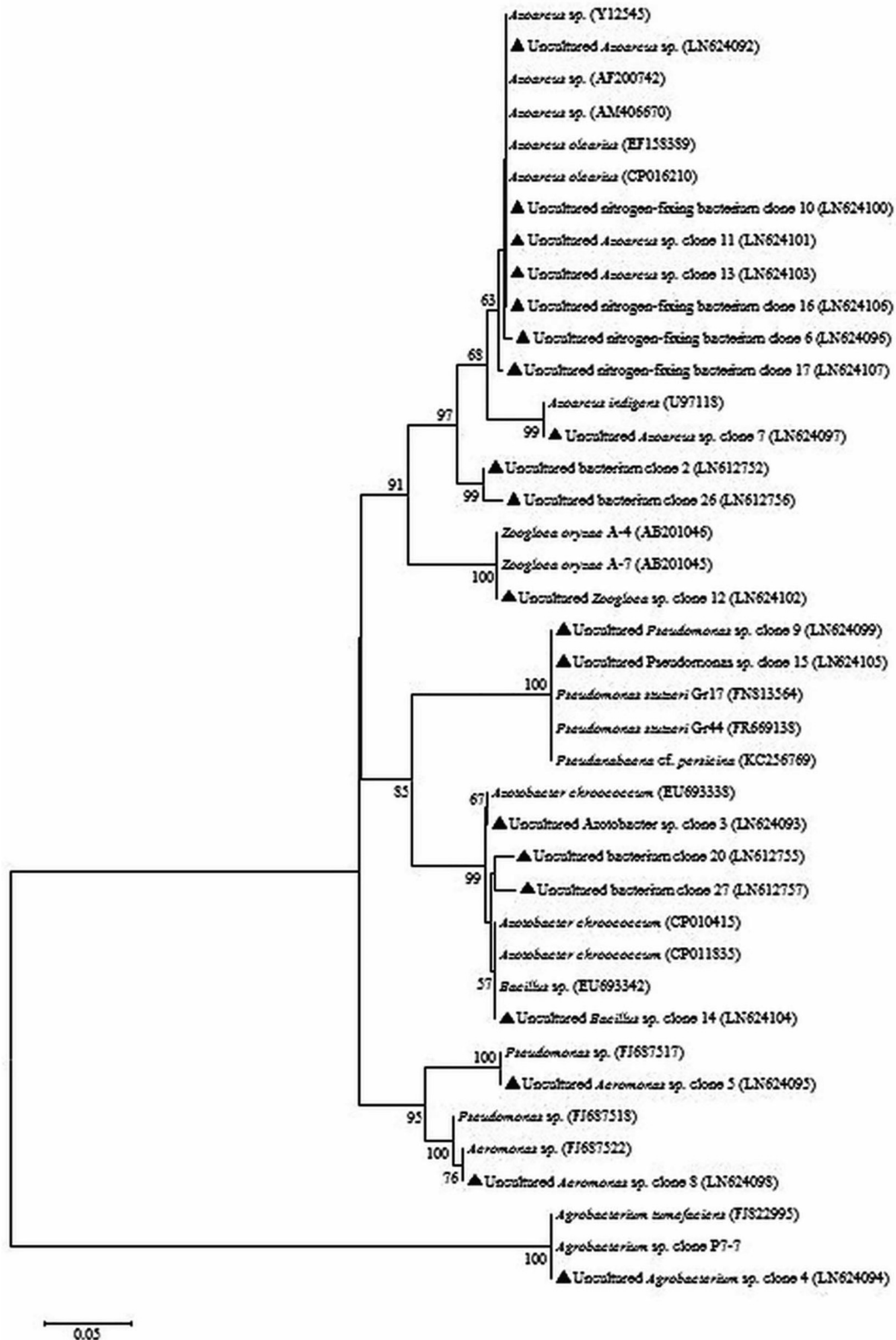

Fig. 3. *nif*H clone library of maize variety FSH-810 rhizosphere soil showing sequences related to culturable and non-culturable bacteria.

them. In the *16S rRNA* clone library, sequences related to *Bacillus* sp., Syntrophaceae, *Kaistobacter* sp. *Sphingomonadales*, *Streptomyces* sp. *Janibacter* sp., *Nocardioides* sp., *Azospirillum* sp., *Sphingomonas* sp., *Rubrobacteraceae* and *Nonomuraea* were detected. Members of these genera have been earlier known as PGPR, exhibiting one or more plant-beneficial traits including enzyme production like ACC-deaminase, bio-control potential against *Fusarium* and improve the phytoremediation ability of *Brassica juncea* grown-up in contaminated soil with glyphosate (Qaisrani et al., 2014; Ermakova et al., 2010). *Bacillus* strains produce antifungal compounds, siderophores and HCN that help plant for optimum growth and exhibit bioremediation potential for Chromium (Cr)

contaminated soils (Kathiravan et al., 2011). The *Burkholderia* strains have been described as to produce ACC-deaminase, siderophores and anti-fungal compounds for maize growth promotion (Byrt et al., 2011). Herbicide resistant characters in *Kaistobacter* and *Nocardioides* strains have also been described. *Sphingomonas* and *Streptomyces* have been reported as biocontrol agents and produce siderophores and enzymes. Bioremediation potential of *Streptomyces* for Cr has been reported (Sheng et al., 2012). The sequences associated to *Terribacillus* sp. *Acidobacteria*; *Gemmata* sp., *Gemmatimonas* sp. *Chloroflexi* and *Actinobacterium* were also detected during this study but no PGPR activity has been reported for the members of these groups so far.

When the culture-dependent data was compared with the culture-independent data, *Azospirillum, Achromobacter, Rhodococcus* and *Bacillus* genera were detected in the clone library but sequences related to *Pseudomonas* were not detected using culture-independent technique. Although, the number of clones sequenced were not in large quantity and were randomly selected but *Pseudomonas* were lacking among the 70 clones. From the rhizosphere of maize, using ITS the presence of acidobacteria, actinobacteria, bacteroidetes, chloroflexi, firmicutes, gemmatimonadetes and proteobacteria have been reported (Chauhan et al., 2011). Moreover, *Enterobacter*, *Erwinia*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas* and *Bacillus* were reported as predominant while *Achromobacter*, *Lysinibacillus* and *Paenibacillus* as rare genera in maize rhizosphere (Paola et al., 2011). Comparing the data of present study with those of published on this subject it is clear that proteobacteria, actinobacteria, bacteroidetes acidobacteria, firmicutes, chloroflexi, planctomycetes, gemmatimonadetes are the most dominant bacteria in the rhizosphere of maize.

Regarding the diversity of functional gene *nif*H, the sequences related to *nif*H of *Azoarcus* sp. (25%). *Pseudomonas stutzeri* (10%), *Aeromonas* (10%), *Azotobacter* (5%), *Agrobacterium* (5%), *Zoogloea oryzae* (5%) and *Bacillus* (5%) were detected. Moreover 35% sequences showed no similarity with the *nif*H of cultured bacteria. These results suggest that maize rhizosphere favors the growth and presence of diverse diazotrophs that can have the potential to enhance the crop productivity. The *nif*H sequences in soil DNA related to those of *Pseudomonas* were detected but *nif*H gene could not be amplified from the pure cultures of *Pseudomonas* retrieved from rhizosphere of maize. The incidence of nitrogen-fixation in *Pseudomonas* genus has been long discussed. *P. stutzeri* strain are rare nitrogen-fixer (Mirza et al., 2006) and in most cases positive identification of these strains based on DNA-techniques were not engaged at the time of their isolation. *nif*H sequences related to *Zoogloea* genus from maize rhizosphere were found. The presence of nitrogen fixing *Zoogloea* has been reported from the soils of Pakistan and was used as PGPR for sugarcane (Mirza et al., 2001).

Among the clones of *nif*H gene obtained in the present study, 25% were related to *Azoarcus*. Isolation of *Azoarcus* strains from kallar grass of Pakistani saline soils was initially reported by Reinhold-Hurek et al. (1993) and was extensive studied there within host plant. Since then, no *Azoarcus* could be isolated from rhizosphere although extensive work was carried out on the isolation of diazotrophs and other PGPR. As a result, *Azoarcus* was considered as of rare occurrence and uncommon among the diazotrophic population in Pakistani soils. However presence of *nif*H sequences related to *Azoarcus* in maize rhizosphere necessitate intensification of isolation attempts to obtain pure cultures of this important bacterium for inoculum production for maize or other crops grown in the country.

Contrary to the *Azoarcus*, four *Azospirillum* strains were identified from the rhizosphere of maize using culture-dependent technique but no *16S rRNA* or *nif*H sequence related to this diazotrophic genus was detected among both the libraries (*nif*H, *16S rRNA*) constructed from soil DNA. This reflects the biasness of the growth medium used in this study rather than the relative abundance of *Azospirillum* in the rhizosphere. Future studies based on next-generation sequencing technology may enable detection of these and other important PGPR in the maize rhizosphere of maize.

5. Conclusion

This study has provided a basis for the future research on "non-culturable" PGPRs and the diazotrophic population present in the rhizosphere of maize especially a rarely cultivated but frequent colonizer *Azoarcus*. More focused approach should be used for targeted cultivation of this diazotroph and exploit its potential to enhance nitrogen acquisition of plant. The information will help to identify potential PGPR for maize inoculation as many of the strains identified might have direct or indirect part in plant stimulation. Moreover, several other bacteria were detected that might have potential for bio-remediation of contaminated-soils or the production of useful enzymes for industrial purposes.

Declaration conflicts of interest

None.

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