Research Paper

Uncultured bacterial diversity in tropical maize (*Zea mays* L.) rhizosphere

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Structure of maize (*Zea mays* L.) rhizosphere bacteria was evaluated to explore the feasibility of identifying novel rhizosphere bacteria using culture-independent method based on direct amplification and analysis of 16S rRNA gene (rRNA) sequences and especially to obtain a better understanding of bacterial community structure and diversity from maize. A total of 274 sequences were analyzed and assigned 48.00% *Proteobacteria*, 10.30% *Actinobacteria,* 9.90% *Bacteroidetes,* 6.60% *Verrucomicrobia,* 4.80% *Acidobacteria*, 1.80% *Firmicutes,* 1.50% *Chloroflexi,* 1.50% *TM7*, 1.10% *Deinococcus-Thermus*, 0.70% *Planctomycetes*, 0.70% *Gemmatimonadetes* and 0.40% *Cyanobacteria*. Economically important phyla *Actinobacteria* was second most dominant group after *Proteobacteria*, in our clone library. It would be interesting to hypothesize that root exudates from maize rhizosphere favors growth of *Actinobacteria* like microbes to eliminate pathogenic bacteria and decompose plant matter, for enhanced plant and soil health. An additional 12.8% of clone library (35 operational taxonomical units (OTUs) from 43 clones) with less than 94% similarity to any GenBank sequence could not be assigned to any known phylum and may represent unidentified bacterial lineages and suggests that a large amount of the rhizobacterial diversity remains to be characterized by culturing.

Abbreviations: OTUs: Operational taxonomical units; DGGE: Denaturing gradient gel electrophoresis; TGGE: Temperature gradient gel electrophoresis; LH-PCR: Length heterogeneity-PCR; SSCP: Single strand conformation polymorphism; tRFLP: Terminal-restriction fragment length polymorphism; SSU rRNA: Small subunit ribosomal rRNA; ACE: Abundance-based coverage estimator; DOTUR: Distance Based Operational Taxonomical Units and richness

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Introduction

The importance of the rhizosphere, defined as the volume of soil adjacent to and influenced by plant roots for plant growth and soil microbiology had already been realized in the very pioneer times of microbiology in the late 19th century as an environment rich in diversified microbiological populations [1]. The rhizosphere of plants is one of the most fascinating microbial habitats for basic and applied studies in the field of environmental microbiology, as it is shaped by the soil, the plant and the microorganisms. The diversity of microorganisms in soil is critical to the maintenance of good soil health, because microorganisms are involved in many important functions such as soil formation, toxin removal, and elemental cycles of carbon, nitrogen, phosphorus, and others [2]. Analysis of bacterial communities and diversity has traditionally begun with cultivating microorganisms from the environment. This technique is limited because of the inability of a wide range of bacteria to be efficiently cultured in the laboratory, due to the unknown conditions for growth requirements of many bacteria and the presence of cells which are in a viable but noncultivable state, the portion of microbial diversity which has been obtained by conventional cultivation techniques is less than 1% of the bacterial species present [2, 3]. This disadvantage

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can be overcome by culture-independent molecular approaches based on direct amplification and sequence analysis of cloned microbial small subunit ribosomal RNA genes (16S ribosomal RNA (rRNA)) to compare the composition, richness, and structure of 99% of the prokaryotes in most natural communities [4]. Estimating microbial phylogenetic diversity is intrinsically interesting to many microbiologists, but it also plays a crucial role in the functional analysis of microbial communities [3].

Molecular fingerprinting techniques including denaturing and temperature gradient gel electrophoresis (DGGE/TGGE), single strand conformation polymorphism (SSCP), length heterogeneity-PCR (LH-PCR), and terminal-restriction fragment length polymorphism (tRFLP) can be used to explore diversity of microbes in ecosystem. However by constructing clone libraries of 16S rRNA gene of microbes, more new microbes can be discovered and identified [4]. Knowledge of the extent of phylogenetic diversity can indicate how many functional groups have not yet been accounted for [5]. Given the tremendous diversity of soil microbes, soil fauna, and plants, it is virtually impossible to investigate the intricacies of every potential rhizosphere interaction in every environmental circumstance. However, environmental engineers are searching for specific rhizosphere organisms capable of degrading/transforming soil pollutants and within agricultural research, efforts are ongoing to detect and biologically control specific plant growth promoting and disease organisms around roots [6]. In addition, soil microbial and ecosystems ecologists have documented remarkable soil biodiversity and the potential for individual plants, plant species, soil types, and management regimes to influence rhizosphere microbial diversity [2].

In this study, we have opted to analyse rhizosphere of maize (*Zea mays* L.) as it is one of the three most important grain crops after wheat and rice. Maize provides nutrients for humans and animals and is serving as a basic raw material for the production of starch, oil and protein, alcoholic beverages, food sweeteners and, fuel [7]. Earlier studies on bacterial diversity as well as fungal communities in tropical maize rhizosphere using TGGE and 18S rDNA respectively have been carried out by Gomes et al. [8, 9]. We have evaluated the structure of maize rhizosphere bacteria to explore the feasibility of identifying novel rhizosphere bacteria using culture-independent method and especially to obtain a better understanding of bacterial community structure and diversity from maize. Cloning and sequencing of 16S rRNA fragments amplified from community DNA were used to provide information on the phylogeny of ribotypes to evaluate the diversity of the bacterial community in the maize rhizosphere.

Materials and methods

Plant cultivation conditions

Micro plot trial for Maize (*Zea mays* cv. Arkil) was carried out at the farm of National Botanical Research Institute, Lucknow, India (11°24′ N/79°44′ E). Soil properties were texture, sandy loam; pH, 7.28 ± 0.11; moisture content %, 1.82 ± 0.14; total organic carbon %, 0.52 ± 0.08; total organic nitrogen %, 0.051 ± 0.004, and C/N ratio, 10.19. Two months old 3 plants each from 5 sites (4 corners and 1 centre) of maize were harvested and rhizosphere soil from 3 plants were collected and pooled as 1 sample for isolating DNA as described earlier by Nautiyal *et al.* [10].

Isolation of DNA from rhizosphere soil

UltraClean™ Soil DNA Isolation Kit (Mo Bio, Solana Beach, CA, USA) was used to isolate the rhizosphere soil DNA from 5 samples, according to the manufacturer's instruction.

PCR amplification of bacterial 16S rRNA gene and sequencing

For 16S rRNA gene clone library of Maize rhizosphere, a partial sequence of the 16S rRNA gene was PCR amplified. This sequence was located between positions 519 to 926, according to the numbering of the respective gene of *Escherichia coli.* The PCR product, which included the variable regions V4 and V5 and hybridize to phylogenetically highly conserved regions of the SSU rRNA gene, was amplified by PCR with primers, universal for Bacteria (Com1, 5′-CAGCAGCCGCGGTAATAC-3′ and Com2, 5′-CCGTCAATTCCTTTGAGTTT-3′) as described by Dohrmann and Tebbe [11]. A reaction mixture of 50 μl contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.5 μM each primer, 2.5 U Platinum *Taq*-Polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA) and 1 μl of template (10 ng of extracted DNA from maize rhizosphere). The PCR conditions were as follows: initial denaturation for 3 min at 94 °C; 30 cycles, each consisting of denaturation for 1 min at 94 °C, primer annealing for 1 min at 50 °C, and 70 s extension at 72 °C; and a final elongation step of 5 min at 72 °C. PCR product was analyzed for its expected size and purity by electrophoresis on 1% agarose gels stained with ethidium bromide. The amplified PCR product was ligated into a PTZ5R/T vector (InsT/AClone TM PCR product cloning kit from Fer-

mentas Life Sciences, Burlington, Canada) as per the manufacturer instructions and transformed in to competent cells of *E. coli* DH5α. Transformed colonies were screened by colony PCR to confirm the presence of inserted fragments. Positive colonies were sequenced at Bangalore GeNei Pvt. Ltd., Bangalore, India.

Phylogenetic affiliation of sequences

All sequences obtained were checked for chimeric artifacts by the Check–Chimera program (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). Presence of same sequence from 2 independent libraries was considered as non chimeric sequence. Sequences were subjected to the SEQUENCE MATCH at RDPII release 9.1 (http://rdp.cme.msu.edu/) and BLAST in GenBank to compare the clone sequences to known 16S rRNA gene sequences for assignment to the nearest taxon. Sequences were aligned by using ClustalX, alignment was checked and manually edited by using BioEdit version 7.0.5.3. DNA distance matrices were calculated according to Jukes-cantor model by using DNAdist programme from Phylip package (http://evolution.genetics-washington.edu/phylip.html). To confirm the phylogenetic assignment phylogenetic tree was constructed using the neighbor-joining algorithm. The statistical significance of branches was estimated by 100 replicates bootstrap analysis, CONSENCE and TREEVIEW programs were used to generate consensus tree and to draw trees respectively.

Phylotypes determination

Distance matrices calculated by DNADIST with Jukes and Cantor correction in PHYLIP package on internet (http://evolution.genetics-washington.edu/phylip.html) was used as INPUT file in DOTUR (Distance Based Operational Taxonomical Units and richness) This program have been used to calculate phylotype at different distance intervals [5]. By using furthest-neighbor assignment algorithm, sequences were grouped in to phylotype at 3, 5, 10 and 20% distances, representing species, genera, family/class and phylum [12].

Nucleotide sequence accession numbers

Sequence data reported in present study has been deposited in the GenBank, nucleotide sequence database under the accession numbers EU441984-EU442179.

Statistical analyses

Rarefaction curves of observed phylotype richness were generated by plotting number of sequences sampled at *x*-axis and number of OTUs observed at *y*-axis, Estimates of phylotype richness according to the abundance-based coverage estimator (ACE) [13], bias corrected Chao1 estimator [14] were calculated in DOTUR, Shannon weaver and reciprocal Simpson diversity index were also calculated by DOTUR [12]. The estimation of diversity coverage of clone library was calculated by the formula (1-(n/N)), where n is the number of singletons and N is the total number of clones [15].

Results

Characterization of 16S rRNA clones

Among 302 transformants, 28 were not suitable for further sequence analysis, because of lack of transformed sequence, low signal in sequencing or chimera. Finally 274 sequences were successfully obtained and were further analyzed. Out of 274 clones 196 OTUs rRNA gene sequences used in phylogenetic analyses have been deposited in the GenBank database with accession numbers EU441984-EU442179. Sequences were subjected to BLAST search against GenBank to determine phylogenetic affiliation of the respective clones. Sequences were subjected to Ribosomal data base Project II (http://rdp.cme.msu.edu/classifier/classifier.jsp) Seqmatch and classifier. Classifier tool of the Ribosomal Data Base Project II [16] assigned 48.0% *Proteobacteria*, 10.3% *Actinobacteria,* 9.9% *Bacteroidetes,* 6.6% *Verrucomicrobia,* 4.8% *Acidobacteria*, 1.8% *Firmicutes,* 1.5% *Chloroflexi,* 1.5% *TM7*, 1.1% *Deinococcus-Thermus*, 0.7% *Planctomycetes*, 0.7% *Gemmatimonadetes*, 0.4% *Cyanobacteria*, and 12.8% were categorized as "unclassified bacteria" by RDP (Fig. 1).

Phylum analysis of the maize rhizosphere clone libraries

Sequences related to *Proteobacteria* phylum made up the largest fraction of clone library and were distributed in *Alpha-, Beta-*, *Gamma-* and *Delta- proteobacteria* sub groups, *Alphaproteobacteria* comprising 31 OTUs were the most dominant subclass of *Proteobacteria* (Fig. 2) with 45 clones, genera *Devosia* were represented by 3 OTUs (9 clones). *Gammaproteobacteria* (Fig. 3) comprised of 30 OTUs (45 clones), genera *Pseudomonas* was represented by 2 OTUs (6 clones), other dominant genres *Pseudoxanthomonas*, *Cell Vibrio*, *Dokdonella* and *Citrobacter. Betaproteobacteria* (Fig. 4) was the third largest abundant subclass with 20 OTUs (30 clones), phylogenetic diversity of *Deltaproteobacteria* (Fig. 4) was much less as only 6 OTUs (6 clones) belonged to this subgroup. Twenty three OTUs (26 Clones) belonged to phylum *Bacteroidetes* (Fig. 5), *Flavobacterium*, *Dyadobacter*, *Chitinophaga*, *Chryseobacterium* were the known cultivable genera, rest were

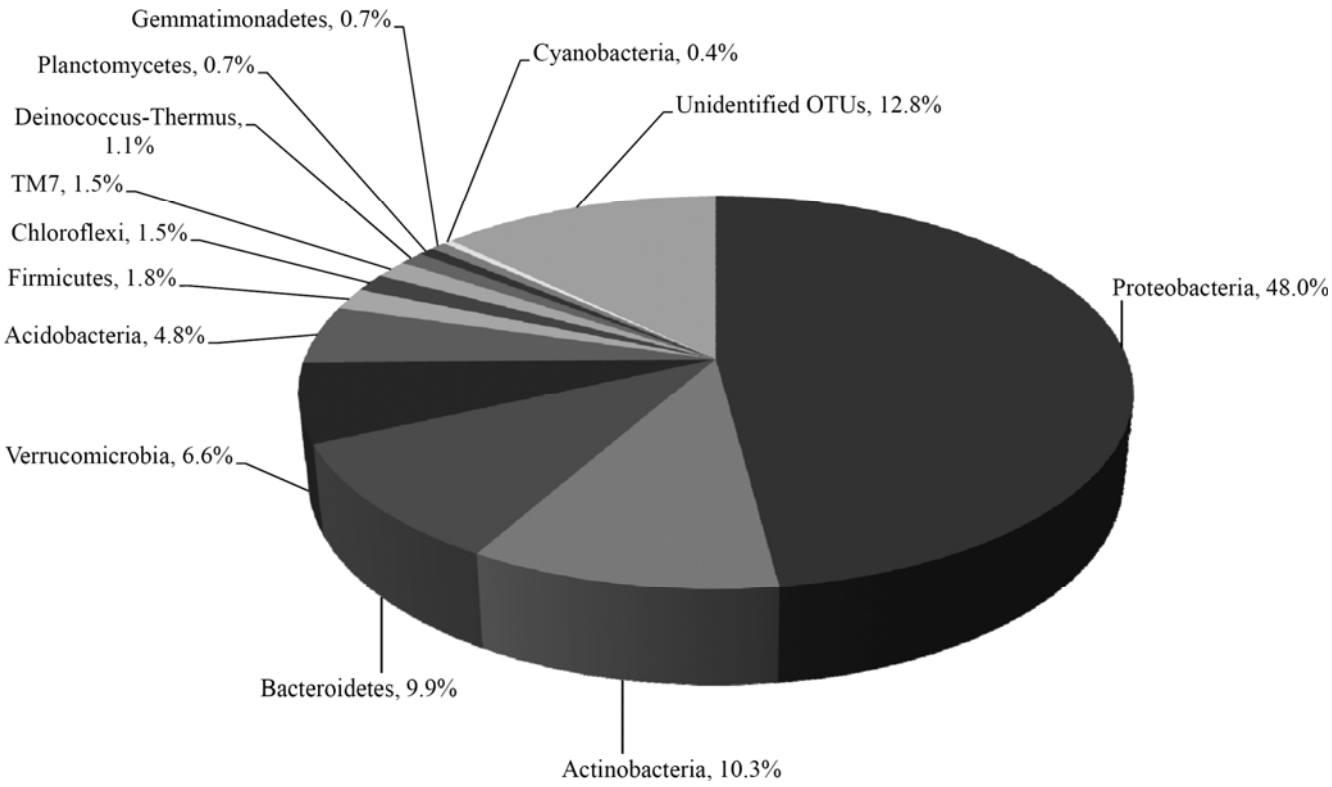

Figure 1. Phylum level distribution of cloned 16S rRNA sequences from maize rhizosphere.

related to uncultured bacteria. Economical important phyla *Actinobacteria* (Fig. 6) was the next dominant group in our clone library, 14 OTUs (22 clones) were related to this phyla, most dominant OTUs with 5 clone sequences was close relative of genera *Streptomyces*. Thirteen OTUs (18 clones) belong to phylum *Verrucomicrobia* (Fig. 7), all OTUs were related to uncultured bacteria. Ten OTUs (15 clones) belong to phylum *Acidobacteria* (Fig. 7), all OTUs were related to uncultured bacteria. Remaining OTUs belonged to, *Deinococcus-Thermus* (Fig. 2), *Cyanobacteria, TM7_genera_incertae_sedis* (Fig. 5) *Firmicutes* (Fig. 6), *Planctomycetes* (Fig. 7), *Gemmatimonadetes* and *Chloroflexi* (Fig. 8). Only one sequence related to *Crenarchaeon* was from lineage *Archeaea*.

Diversity measures

To calculate the diversity in clone library, sequences were grouped in OTUs at different distances (operation taxonomical units), DOTUR calculated 196 OTUs at species level cut off value (3% differences), 167 at genera level (10% differences), 111 at family/class level (15% differences) and 48 OTUs at phylum level (20% differences) cut off value. Rarefaction curve (Fig. 9) was generated by plotting the number of phylotypes observed against the number of clones sequenced, as cut off value increase towards phylum level, number of OTUs observed keep on decreasing and reaches a complete plateau at 20% distance, it indicates that most of the diversity at family or phylum level has been detected. The rarefaction curves of the richness of OTUs with the function of sequences sampled indicate that at difference levels 3, 5, 10 and 20%) the estimated OTUs revealed 626, 431, 229 and 65 respectively. For the 20% level the estimated number of OTUs ($n = 65$) is very close to the observed number of OTUs ($n = 48$). The rarefaction curves of the ChaoI estimator of the richness of OTUs ChaoI estimator on the *y*-axis against the number of sequences samples on the *x*-axis) for different cut-off levels (3, 5, 10 and 20%) are indicated (Fig. 10A). The rarefaction curves of the richness of OTUs with the function of sequences sampled indicate that at difference levels 3, 5, 10 and 20%) the estimated OTUs revealed 723, 538, 215 and 59. respectively. The rarefaction curves of the ChaoI estimator of the richness of OTUs ACE estimator on the *y*-axis against the number of sequences samples on the *x*-axis) for different cut-off levels (3, 5, 10 and 20%) are indicated (Fig 10B).

Using the formula of good coverage of library were 28.46, 39.05, 59.48 and 82.48 at species, genera, family and phylum level respectively (Table 1). Evenness calculated from Shannon weaver index showed no dominance of any particular group of bacteria. We construct-

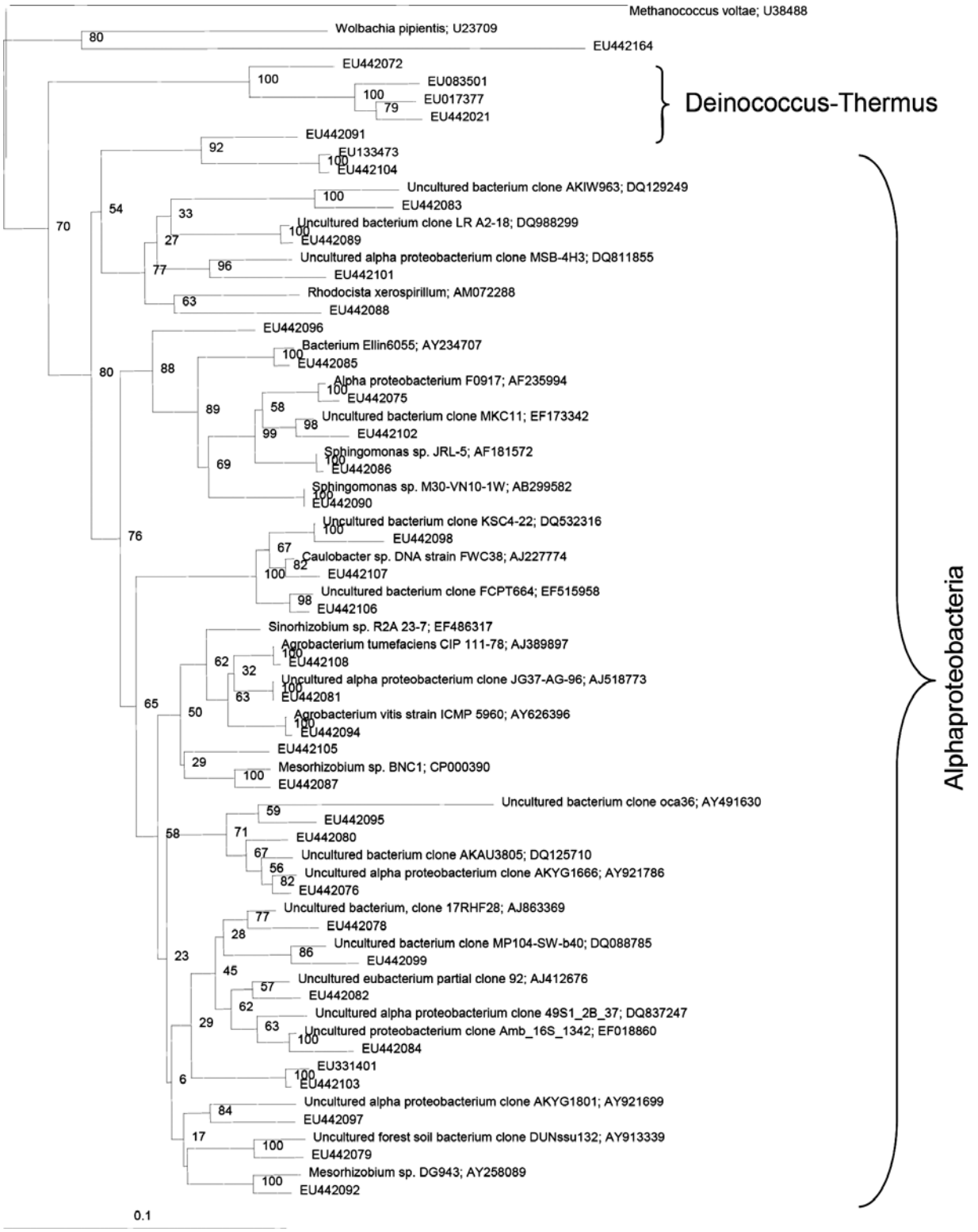

Figure 2. Weighbor weighted neighbor-joining phylogenetic tree showing clones from *Deinococcus-Thermus* and *Alphaproteobacteria* obtained from maize rhizosphere. Tree was constructed by the use of neighbor-joining analysis based on 16S rRNA sequences. When two or more identical sequences were detected only one was used to generate tree. Bootstrap values (based on 100 replication) are represented on each node when >50% and the branch length index is represented below the dendrogram. The scale bar represents 0.1 substitution per nucleotide position.

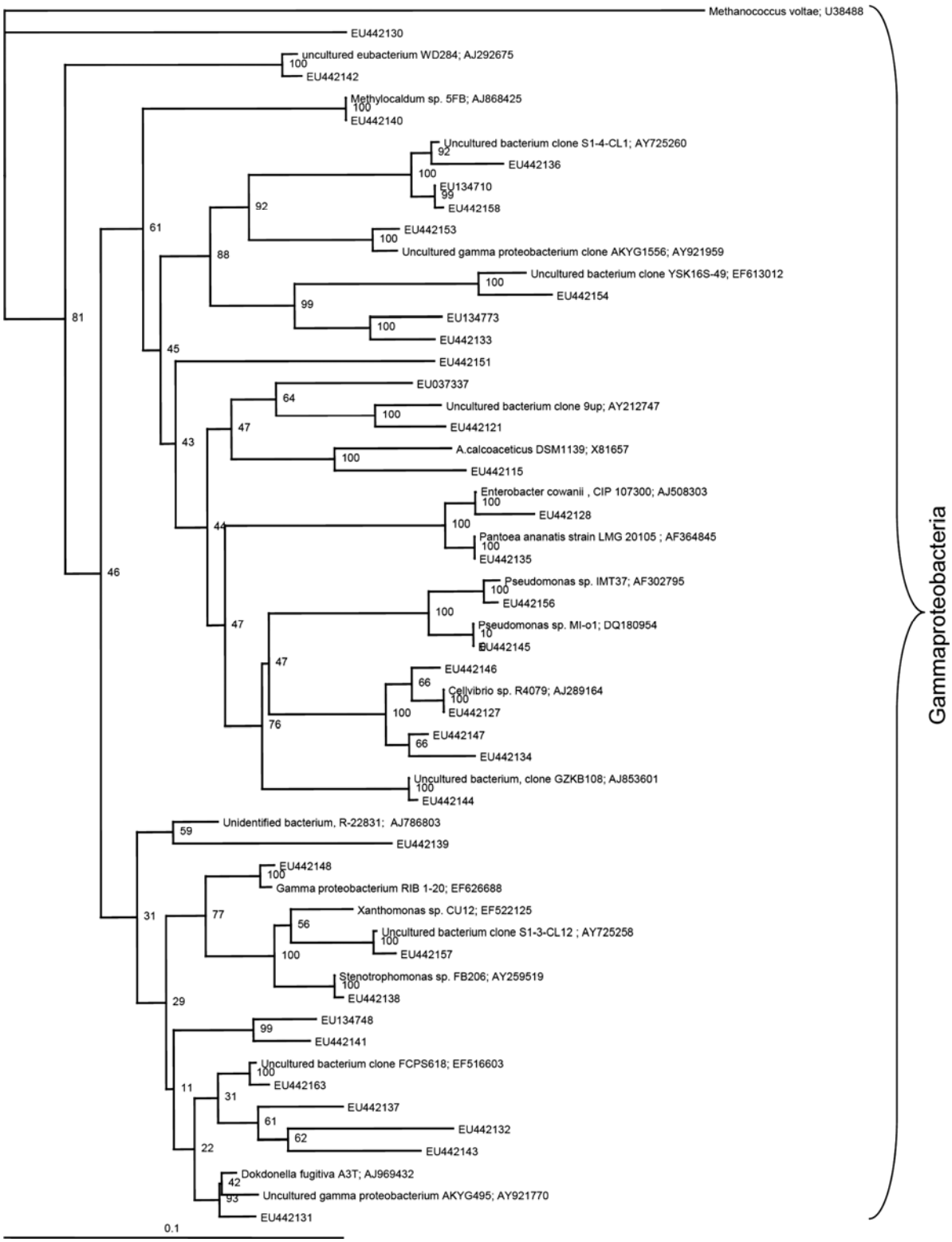

Figure 3. Weighbor weighted neighbor-joining phylogenetic tree showing clones from *Gammaproteobacteria* obtained from maize rhizosphere. Tree was constructed by the use of neighbor-joining analysis based on 16S rRNA sequences. When two or more identical sequences were detected only one was used to generate tree. Bootstrap values (based on 100 replication) are represented on each node when >50% and the branch length index is represented below the dendrogram. The scale bar represents 0.1 substitution per nucleotide position.

Figure 4. Weighbor weighted neighbor-joining phylogenetic tree showing clones from *Beta*- and *Deltaproteobacteria* obtained from maize rhizosphere. Tree was constructed by the use of neighbor-joining analysis based on 16S rRNA sequences. When two or more identical sequences were detected only one was used to generate tree. Bootstrap values (based on 100 replication) are represented on each node when >50% and the branch length index is represented below the dendrogram. The scale bar represents 0.1 substitution per nucleotide position.

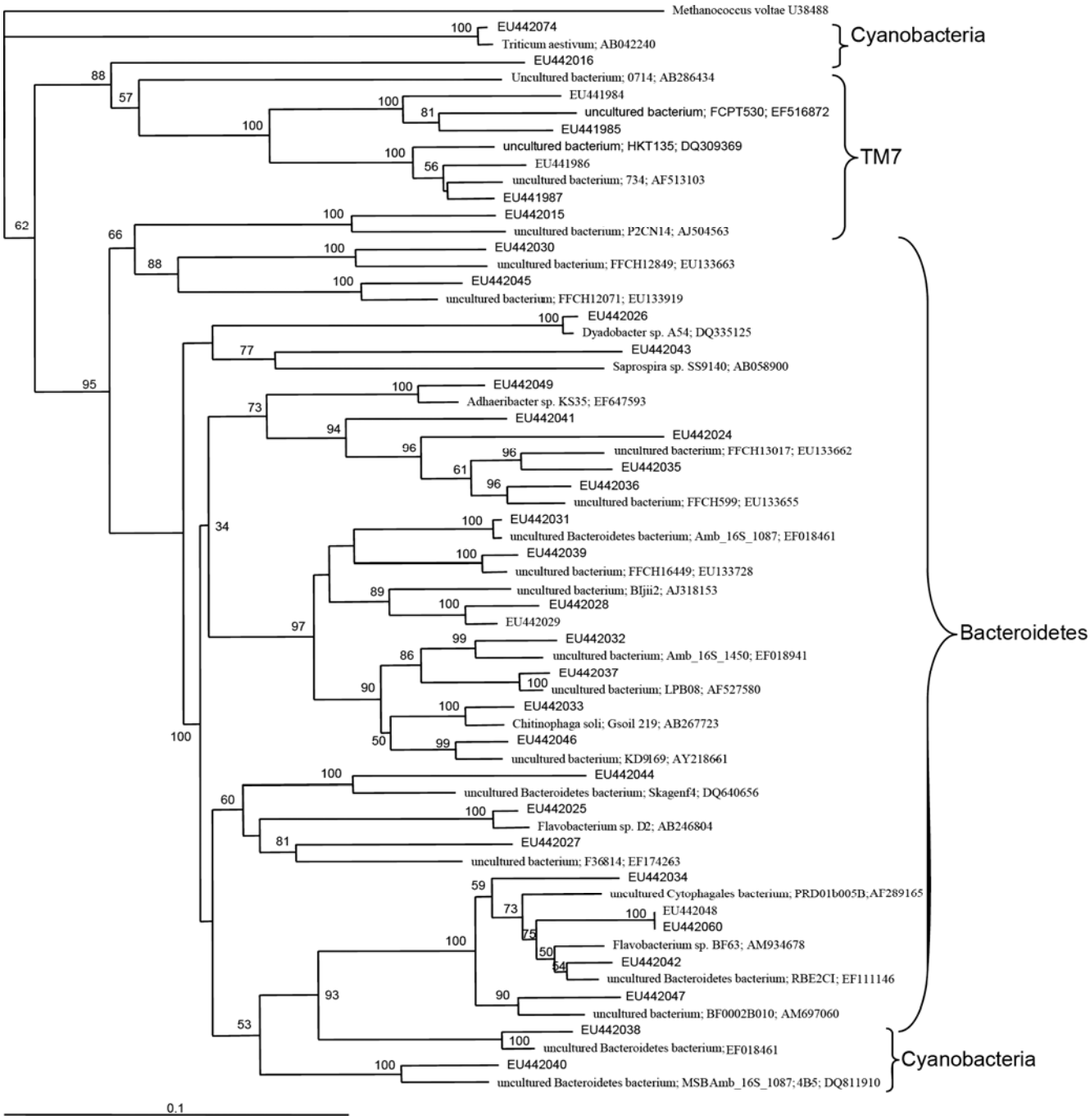

Figure 5. Weighbor weighted neighbor-joining phylogenetic tree showing clones from *Cyanobacteria*, *TM7* and *Bacteroidetes* obtained from maize rhizosphere. Tree was constructed by the use of neighbour-joining analysis based on 16S rRNA sequences. When two or more identical sequences were detected only one was used to generate tree. Bootstrap values (based on 100 replication) are represented on each node when >50% and the branch length index is represented below the dendrogram. The scale bar represents 0.1 substitution per nucleotide position.

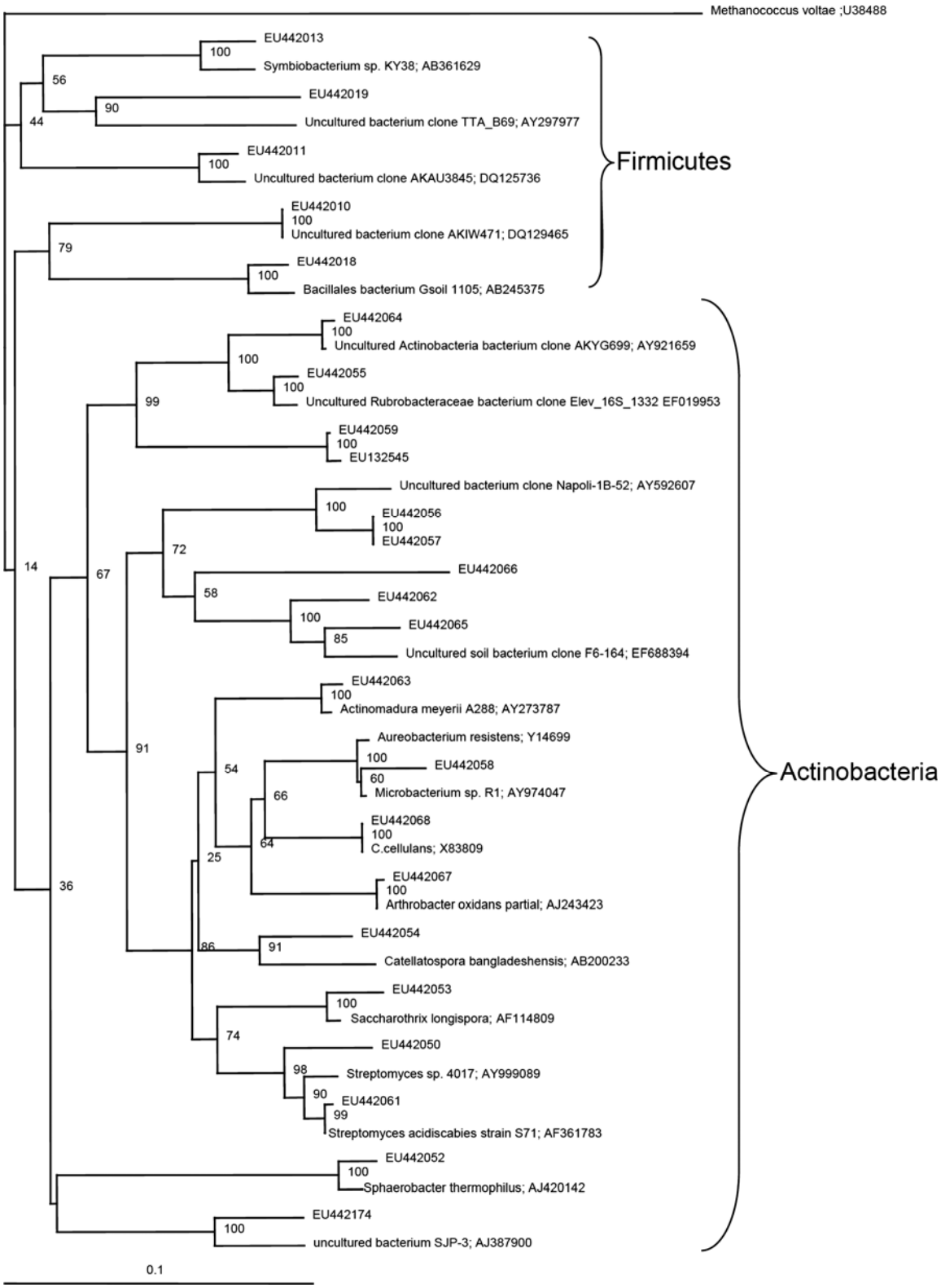

Figure 6. Weighbor weighted neighbor-joining phylogenetic tree showing clones from *Firmicutes* and *Actinobacteria* obtained from maize rhizosphere. Tree was constructed by the use of neighbor-joining analysis based on 16S rRNA sequences. When two or more identical sequences were detected only one was used to generate tree. Bootstrap values (based on 100 replication) are represented on each node when >50% and the branch length index is represented below the dendrogram.

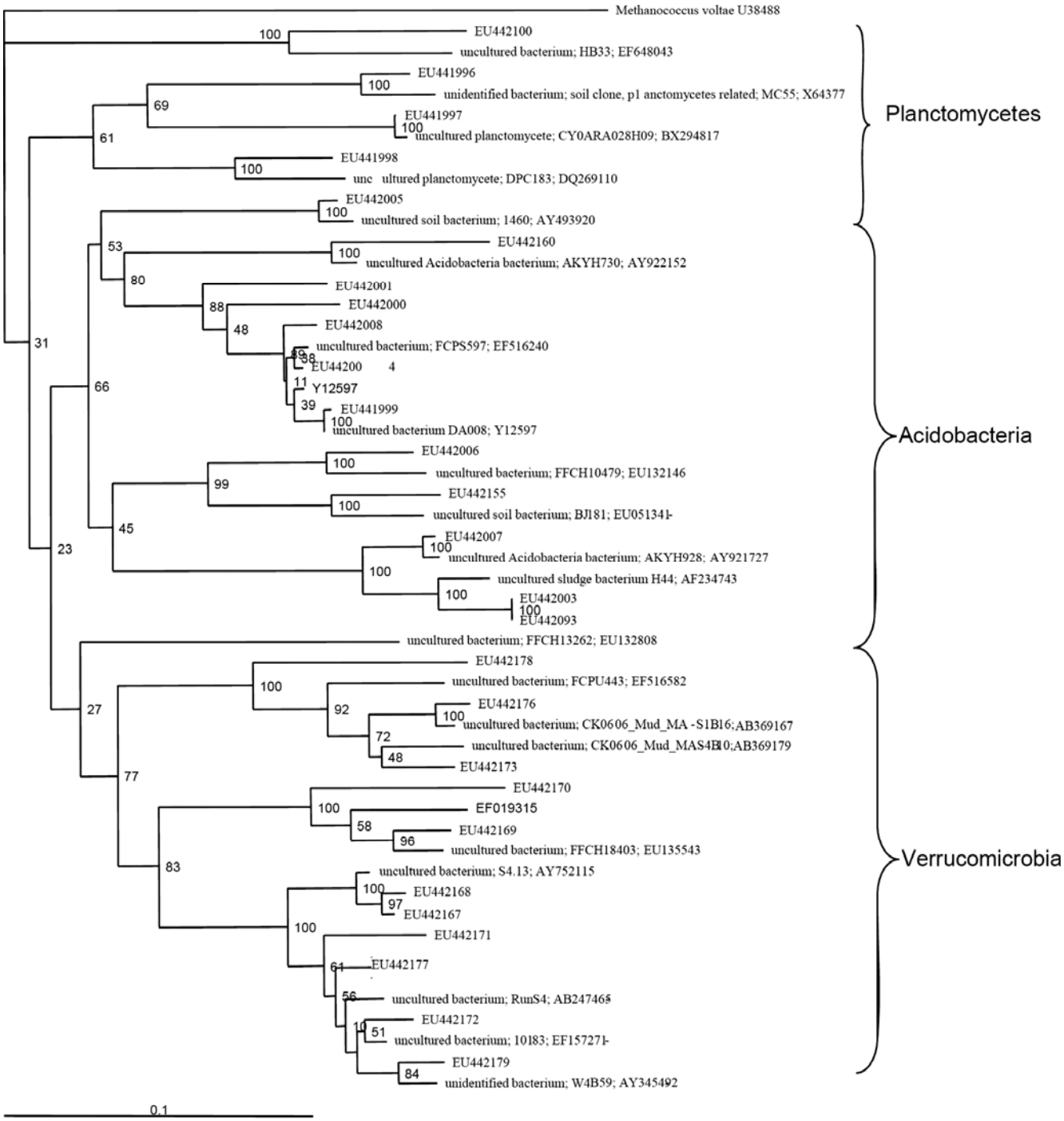

Figure 7. Weighbor weighted neighbor-joining phylogenetic tree showing clones from *Planctomycetes*, *Acidobacteria* and *Verrucomicrobia* phylotypes obtained from maize rhizosphere. Tree was constructed by the use of neighbour-joining analysis based on 16S rRNA sequences. When two or more identical sequences were detected only one was used to generate tree. Bootstrap values (based on 100 replication) are represented on each node when >50% and the branch length index is represented below the dendrogram. The scale bar represents 0.1 substitution per nucleotide position.

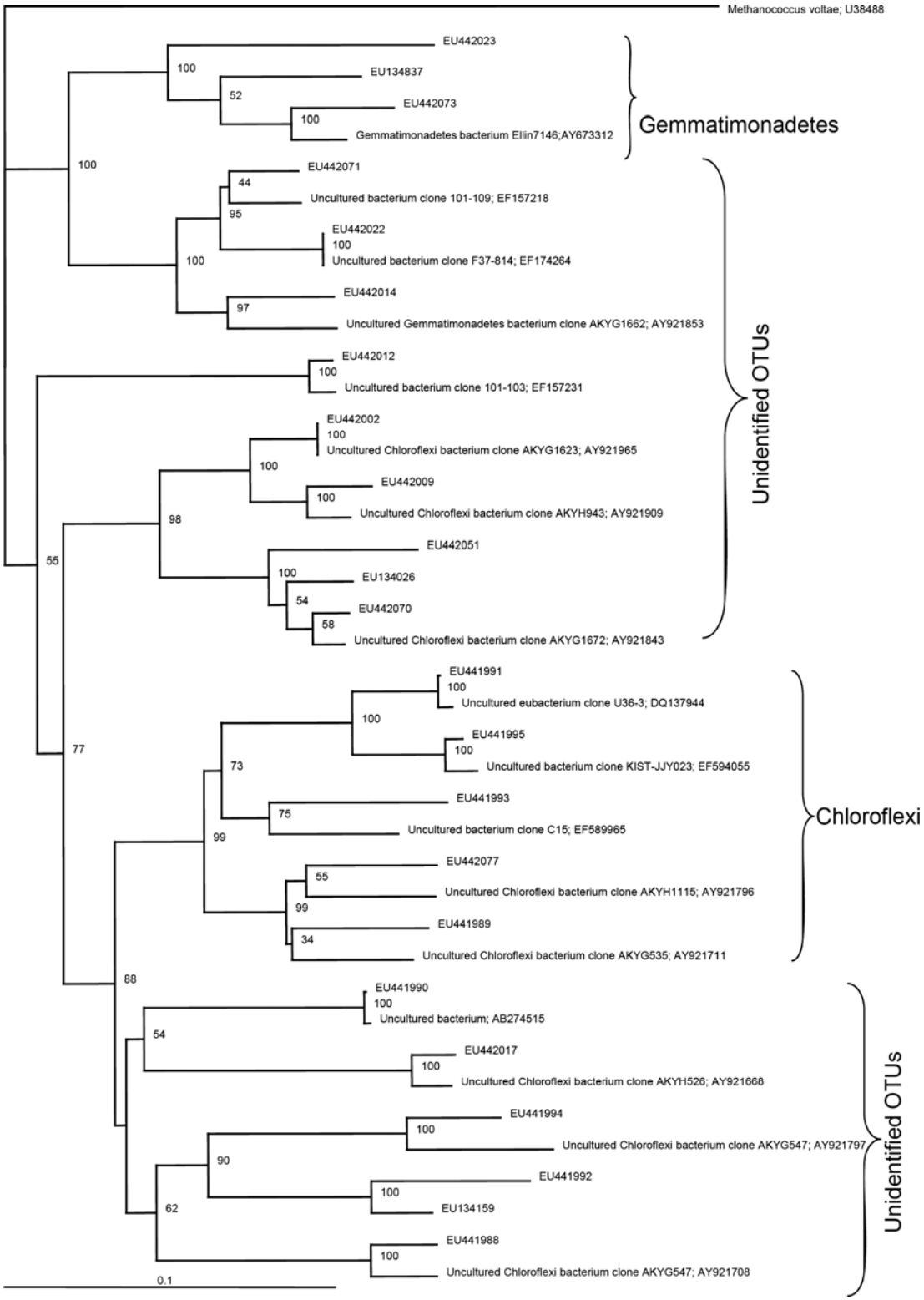

Figure 8. Weighbor weighted neighbor-joining phylogenetic tree showing clones from *Chloroflexi* and putative unidentified bacterial lineages with less than 94% similarity to any GenBank sequence database obtained from maize rhizosphere. Tree was constructed by the use of neighbor-joining analysis based on 16S rRNA sequences. When two or more identical sequences were detected only one was used to generate tree. Bootstrap values (based on 100 replication) are represented on each node when >50% and the branch length index is represented below the dendrogram. The scale bar represents 0.1 substitution per nucleotide position.

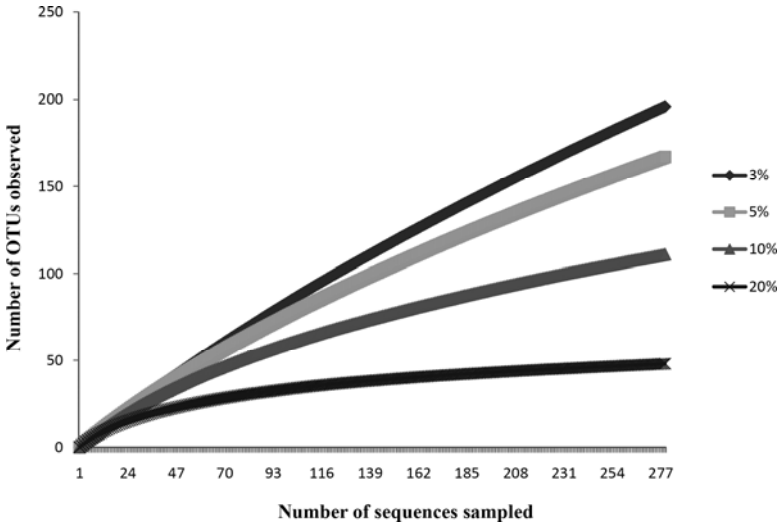

Figure 9. Rarefaction curve of rhizospheric bacterial 16S rRNA clone library of maize root at species (3%), genera (5%), family/class (10%) and phylum level (20% differences).

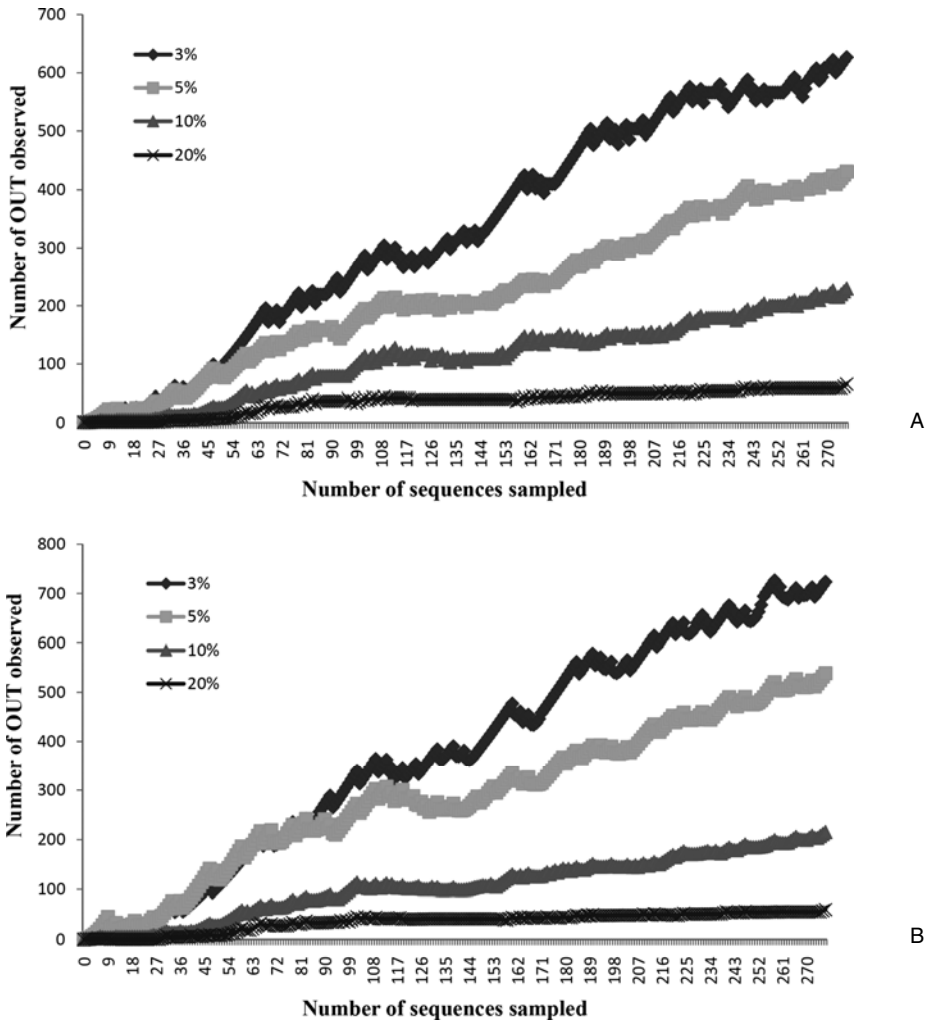

Figure 10. Rarefaction curves of the Chaol estimator (A) and ACE (abundance-based coverage estimator) (B) from 60 days old maize rhizosphere 16S rRNA clone library.

Table 1. Statistical indicators from 16S rRNA clone library generated from maize rhizosphere.

	3%	5%	10%	20%
Number of OTUs	196	167	111	48
Singleton	153	120	62	15
Chao1 estimate of OTUs richness	626.667	431.444	229.188	65.5
ACE estimate of OTUs richness	723.56	538.696	215.354	59.823
Shannon index of diversity (H)	5.10235	4.85178	4.32821	3.30151
Simpson's index of diversity (1/D)	236.23	136.05	66.22	19.23
Evenness	0.96	0.94	0.91	0.85
Goods coverage	28.46	39.05	59.48	82.48

ed collectors curve of observed and non parametric richness estimators such as Chao1, ACE at different distances. Gap between the observed phylotypes and the number of phylotypes estimated by Chao1 and ACE is represented by unseen phylotypes, and it is interesting to note that this gap decreased towards phylotypes definition (Table 1).

Unidentified bacterial lineages

Thirty five OTUs were selected which were showing less than 95% similarity against published database. Out of 35, 13 OTUs from phylum *Proteobacteria* showing less than 94% similarity against published database, contributed by *Alpha-, Beta-, Gammaproteobacteria* sub groups 7, 2 and 4 OTUs respectively, whereas 5, 4, 3, 2, 2, 2, 1, 1, 1, from *Chloroflexi, Firmicutes, Actinobacteria, TM7, Acidobacteria, Verrucomicrobia, Gemmatimonadetes, Bacteroidetes,* and *Planctomycetes* respectively (Table 2).

Discussion

Earlier studies have been made regarding microbial diversity in maize rhizosphere using various culture dependent as well as independent methods like SSCP, DGGE, Biolog, FAME (Fatty acid methyl esters), ARISA (Automated Ribosomal Intergenic Spacer Analysis), 16S rDNA restriction fragment length polymorphism (RFLP), cultivation based method etc. [17, 18, 19]. In the present study, 16S rRNA gene sequence analysis was used to estimate the bacterial diversity in the maize rhizosphere. One advantage of sequence analysis is that the generation of sequence data can be used to design group-specific probes and primers for further studies. Therefore so far, cloning of PCR-amplified 16S rRNA is a useful approach to explore microbial diversity and to determine the species composition of mixed microbial communities [2, 5]. We acknowledge that one has to be careful in interpreting phylogenies based on partial 16S rRNA gene. However, it has previously been shown that the V4-V5 region is suitable for consistent phylogenetic assignment compared to full-length sequence information [20, 21]. The data derived maize rhizosphere in this study is particularly important since little 16S rRNA sequence information has been previously reported.

In earlier studies using culture-based methodologies, members of *Actinobacteria* were shown to be the second most abundant bacterial group present on the roots and leaves of maize, after the *Alphaproteobacteria* [22]. Molecular fingerprinting analysis of maize rhizobacteria confirmed that *Actinobacteria* formed dominant populations on maize roots in a tropical soil. It was also found that members of the *Bacteroidetes* group constituted dominant populations in the rhizosphere of maize and of canola [23]. Members of this bacterial group are capable of degrading complex macromolecules, thus contributing to the turnover of carbon, nitrogen and phosphorus [24]. The analysis of a clone library of the maize rhizobacterial community indicated that *Alpha-* and *Betaproteobacteria* were dominant populations [25]. To the best of our knowledge this is the first detailed study to explore the feasibility of identifying maize rhizosphere bacteria using culture-independent method and especially to obtain a better understanding of bacterial community structure and diversity using the method of sequencing of 16S rRNA genes. Sequences related to *Proteobacteria* phylum made up the largest fraction of clone library consisted of phylum *Proteobacteria*, followed by *Actinobacteria, Bacteroidetes, Verrucomicrobia, Acidobacteria*, *Firmicutes, Chloroflexi, TM7*, *Deinococcus-Thermus*, *Planctomycetes*, *Gemmatimonadetes*, *Cyanobacteria*, and 12.8% were categorized as "unclassified bacteria". These 16S rRNA gene sequences may represent members of novel lineages that have not been previously reported. However extra data are necessary, to prove occurrence of new phylotypes, as only a fragment of the 16S rRNA gene was analyzed.

The observation that sequences related to *Proteobacteria* phylum made up the largest fraction of clone library in this study is consistent with previous findings that the phylum *Proteobacteria*, which is metabolically versatile and genetically diverse, comprises the largest frac-

Table 2. Similarity values of 16S rRNA gene sequences retrieved from the maize rhizosphere of unidentified bacterial lineages represented by 35 OTUs which were showing less than 95% similarity to nearest relative (GenBank accession no.).

S. No.	Clone No. (GenBank accession no.)	Nearest relative (GenBank accession no.)	Description	Similarity (%)	Phylum	Source
1.	CSN-NBRI001 (EU441984)	EF075382.1	Uncultured bacterium clone GASP-WC2W3_F04 16S ribosomal RNA gene, partial sequence	93%	*TM7* (100%)	Pasture
2.	CSN-NBRI002 (EU441985)	AF269020.1	Uncultured bacterium NoosaAW69 16S ribosomal RNA gene, partial sequence	92%	*TM7* (100%)	Sewage treatment plant
3.	CSN-NBRI006 (EU441989)	AY921711.1	Uncultured Chloroflexi bacterium clone AKYG535 16S ribosomal RNA gene, partial sequence	92%	*Chloroflexi* (43%)	Farm soil
4.	CSN-NBRI009 (EU441992)	AJ607261.1	Uncultured bacterium partial 16S rRNA gene, clone MINGYW57	94%	*Chloroflexi* (64%)	Mine soil
5.	CSN-NBRI010 (EU441993)	AM086087.1	Uncultured bacterium partial 16S rRNA gene, clone c1LKS48	90%	*Chloroflexi* (95%)	Lake profundal sediment
6.	CSN-NBRI011 (EU441994)	AY921797.1	Uncultured Chloroflexi bacterium clone AKYG547 16S ribosomal RNA gene, partial sequence	93%	*Chloroflexi* (59%)	Farm soil
7.	CSN-NBRI015 (EU441998)	DQ269110.1	Uncultured planctomycete clone DPC183 16S ribosomal RNA gene, partial sequence	93%	*Planctomycetes* (76%)	Surface of marine macro-alga
8.	CSN-NBRI017 (EU442000)	EF074849.1	Uncultured Acidobacteria bacterium clone GASP-WC1W3_F01 16S ribosomal RNA gene, partial sequence	91%	*Acidobacteria* (57%)	Pasture
9.	CSN-NBRI020 (EU442003)	AF234743.1	Uncultured sludge bacterium H44 16S ribosomal RNA gene, partial sequence	93%	*Acidobacteria* (100%)	Industrial sewage treatment plant
10.	CSN-NBRI031 (EU442014)	AY921853.1	Uncultured Gemmatimonadetes bacterium clone AKYG1662 16S ribosomal RNA gene, partial sequence	93%	*Firmicutes* (25%)	Farm soil
11.	CSN-NBRI032 (EU442015)	DQ395409.1	Uncultured organism clone ctg_CGOAB90 16S ribosomal RNA gene, partial sequence	93%	*Firmicutes* (55%)	Deep-sea octacoral
12.	CSN-NBRI033 (EU442016)	AJ607268.1	Uncultured bacterium partial 16S rRNA gene, clone MINGYW64	90%	*Firmicutes* (46%)	Mine soil
13.	CSN-NBRI044 (EU442027)	AB244310.1	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone:LCFA-B03	90%	*Bacteroidetes* (100%)	Mesophilic Methanogenic Consortium
14.	CSN-NBRI062 (EU442045)	AB196088.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:TB003-112	92%	*Bacteroidetes* (47%)	Sludge
15.	CSN-NBRI068 (EU442051)	DQ236250.1	Uncultured bacterium clone D125-Chloroflx_sf_1 16S ribosomal RNA gene, partial sequence	88%	*Actinobacteria* (65%)	urban aerosol amplicon
16.	CSN-NBRI071 (EU442054)	AY934797.1	Uncultured bacterium clone GB2a 16S ribosomal RNA gene, partial sequence	93%	*Actinobacteria* (100%)	Earthworm cast
17.	CSN-NBRI075 (EU442058)	AB176173.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:SSmCB08-6	91%	*Actinobacteria* (100%)	Vent area water
18.	CSN-NBRI090 (EU442073)	AY673312.1	Gemmatimonadetes bacterium Ellin7146 16S ribosomal RNA gene, partial sequence	93%	*Gemmatimonadetes* (98%)	Soil

Table 2. (continued)

S. No.	Clone No. (GenBank accession no.)	Nearest relative (GenBank accession no.)	Description	Similarity (%)	Phylum	Source
19.	CSN-NBRI094 (EU442077)	AY607181.1	Uncultured bacterium clone X9Ba49 small subunit ribosomal RNA gene, partial sequence	81%	*Chloroflexi* (56%)	Rice field soil
20.	CSN-NBRI101 (EU442084)	EF074440.1	Uncultured Methylocystaceae bacterium clone GASP-WC1S2_G12 16S ribosomal RNA gene, partial sequence	89%	*Alphaproteobacteria* (100%)	Pasture
21.	CSN-NBRI108 (EU442091)	AY726918.1	Uncultured alpha proteobacterium clone JL-ETNP-Z1 16S ribosomal RNA gene, partial sequence	89%	*Alphaproteobacteria* (97%)	North Pacific Ocean
22.	CSN-NBRI110 (EU442093)	DQ300745.1	Uncultured bacterium clone HF770_A2_P1 16S ribosomal RNA gene, partial sequence	89%	*Alphaproteobacteria* (98%)	Ocean's interior
23.	CSN-NBRI113 (EU442096)	AB176206.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:SSmCB08-42	92%	*Alphaproteobacteria* (100%)	Vent area water
24.	CSN-NBRI115 (EU442098)	DQ532316.1	Uncultured bacterium clone KSC4-22 16S ribosomal RNA gene, partial sequence	92%	*Alphaproteobacteria* (98%)	Kennedy Space Center
25.	CSN-NBRI116 (EU442099)	DQ088785.1	Uncultured bacterium clone MP104-SW-b40 16S ribosomal RNA gene, partial sequence	89%	*Alphaproteobacteria* (97%)	Crustal biotome
26.	CSN-NBRI117 (EU442100)	AJ421890.1	uncultured bacterium partial 16S rRNA gene, clone Alt9-K9	87%	*Alphaproteobacteria* (59%)	Altamira Cave
27.	CSN-NBRI132 (EU442115)	AF143837.1	Uncultured bacterium clone AF1.2 16S ribosomal RNA gene, partial sequence	93%	*Betaproteobacteria* (100%)	Aerobic freshwater and marine enrichment cultures
28.	CSN-NBRI146 (EU442129)	AY898045.1	Uncultured organism clone M8907A10 small subunit ribosomal RNA gene, partial sequence	86%	*Betaproteobacteria* (70%)	Water and air of a hospital therapy pool
29.	CSN-NBRI147 (EU442130)	DQ973207.1	Uncultured bacterium clone YC42 16S ribosomal RNA gene, partial sequence	91%	*Gammaproteobacteria* (73%)	Grassland
30.	CSN-NBRI154 (EU442137)	EF072606.1	Uncultured Xanthomonadaceae bacterium clone GASP-WA2S2_G08 16S ribosomal RNA gene, partial sequence	93%	*Gammaproteobacteria* (100%)	Soil
31.	CSN-NBRI156 (EU442139)	AM403231.1	Aquimonas sp. D11-34A partial 16S rRNA gene, isolate D11-34A	92%	*Gammaproteobacteria* (100%)	Marine aquaculture
32.	CSN-NBRI163 (EU442146)	AY328792.1	Uncultured bacterium DSSD95 16S ribosomal RNA gene, partial sequence	89%	*Gammaproteobacteria* (99%)	Drinking water
33.	CSN-NBRI177 (EU442160)	AY922152.1	Uncultured Acidobacteria bacterium clone AKYH730 16S ribosomal RNA gene, partial sequence	81%	*Firmicutes* (55%)	Farm soil
34.	CSN-NBRI194 (EU442177)	AY212657.1	Uncultured bacterium clone 210ds10 16S ribosomal RNA gene, partial sequence	93%	*Verrucomicrobia* (100%)	Water
35.	CSN-NBRI195 (EU442178)	AY622244.1	Uncultured Verrucomicrobia bacterium clone B-I12 16S ribosomal RNA gene, complete sequence	87%	*Verrucomicrobia* (38%)	Subsurface soil

tion of the bacterial community in soil ecosystems, including the rhizosphere [26]. The representation of genera *Devosia* is interesting as, previously these genera have been detected as a nitrogen fixing nodule former in *Neptunia natans* (L.f.) Druce [27], other dominant phylotypes were relatives of well known genera *Rhizobium*/ *Mesorhizobium*/ *Rhodoplanes*. 16S rRNA gene fragments related to *Rhizobium* was also recovered from maize roots and it was noted that pea was grown in the field site prior to maize cultivation [28]. It is also possible that *Rhizobium* is a native member of non-legume rhizospheres [29]. *Pseudomonas* is also recognized as a common rhizosphere colonizing bacteria. Cloned 16S rRNA genes affiliated with *Pseudomonas* were also infrequently recovered from rhizosphere soil samples [30]. *Pseudoxanthomonas* has been known to use as bioremediation as reported earlier by Sanchez *et al.* [31]. Most interestingly, the economically important phyla *Actinobacteria* was the second dominant group in our clone library, and among most dominant OTUs with 5 clone sequences was close relative of genera *Streptomyces*. This genera is of economical importance because of its antibiotic producing ability. *Streptomyces*, act as antagonists to many different phytopathogenic fungi, detection of chitinolytic activity of *Actinomycetes* also makes them promising candidates as biocontrol agents for diverse fungal diseases. Presence of phyla *Actinobacteria* in the rhizosphere opens the possibility of exploring them to identify members with antagonistic properties toward some economically hazardous plant pathogens. An important decomposer of plant matter in the soil, this phylum has previously been shown to be abundant in some of these soils by culture-dependent and independent techniques [32]. It would be interesting to hypothesize that root exudates from maize rhizosphere favors growth of *Actinobacteria* like microbes to eliminate pathogenic bacteria and decompose plant matter, for enhanced plant and soil health. Likewise bacteria within the *Bacteroidetes* are known to be chemoorganotrophic and especially proficient in degrading various polymers and complex substrates in media such as cellulose, chitin, and pectin [33]. It has been reported earlier that the members of the phylum *Verrucomicrobia* have been shown to make up 1 to 10% of the bacterial 16S rRNA in soils, although most members of this phylum remains uncultivated. Some members of this group are among the most successful human pathogens, others are abundant soil microbes, and others still are of major importance for the marine nitrogen cycle and hold much promise for sustainable wastewater treatment [34]. In the maize rhizosphere 10 OTUs belong to phylum *Acidobacteria* were related to uncultured bacteria. It has been reported earlier that phylum *Acidobacteria* occupied a large proportion of DNA- and RNA-derived clone libraries, as well as metabolically active in the soil sample, implying that the phylum *Acidobacteria* might be highly involved in the biogeochemical cycles of the rhizosphere soil [35].

It is interesting to note that 35 OTUs were showing less than 95% similarity against published database. To the best of our knowledge these OTUs, using GenBank sequence database could not be assigned to any known phylum and may represent unidentified bacterial lineages and suggests that a large amount of the rhizobacterial diversity remains to be characterized by culturing. The combined data demonstrated that the clone libraries represented a phylogenetically broad spectrum of organisms. Our data correlates well with a recent meta-analysis of 16S rRNA data from rhizospheres of 2 woody dicots, 9 herbaceous dicots, and three grasses. At least 35 taxonomic orders of bacteria were present, including abundant *Proteobacteria* (often *Gammaproteobacteria*, though *Alpha-* and *Betaproteobacteria* also were present), gram-positive members of the CFB (*Cytophaga-Flavobacterium-Bacteroides*) group, and smatterings of Actinobacteria among others. Known capabilities of these groups hint at complex rhizosphere biogeochemistry; methanotrophy, nitrification, and diazotrophy are known in *Proteobacteria*, and the CFB group can use complex substrates [36].

We used various methods to measure species richness in our clone library Simpson index of diversity (1/D) which considers both richness and evenness was used to measure diversity. This index has been used previously for microbial communities [37]. It has been suggested by Zhou *et al*. [38], the values for 1/*D* below about 50 indicate typical dominance profiles; therefore, high value of 250 shows no dominance of any bacterial clones.

In conclusion, in this study we could show that molecular techniques based on ribosomal genes allow a detailed description of microbial communities independent of the uncertainties of classical cultivation. However, several limitations have to be considered. The specificity of broad-range primers is limited, and PCR-based methods in general are susceptible to amplification bias. Although our statistical analysis showed that the number of clones is sufficient to cover the full richness of the clone library, the results should not be interpreted to indicate absolute bacterial representation. Furthermore, due to inherited limitations of the experimental approach (species-specific number of rRNA copies, small amount of detectable 16S rRNA), no quantitative representation of bacterial signatures/phy-

lotypes can be achieved. Nevertheless this study established a library of diverse 16S rRNA gene fragments from maize rhizosphere soil which can be used to construct specific DNA primers and probes to target bacterial groups of interest. Microbial indicators could prove valuable for assessments of soil quality relating to soil management. This study revealed a high phylogenetic diversity of bacteria in maize. Knowledge on the abundance and composition of bacteria is an indispensable precondition for future applications in areas such as soil health, plant health promotion and phytoremediation. To our knowledge, this is the first report that 35 OTUs have been identified as phyla associated with rhizosphere by the culture-independent approach. The results suggest that the diversity of rhizosphere bacteria is abundant in maize. It was also found that the maize rhizosphere community was very complex and a lot of uncultured bacteria could be interesting subjects for further exploration.

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