Large Rhizosphere Bacterial Diversity Exits Among Wild Progenitor Species of Modern Sugarcane (Saccharum Spp. Inter-Specific Hybrids)

Mukesh Kumar Malviya
Guangxi Academy of Agricultural Science
Chnag-Ning Li
Guangxi Academy of Agricultural Science
Manoj Kumar Solanki
Agricultural Research Organization Volcani Center
Prakash Lakshmanan
Guangxi Academy of Agricultural Science
Rajesh Kumar Singh
Guangxi Academy of Agricultural Science
Krishan K Verma
Guangxi Academy of Agricultural Science
Pratiksha Singh
Guangxi Academy of Agricultural Science
Anjney sharma
Guangxi Academy of Agricultural Science
Qi-Qi Song
Guangxi Academy of Agricultural Science
Qian Nong
Guangxi Academy of Agricultural Science
Xiu-Peng Song
Guangxi Academy of Agricultural Science
Yang-Rui Li (liyr5745@126.com)
Guangxi Academy of Sciences https://orcid.org/0000-0002-7559-9244

Research article

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Abstract

Background

Rhizosphere is rich in highly diverse and complex microbial communities. Plant growth promoting rhizpbacteria and diazotrops are played crucial role in plant growth and development. In this study, rhizosphere soils were collected from five wild *Saccharum* species- *S. officinarum* L. cv Badila (BRS), *S. barberi* Jesw. cv Pansahi (PRS), *S. robustum* (RRS), *S. spontaneum* (SRS), and *S. sinense* Roxb. cv Uba (URS) for studied of rhizosphere and diazotroph bacterial diversity using 16S rRNA and *nifH* gene amplification and sequencing.

Results

We detected a total of 6202 operational taxonomic units (OTUs) specific to the bacterial communities from all species combined. Out of the 107 bacterial communities detected among all samples, we found a core microbiome of 31 rhizobacterial families spread across all the species analyzed. A total of 1099 OTUs were identified for diazotrophs with a core microbiome of 9 families distributed among all the sugarcane species. The core microbiomes were distributed across twenty genera-*Bradyrhizobium*, *Dechloromonas*, *Desulfovibrio*, *Stenotrophomonas*, *Xanthobacter*, *Anaeromyxobacter*, *Azospirillum*, *Pseudoacidovorax*, *Methylobacterium*, *Azoarcus*, *Paenibacillus*, *Ideonella*, *Beijerinckia*, *Paraburkholderia*, *Burkholderia*, *Ruficoccus*, *Geobacter*, *Sinorhizobium*, *Kosakonia,* and *Azotobacter.*

Conclusion

The results presented here advance our understanding of rhizosphere associated bacterial diversity among genetically closely related wild species and provide a knowledge base for studying the evolution of rhizobacteria-host plant association during crop domestication.

Background

Rhizosphere is rich in highly diverse and complex microbial communities. These diverse microbial communities include saprophytes, epiphytes, endophytes, pathogens, and also plant growth promoting microorganisms [1]. Bacteria are the most abundant rhizospheric microbiota that other microbes, covering up to 15% of the total root surface [2]. It is reported that 2 to 5% of rhizobacteria are plant growth promoting in nature [3]. Many plant growth promoting rhizobacteria (PGPR) have been found to be associated with nitrogen fixation, solubilization of inorganic molecules like phosphate, production of plant growth regulators/hormones, siderophores and compounds that control phytopathogens [4,5,6]. Thus, PGPRs and other rhizosphere bacteria are now well recognized as essential parts of sustainable agriculture. The most commonly found rhizospheric bacterial genera are *Bacillus, Pseudomonas*, *Rhizobia*, *Arthobacter*, *Agrobacterium*, *Micrococcus*, *Cellulomonas*, *Azotobacter*, *Alcaligenes*, *Mycobacterium* and *Flavbacter* [7,8]. As the majority of PGPR are not culturable, studying them using traditional lab-based methods is highly cumbersome and time-consuming [7,9]. Advancements in

molecular biology, especially the advent of high-throughput DNA sequencing technologies and the associated data analytics has helped understand the rhizosphere micro flora by culture-independent studies rapidly and at relatively low cost [10,9]. The next-generation sequencing approaches provide an efficient and comprehensive approach to identify microbial species in the rhizosphere irrespective of microbial abundance [11]. As a result, through the sequencing of 16S rRNA gene, the taxonomic characterization of highly diversified rhizospheric bacteria has increased remarkably [12,13]. Further, modern molecular techniques permit an in-depth analysis of soil bacterial communities' compositional and functional dynamics in changing soil environmental conditions, a recurring feature of agricultural soil [9,14].

Sugarcane is an important agricultural crop grown in nearly 110 countries world wide. China is the third largest sugarcane producer (a collective term for *Saccharum* species, but more commonly applied to *Saccharum officinarum* L. and *Saccharum* spp. inter-specific hybrids) and it is a major crop in southern China, accounting for ~90% of Chinese sugar production [15]. Over the years, sugarcane has been developed as a multi-purpose agro-industrial crop as it provides the raw material for different industries like food, thermal, energy/fuel, paper etc. [16,17]. Sugarcane is mostly grown as a monoculture for extended periods resulting in yield decline which is attributed to degraded soil, imbalanced soil biology, and build-up of pests and diseases. Restoration of soil biology and soil fertility is now emerging as a priority for improving soil health, reducing yield gap and sustaining profitable green agriculture. Hence, significant research is now being carried out on sugarcane rhizobacteria to understand their diversity and role in crop improvement. Several novel PGPRs from the sugarcane microbiome have been identified and used to improve crop production [18,19]. They are mostly involved in nitrogen fixation and plant hormone production, thus positively affecting sugarcane growth [16,20,21]. Rhizobacteria play a significant role in nitrogen fixation in sugarcane crops [22,23]. However, much remains to be learned about these diazotrophic rhizobacteria, a key driver of soil health and fertility. Our present study aims to understand the role of rhizosphere bacterial communities and identify new species of nitrogen fixing bacteria using high-throughput 16S rRNA and *nifH* gene sequencing by the Illumina platform. The current study reports interesting and novel findings on the diversity of bacterial communities in five *Saccharum* species namely, *S. officinarum* L. cv Badila (BRS), *S. barberi* Jesw. cv pansahi (PRS), *S. robustum* (RRS), *S. spontaneum* (SRS), and *S. sinense* Roxb. cv Uba (URS), and provides a knowledge base to study the influence of sugarcane genotype on rhizosphere bacteria in this necessary sugar and energy crop.

Results

This study analyzed the rhizosphere soil microbiota of five different critical ancestral sugarcane species using 16S rRNA and *nifH* gene sequencing to understand their bacterial community diversity, especially that ofdiazotrophs.

Data filtration, quality evaluation, and sequence optimization

A total of 233,455 effective sequences number with an average length of 413 bp were obtained by sequencing 16S rRNA from different sugarcane species samples. *nifH* gene sequencing resulted in a total of 182,185 effective sequences with 357 bp average length from all the samples. Filtration of raw reads of 16S rRNA and *nifH* genes was done using QIIME quality filters, followed by OTU identification, clustering, and analysis, respectively (Additional file 2: Table S1 and S2).

Identification and analysis of Operational Taxonomic Units (OTUs)

Rarefaction curves

To assess microbial diversity among all the sugarcane samples, a Rarefaction curve was drawn. The presence of OTUs across all the 16S samples is shown in Additional file 1: Figure S1A. The highest number of OTUs was observed in *S. sinense* followed by *S. robustum*, *S. barberi*, *S. officinarum* and *S. spontaneum.* Presence of the OTUs identified in *nifH* sequence data across all the samples is shown in Additional file 1: Figure S1B. The highest number of OTUs was observed in *S. barberi* and *S. sinense* followed by *S. robustum* and *S. spontaneum.* The clustering of OTUs was done based on 97% sequence similarity.

Rank abundance curves (RACs)

Comparison of rank abundance based on OTU ranks derived from 16S rRNA and *nifH* sequences (Additional file 1: Figure S2 A and B) was performed to visualize the relative species abundance across all the samples. RACs depict the species richness and species evenness, which help identify common and rare rhizobacterial species in the sugarcane species studied. Figure S2A, revealed that all the sugarcane samples showed high abundance at least OTU ranks i.e. 1, following this at 500 OTU rank equal species richness and evenness was displayed. At the highest OTU rank, i.e. 1000, all the samples showed least species abundance and high species evenness. *S. sinense* samples displayed the highest species richness among all the samples at highest OTU rank. But in (Additional file 1) Figure S2B, *S. barberi* samples displayed the highest species richness among all the samples at highest OTU rank. The *S. spontaneum* sample, compared to all the other samples, showed less species evenness at low OTU rank, i.e. approximately 180.

Venn diagrams

We analyzed common and unique OTUs based on 16S rRNA and *nifH* gene sequences for each sample in the Venn diagram (Figure 1A & B). In the 16S rRNA sequence data, 6202 OTUs were identified collectively, of which 519 OTUs were common across all samples. According to the Venn diagram, the relative frequency of OTUs in the studied species is as follows: *S. sinense*>*S.robustum*>*S.barberi*>*S. officinarum*>*S. spontaneum*. The highest number of OTUs was recorded in *S. sinense*. A total of 1099 OTUs were identified in all the samples of *nifH* gene sequence data. Among them, 14 were common OTUs found across all the samples. According to the Venn diagram, occurrence of OTUs in the plant samples was as follows: *S.barberi*>*S. robustum*>*S. officinarum*>*S. sinense*>*S. spontaneum*. The highest numbers

of OTUs were found in *S. barberi* and, *S. spontaneum* had the lowest number of OTUs for both 16S rRNA and *nifH* gene sequence data.

Principal component analysis (PCA)

To understand the rhizobacterial community composition, PCA plots were generated based on 16S rRNA and *nifH* gene samples OTUs data (Figure S3 A and B). The OTUs present in the 16S samples shows that *S. officinarum* and *S. robustum* are not identical, *S. sinense* and *S. robustum* are more similar, and *S. spontaneum* is distinct from all the other samples (Figure S3 A). PCA of *nifH* gene samples showed a close identity between *S. barberi* and *S. spontaneum* whereas *S. sinense, S. officinarum* and *S. robustum* remained distinct to one another.

Alpha diversity

Alpha diversity refers to the diversity within a particular sample individually, and it is usually represented by the species (i.e. species richness) enumerated in the test samples. Alpha diversity analysis was done using Shannon, Simpson and Chao Indices Rarefaction curves for both 16S and *nifH* sequence data. Additional file 1: Figure S4 A consists of two plots displaying Shanon, Simpson, and Chao Indices, built using 16S samples. The Shannon Index increased as both the species richness and the evenness in the community increase. Among the 16S rRNA data of all the samples analyzed, Shanon and Chao Index of *S. sinense* sample was the highest as compared to the other four samples with little variation among themselves. Whereas, Simpson Index increased as diversity increased. In the Simpson Index plot, *S. officinarum* sample showed the highest value implying that it has more species diversity compared to the other four samples. Shannon and Chao Index of *nifH* gene data of all the samples showed more species diversity in *S. barberi* sample than others. According to the Simpson Index, of *S. officinarum* sample was the highest compared to other (Additional file 1: Figure S4 B).

Community composition analysis

The relative abundance of the microbial communalities differed among five sugarcane species analyzed.The abundant phyla identified in 16S samples in all sugarcane species are Proteobacteria, Firmicutes, Actinobacteria, Acidobacter, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Planctomycetes and Nitrospirae (Figure 2A). Firmicutes were the highest phyla present in *S. officinarum* compare to other samples. Gemmatimonadetes was the highest in *S. barberi*, Acidobacter was highest in *S. spontaneum*, and Bacteroidetes was highest in *S. sinense*. Based on *nifH* gene samples, we observed the occurrence of Proteobacteria and Verrucomicrobia (Figure 2B). Firmicutes were abundant in *S. spontaneum* samples compare to other samples. Many unclassified phyla were also represented abundantly in *S. spontaneum* followed by *S. sinense* and *S. barberi* samples.

Genus distribution using 16S rRNA sequence data (Figure 3A) showed *Bacillus*, *Pseudomonas*, *Pseudoarthobacter*, *Massilia*, *Lysobacter*, *Nitrospira*, *Gemmatimonas*, *Streptomyces,* and *Rhizobium*weremost abundant in *S. officinarum* whereas *S. barberi* sample was dominated by *Bacillus*,

Pseudomonas, *Pseudoarthobacter*, *Lysobacter*, *Gemmatimonas*, and *Sphingomonas* species. *Bacillus*, *Pseudomonas*, *Pseudoarthobacter*, *Massilia*, *Nitrospira*, *Gemmatimonas*, *Streptomyces, Paenibacillus*, and *Dechloromonas*were reported in *S. robustum*. *S. spontaneum* sample contained *Pseudomonas*, *Pseudoarthobacter*, *Massilia*, *Tumebacillus, Remibacter, Sphingomonas*, and *Skermanelia. Bacillus*, *Pseudomonas*, *Pseudoarthobacter*, *Massilia*, *Lysobacter*, *Nitrospira*, *Faecalibacterium and Streptococus*were detected in *S. sinense. Bacillus* was the most abundant genus in *S. officinarum, S. robustum* and *S. sinense* while *Pseudomonas* became the number one genus in *S. barberi* and *S. spontaneum*.

Genus distribution using *nifH* gene data is presented in Figure 3B. *Bradyrhizobium*, *Dechloromonas*, *Desulfovibrio*, and *Stenotrophomas* were abundant in *S. officinarum*, while *Bradyrhizobium*, Desulfovibrio, *Xanthobacter* and *Anaeromyxobacter* were the leading genera in *S. barberi*. *Bradyrhizobium*, *Dechloromonas*, *Desulfovibrio*, and *Anaeromyxobacter* were the dominant groups in *S. robustum. S. spontaneum* contained *Bradyrhizobium*, *Azospirillum*, *Methanobacterium*, and *Paenibacilllus* species. *Bradyrhizobium*, *Dechloromonas*, *Xanthobacter* and *Anaeromyxobacter* were present in *S. sinense*. Genus *Burkolderia* was found in *S. barberi*, *S. spontaneum*, and *S. sinense* while *Beijerinckia* was recorded in *S. barberi, S. spontaneum*, and *S. sinense*. Genus *Idenella* was commonly present in all the samples except *S. spontaneum* and *Kosakonia* was commonly present in *S. barberi and S. spontaneum*.

KRONA analysis

The KRONA software was used to visualize the species annotation results, in which the circles represent different points from the inside out class level, the size of the fan represented the relative proportions of the results of different OTU comments (Figure 4). Species annotation results visualized with KRONA identified *Bacillus*, *Pseudomonas*, *Pseudoarthobacter*, *Rhizobiales*, *Burkholderiales*, *Massilia* and *Streptomyces* as the most dominant ones (Figure 4A). Similar analyses carried out with *nifH* OTUs showed *Bradyrhizobium*, *Dechloromonas*, *Desulfovibrio*, *Stenotrophomonas*, *Anaeromyxobacter* etc. as the most common genera identified (Figure 4B). The relative abundance of the top twenty diazotrophs at the genus level present in all sugarcane species is shown in Figure 5. Diazotrophs belonging to the genus *Bradyrhizobium* were present in all the samples tested.

Star analysis of all the sugarcane samples

Star analysis was conducted using the top 10 genera of each sample. Star analysis for both 16S and *nifH* sequence data are presented in Additional file 1: Figure S5 A&B. Top 10 genera used for the 16S data based analysis were *Pseudoarthobacter*, *Pseudomonas*, *Bacillus*, *Massilia*, *Gemmatimonas*, *Nitrospora*, *Haliangium*, *Ramibacter*, *Tumebacillus* and *Lysobacter*. From this analysis, *Bacillus* genus was found high in *S. officinarum* and *S. robustum* samples, while *Pseudomonas* was the dominant one in *S. barberi*, *S. robustum*, *S. spontaneum* and *S. sinense. Tumebacillus* genus was found only in *S. spontaneum*. Similarly, star analysis for *nifH* gene samples identified the presence of *Bacillus* in all the samples. *Desulfovibrio* was found in *S. officinarum*, *S. robustum* and *S. barberi* in significant numbers. *Xanthobacter* was abundant in *S. sinense. Anaeromyxo bacter* was found in all the samples except that

of *S. spontaneum. Pseudoacidovorax* genus was found only in *S. robustum*, *S. barberi* and *S. sinense* samples. *Azospirillum* and *Methylobacterium* were unique to *S. spontaneum*.

Beta diversity

Beta diversity measures species diversity among different samples collected from different or similar environments. We performed the principal coordinated analysis (PCoA) and UniFrac-based cluster analysis to understand the beta diversity of 16S rRNA and *nifH* gene in the sugarcane species studied (Figures 6 & 7). Based on 16S rRNA data, *S. sinense,* along with *S. barberi*, and *S. officinarum*a long with *S. robustum*, formed independent clusters. And, *S. spontaneum* segregated away from all others, displaying higher beta diversity. The same was found true for *nifH* gene analysis (Figure 7A). Hierarchical clustering based on the UniFrac cluster analysis showed similar results for both 16S rRNA and *nifH* gene data, containing similar identical sequences showing 0 (blue colour) distances. *S. sinense* to *S. spontaneum* showed more distance (red colour = 0.3) indicating dissimilarity in sequences of *S. sinense* to *S. spontaneum* samples (Figure 6A and 7B).

The distribution of dominant genera based on their relative abundance performed with Bray–Curtis algorithm showed *Bacillus* being the dominant genus in all the samples except those from *S. Spontaneum* (Figure 8). Second dominant genus identified was *Pseudomonas,* while *Pseudoarthobacter* was the third leading one. *S. spontaneum* showed the presence of most unidentified genera in our analysis. Similarly, distribution of dominant genera in *nifH* samples showed the presence of *Dechloromonas* sp. abundant in all samples except those from *S. barberi*. *Xanthobacter* and *Bradyrhizobiumg* were found to be dominant in *S. Officinarum* and *S. sinense,* respectively (Figure 9).

Discussion

There are several reports of identification and characterization of plant growth promoting rhizobacteria from sugarcane [34,35] and other crops [36-38] and they are also being used for crop productivity improvement. Most of the sugarcane studies are conducted with modern cultivated sugarcane hybrid varieties and there is no previous attempt to understand the diversity of rhizobacteria in its wild progenitors and closely related species such as *S. officinarum, S. spontaneum, S. robustum*, *S. barberi* and *S. sinensis* as we did in this study. Here, we used high-throughput sequencing of 16S rRNA and *nifH* genes to study the rhizobacterial diversity and complexity in sugarcane species. Rhizospheric microorganisms are known to interact with plants for their survival and nutritional requirements [39,40]. Many of them are also beneficial bacteria to help plants with nutrient uptake and to cope with pathogens and abiotic stresses [18,41,42]. Considering the large microbial diversity in the rhizosphere, a large number of PGPRs are yet to be identified for various crops, including sugarcane. Bacteria are the most abundant of all the rhizospheric microbiota, and many are known to promote plant growth [2,3,42]. Our study made an effort to understand the diverse and complex diazotroph bacterial communities present in the rhizospheric soils of progenitors and closely related speciesof modern sugarcane hybrids as nitrogen fixation plays a critical role in its growth and production [43,22].

16S rRNA sequence data revealed 6202 OTUs assigned to different bacterial species colonizing rhizosphere of different sugarcane species studied. Analysis of these OTUs showed that *S. sinense* rhizosphere has the largest number of rhizobacterial communities compared to other related species studied here. Besides this, alpha diversity analysis also predicted the highest bacterial diversity in *S. sinense* samples. However, other sugarcane species also showed considerable species diversity. Phylum level distribution studies identified the dominance of Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Planctomycetes and Nitrospirae. Many of them were reported to be present in sugarcane rhizosphere previously [44]. Out of the total OTUs identified, 1099 were from diazotrophs based on *nifH* gene data. Analysis of *nifH* gene sequence variation helped identify the top twenty genera (*Bradyrhizobium*, *Dechloromonas*, *Desulfovibrio*, *Stenotrophomonas*, *anthobacter*, *Anaeromyxobacter*, *Azospirillum*, *Pseudoacidovorax*, *Methylobacterium*, *Azoarcus*, *Paenibacillus*, *Ideonella*, *Beijerinckia*, *Paraburkholderia*, *Burkholderia*, *Ruficoccus*, *Geobacter*, *Sinorhizobium*, *Kosakonia* and *Azotobacter*). Out of these, ten were found to fix nitrogen in sugarcane and other plants. *Bradyrhizobium* is known to fix nitrogen in sorghum and sugarcane [45, 46]. Members of *Sinorhizobium* genus are also known to fix nitrogen symbiotically in leguminous alfalfa plants [47]. *Azotobacter* genus consists of seven different species and they are involved in atmospheric nitrogen fixation in different crop plants [48]. *Azotobacter* and *Beijerinckia* were studied for diazotrophic attributes in early 1960s in rice and other cereal crops [49,50]. Contrary to *Azotobacter*, *Beijerinckia* genus is largely restricted to the tropics and its nitrogen fixation ability has been reported in a variety of plants [51]. *Kosakonia* spp. fix nitrogen on cucumber roots [52]. Roots of switchgrassare inhabited with nitrogen-fixing bacteria belonging to *Dechloromonas*, *Desulfovibrio*, *Azoarcus*, *Ideonella*, *Paraburkholderia* and *Burkholderia* [53]. It is hard to identify and classify most of the bacteria in culture because of their morphological similarities. But, culture-independent methods like 16S rRNA sequencing and highly efficient, cost-effective and provide accurate identification and classification of rhizobacteria. Overall, the dominant genera identified in this study are known to fix atmospheric nitrogen for plant growth.

In the present study, we observed a few taxa such as *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Firmicute*, *Sphingomonas*, *Bradyrhizobium*, and *Gemmatimonas* were dominant while genus such as *Bacillus*, *Pseudomonas*, *Bradyrhizobium*, *Burkholderia*and *rhizobium* were dominant. In our previous studies, we observed that some taxa such as *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Firmicute*, *Bacteroidetes, Sphingomonas*, *Bradyrhizobium*, *Bryobacter*, and *Gemmatimonas* were always present in the sugarcane rhizosphere [54,55]. Some genus such as *Bacillus*, *Pseudomonas*, *Burkholderia*, known for their plant growth promoting and nitrogen fixating properties, were found to be enriched in the sugarcane rhizosphere [56,22,23]. Community composition analysis of 16S rRNA sequence data helped in tracking phylum and genus level distribution of rhizobacteria among different sugarcane species studied. Interestingly, we observed the presence of a few genera, namely *Streptococcus*, *Rhodanbacter*, *Anaeromyxo bacter* and *Prevotella* common among all the species studied here. The members of these genera were found commonly in soil and can colonize crop plants. From the previous reports it appears that *Rhodanbacter*, *Anaeromyxobacter* and *Prevotella* genera were isolated from different soil and plant sources and they were found to have *nifH* gene and nitrogen fixation abilities [57-59]. Thus, we believe

that more characterization of these bacteria colonizing the sugarcane rhizosphere will be beneficial for developing bio-based crop products to improve sugarcane crop productivity.

Diversity among these nitrogen fixing bacteria was revealed by alpha and beta diversity analyses. The top ten genera with the highest abundance were found to be *Bacillus*, *Desulfovibrio*, *Xanthobacter*, *Anaeromyxobacter*, *Pseudoacidovorax*, *Azospirillum* and *Methylobacterium.* Among these *Bacillus* is a common bacterial diazotroph in sugarcane [60]. *Desulfovibrio*, *Anaeromyxobacter*, *Azospirillum* and *Xanthobacter* are nitrogen fixers in rice [61-64]. *Methylobacterium* is a nitrogen fixing bacterium, found in legumes [65], but such a function was not reported for *Pseudoacidovorax*.

Conclusions

All the sugarcane species studied here showed a significant number of nitrogen fixing rhizobacteria, which strengthens the contention, that exploring rhizosphere bacteria may help develop a sustainable low sugarcane crop production system meeting its N requirement from atmospheric nitrogen fixation requiring low nitrogen fertilizer input. Substantial genetic variation for rhizobacteria and diazotrophs communities exists among different progenitors and closely related species of modern cultivated sugarcane hybrids. However, considering the wide natural habitats of these wild species spanning both, tropics and sub-tropics, similar studies using accessions sourced from different locations and environmental conditions will greatly advance our understanding of sugarcane rhizo-microbiome. Future research should also focus on isolation and practical application of beneficial plant growth promoting rhizobacteria and diazotrophs. Filling the large knowledge gap on microbiota and sugarcane interactions is critical for exploiting these beneficial microbes for sustainable sugarcane agriculture.

Methods

Sample collection

Rhizospheric soil of five sugarcane species, *S. officinarum* L. cv. Badila (BRS), *S. barberi* Jesw. cv. pansahi (PRS), *S. robustum* (RRS), *S. spontaneum* (SRS), and *S. sinense* Roxb.cv. Uba (URS) were collected from the sugarcane germplasm collection of Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, Guangxi, China. The soil compositions of all the root collection sites are presented in Additional file: 2 Table S3. We sampled three soil samples randomly from each species and were used for DNA isolation.

Bacterial genome isolation and sequencing

Extraction of genomic DNA

We used a culture-independent method [24] to study the bacterial composition of rhizospheric microbiome collected from the test species. Total microbial DNA was extracted using CTAB/SDS isolation method with minor modifications [25]. The purity and concentration of DNA preparations were

determined using 1% agarose gel electrophoresis. DNA samples diluted to 1ng DNA/μL using sterile water and used for further analysis. We mixed three DNA samples of each sugarcane species in the same concentration to get a mixed sample. Mixed samples were used for the sequencing analysis.

Identification of 16S rRNA and *nifH* genes

Microbial 16S rRNA was amplified with the universal primers 341F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT), which target the V3–V4 region. The *nifH* gene was amplified with primers Pol-F (TGCGAYCCSAARGCBGACTC) and Pol-R (ATSGCCATCATYTCRCCGGA), as previously reported [26]. Polymerase chain reaction (PCR) amplification of identified 16S rRNA and *nifH* genes was performed with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Thermal cycling consisted of initial denaturation at 98 ºC for 1 min, followed by 30 cycles of denaturation at 98 ºC for 10 s, annealing at 50 ºC for 30 s, and elongation at 72 ºC for 60 s, finally, 72 ºC for 5 min, following the protocols reported previously by Zhou et al.[27].

PCR product quantification and Library preparation and sequencing

Visualization and quantification of PCR products were conducted by mixing an equal volume of 1× loading buffer containing SYB green dye with the PCR products and electrophoresed on 2% agarose gel. The 400-450bp DNA fragments were isolated and used for further experiments. Equimolar amounts of PCR products from all samples were pooled and the mixture was purified using Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries of purified amplicons were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) in accordance with manufacturer's protocol and index codes were added. The library quality and concentration were assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. To perform sequencing the qualified libraries were fed into the IlluminaHiSeq2500 platform.

Data analysis

Quality control and sequence assembly

Based on their unique barcode, trimming of barcode and primer sequencing were done. Reads were assembled using FLASH (V.1.2.7) (http://ccb.jhu.edu/software/FLASH/) [28] to generate raw tags. To obtain high quality clean tags from raw tags, we performed quality filtration using QIIME (V1.7.0) (http://qiime.org/index.html) [29]. Removal of chimera sequences was done by comparing the tags with the reference database Unite Database (https://unite.ut.ee/) using UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) [30]. The above step is critical to obtain effective tags.

Operational Taxonomic Unit (OTU) cluster and species annotation

OTU identification

Operational Taxonomic Units identification was done with UPARSE software (v.7.0.1001) (http://drive5.com/uparse/) [31]. Based on ≥97% of sequence similarity, all the effective tags were clustered into OTUs. For each OTU cluster, a representative sequence was screened to perform taxonomic annotation.

Species annotation

OTUs were taxonomically annotated following a BLAST analysis against the Unite Database of each identified representative bacterial sequence done in QIIME software.

Phylogenetic relationship construction

Multiple sequence alignment was conducted with MUSCLE software (V.3.8.3) (1http://www.drive5.com/muscle/) [32], and phylogenetic relationship of different OTUs was established to understand the diversity of microbial species in various samples (groups).

Data normalization

Standard sequence number given to samples with least sequences was considered for OTUs abundance information normalization. Subsequently, alpha diversity and beta diversity analysis were conducted based on the normalized data.

Alpha diversity

Alpha diversity analysis is carried out to find the complexity of species diversity for each sample using 6 indices, which include observed species, Chao1, Shannon, Simpson, abundance-based coverage estimator (ACE) and Good's coverage. Indices calculation for all the samples was done using QIIME and visualized in R software (V. 2.15.3). Community richness was identified with two selected indices using Chao - the Chao1 richness estimator (http://www.mothur.org/wiki/Chao). Whereas, the Shannon index (http://www.mothur.org/wiki/Shannon) and Simpson index (http://www.mothur.org/wiki/Simpson) indices were used for identification of community diversity in all the samples. To characterize the sequencing depthandcoverage, the Good’s coverage (http://www.mothur.org/wiki/Coverage) was used.

Beta diversity

To evaluate the differences in bacterial species among all the samples, beta diversity analysis was performed. QIIME software was used to calculate beta diversity using weighted and unweighted UniFrac distances.

Statistical analysis

Cluster analysis followed by principal component analysis (PCA) was applied to reduce the dimensionality and preserve the variability information of the sample data. Using the FactoMineR and ggplot2 packages in R software, results were visualized. Later, Principal Coordinate Analysis (PCoA) was

performed to obtain principal coordinates and to visualize them. To compare microbial diversity present in different samples, the UniFrac method was used to generate weighted or unweighted UniFrac values among samples that were transformed to give uncorrelated/orthogonal axes. Visualization of PCoA results was done using WGCNA package, stat and ggplot2 packages in R software. QIIME software was used for hierarchical clustering by Unweighted Pair-group Method with Arithmetic Mean method (UPGMA) [33] to infer the distance matrix using average linkage. The raw data of 16S rRNA (Accession no. PRJNA678588) and *nifH* gene (Accession no. PRJNA681283) were submitted to the NCBI Sequence Read Archive.

Abbreviations

BRS: *S. officinarum* L. cv Badila; PRS: *S. barberi* Jesw. cv Pansahi; RRS: *S. robustum;* SRS: *S. spontaneum;* URS: *S. sinense* Roxb. cv Uba; OTUs: Operational taxonomic units; PCoA: Principal coordinate analysis; QIIME: Quantitative insights into microbial ecology; PCR: Polymerase chain reaction; RACs: Rank abundance curves

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw data of 16S rRNA (Accession no. PRJNA678588) and *nifH* gene (Accession no. PRJNA681283) are available in NCBI SRA in following links. https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA678588; https://www.ncbi.nlm.nih.gov/sra/PRJNA681283.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions: Conceived and designed the experiments: MKM, CNL, & YRL. Performed the experiments: MKM & CNL. Analyzed the data: MKM, CNL, & MKS. Contributed reagents/materials/analysis tools: MKM, RKS, KKV, PS, AS, QQS, QN and XPS. Wrote the paper: MKM & PL. All authors have read and agreed to the published version of the manuscript.

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Figures

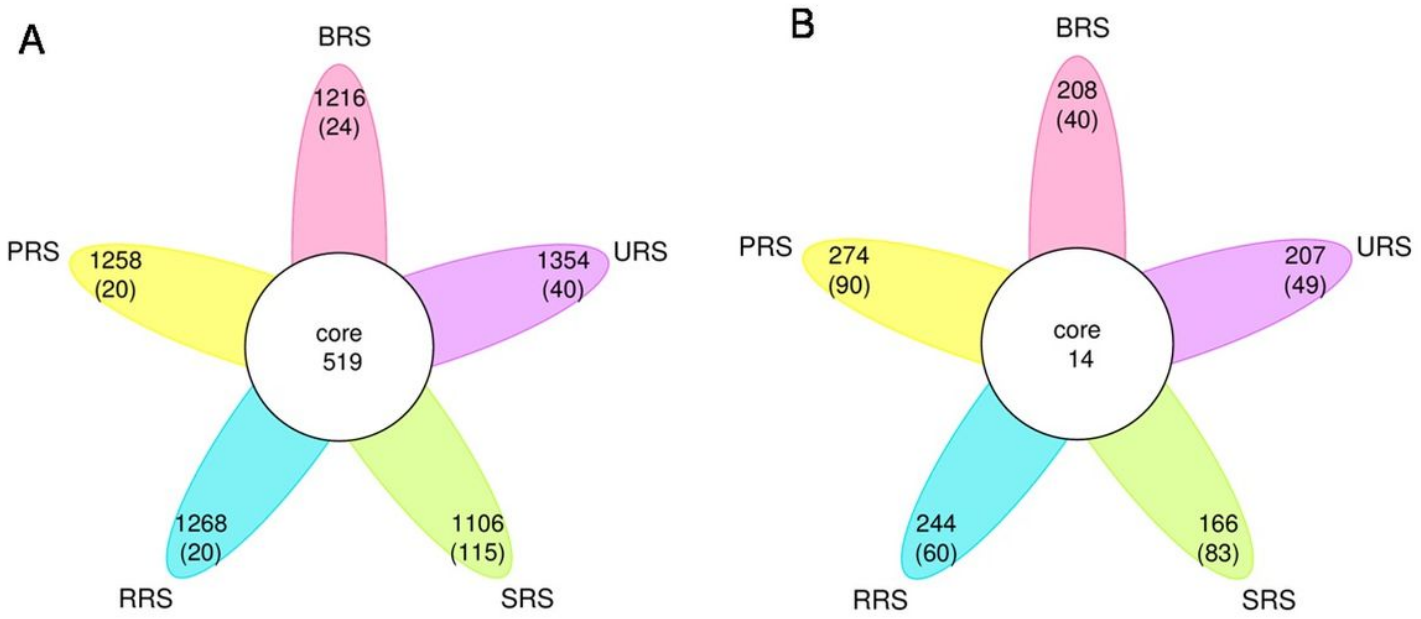

Figure 1

Venn diagram showing the OTUs of all sugarcane species (A) 16S and (B) nifH gene. S. officinarum L. cv Badila (BRS), S. barberi Jesw. cv pansahi (PRS), S. robustum (RRS), S. spontaneum (SRS), and S. sinense Roxb. cv. Uba (URS)

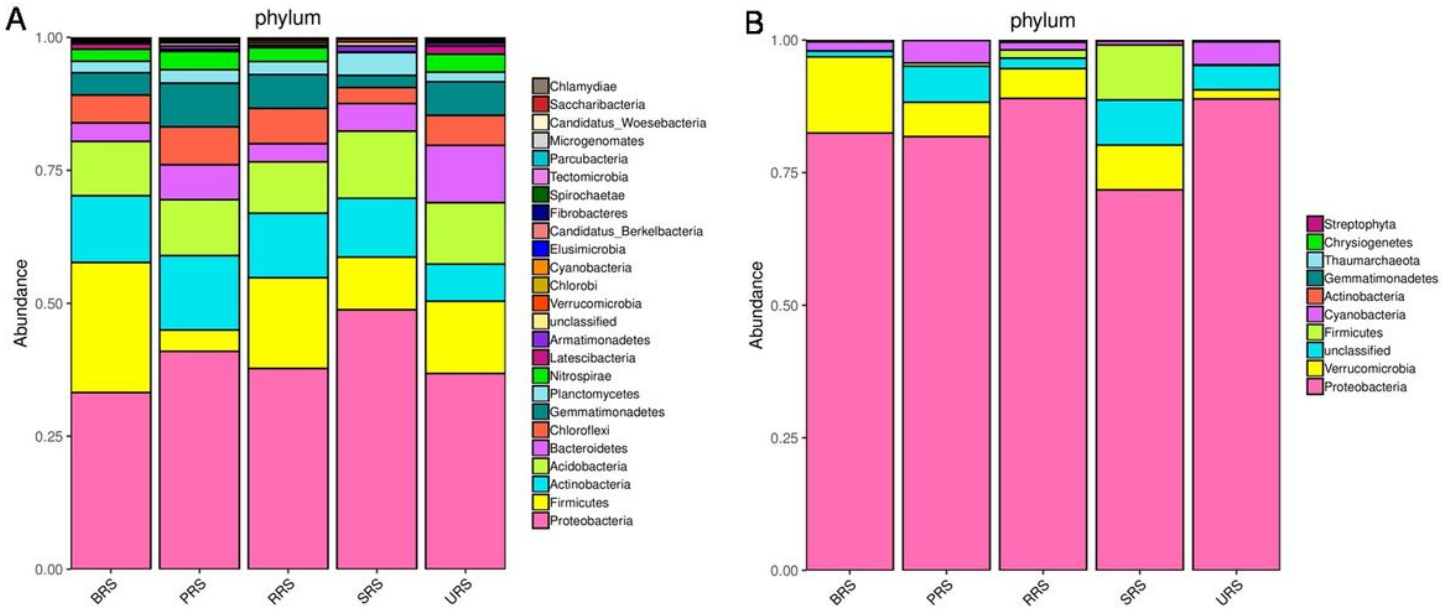

Figure 2

Community composition analysis at phyllum level of all sugarcane species (A) 16S rRNA and (B) nifH gene. S. officinarum L. cv Badila (BRS), S. barberi Jesw. cv pansahi (PRS), S. robustum (RRS), S. spontaneum (SRS), and S. sinense Roxb. cv. Uba (URS)

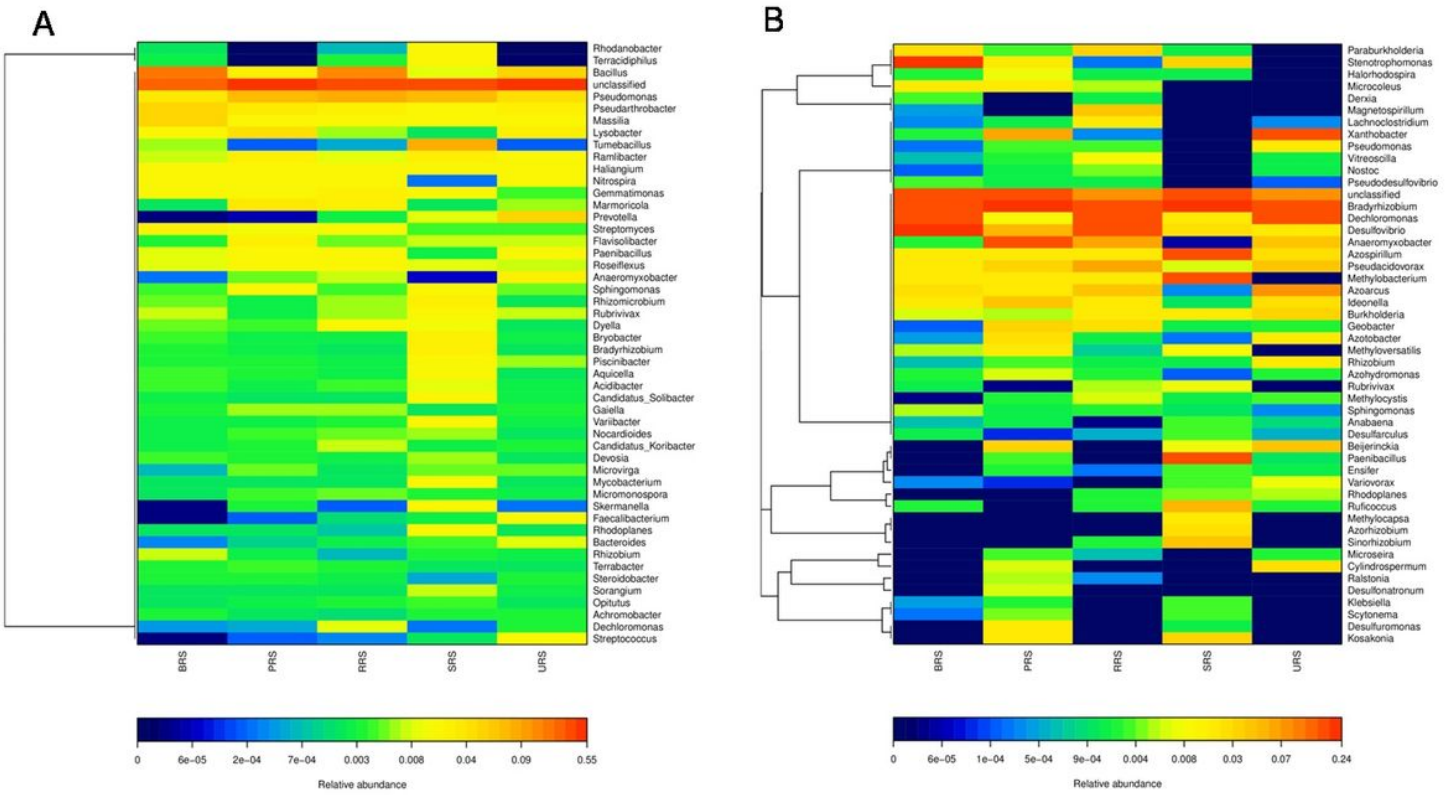

Figure 3

Heat map showing the abundances of bacterial communities at genus level of all sugarcane species (A) 16S rRNA and (B) nifH gene. S. officinarum L. cv Badila (BRS), S. barberi Jesw. cv pansahi (PRS), S. robustum (RRS), S. spontaneum (SRS), and S. sinense Roxb. cv. Uba (URS)

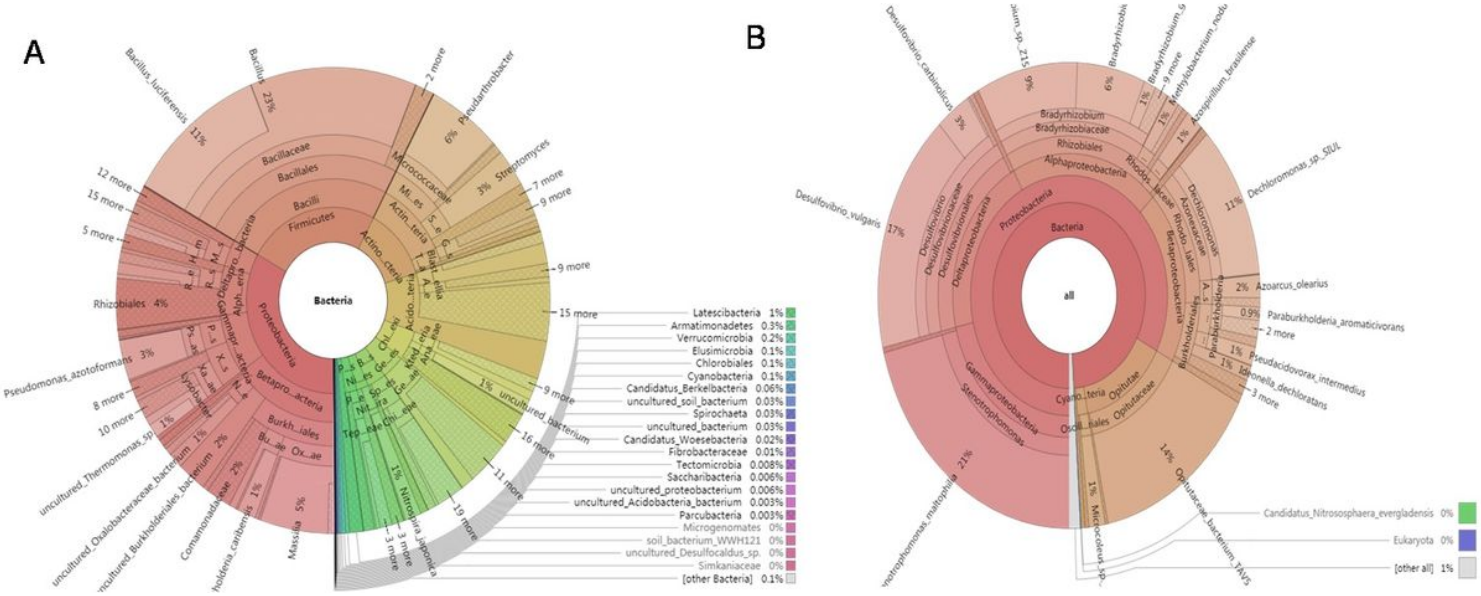

Figure 4

Korona analysis (A) 16S rRNA (B) nifH. Different colored regions represent different species, the circles from inside to out represent the classification levels from the phylum to genus. S. officinarum L. cv Badila

(BRS), S. barberi Jesw. cv pansahi (PRS), S. robustum (RRS), S. spontaneum (SRS), and S. sinense Roxb. cv. Uba (URS)

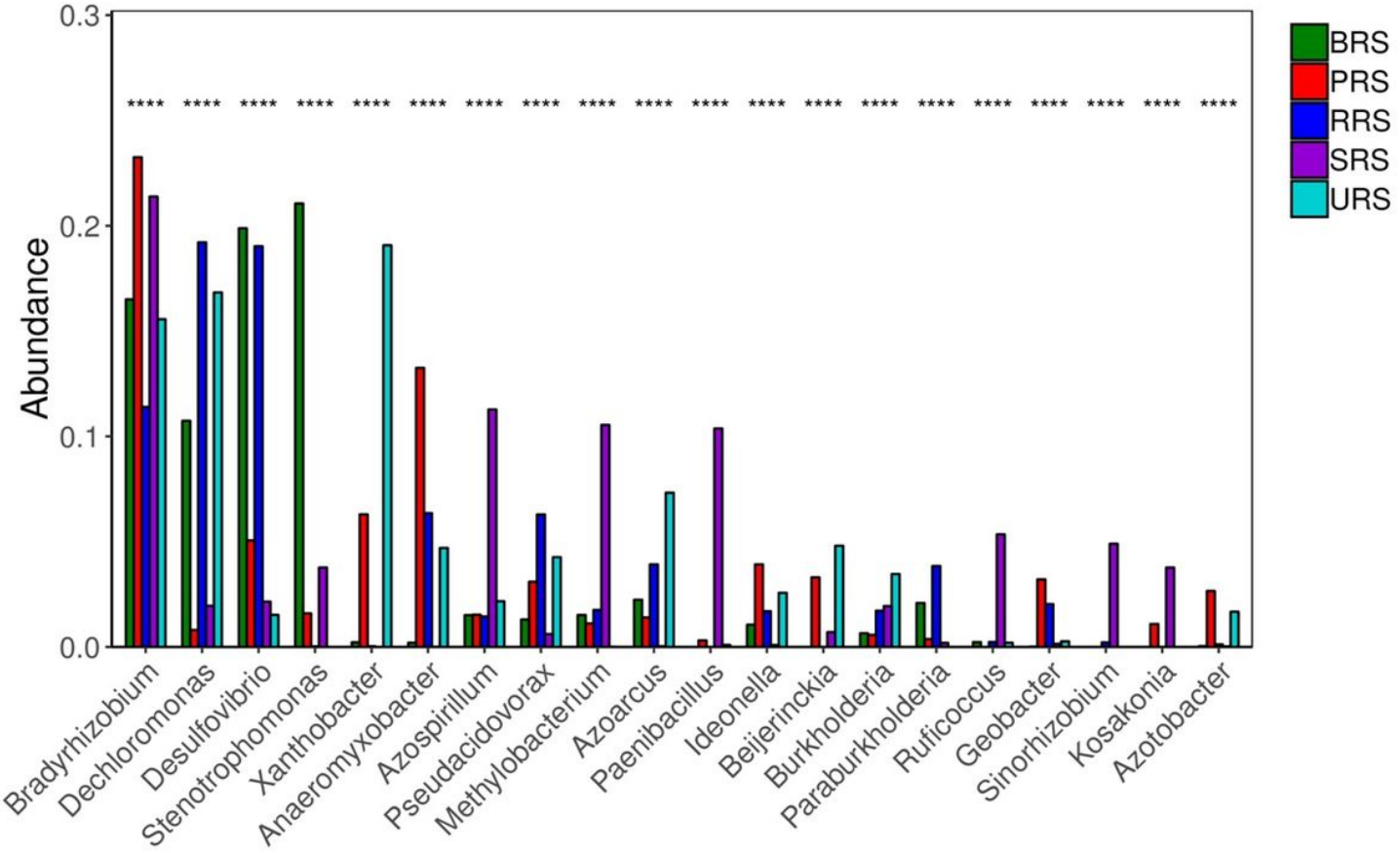

Figure 5

The relative abundances of the top 20 diazotrophs at the genus level of all samples. S. officinarum L. cv Badila (BRS), S. barberi Jesw. cv pansahi (PRS), S. robustum (RRS), S. spontaneum (SRS), and S. sinense Roxb. cv. Uba (URS)

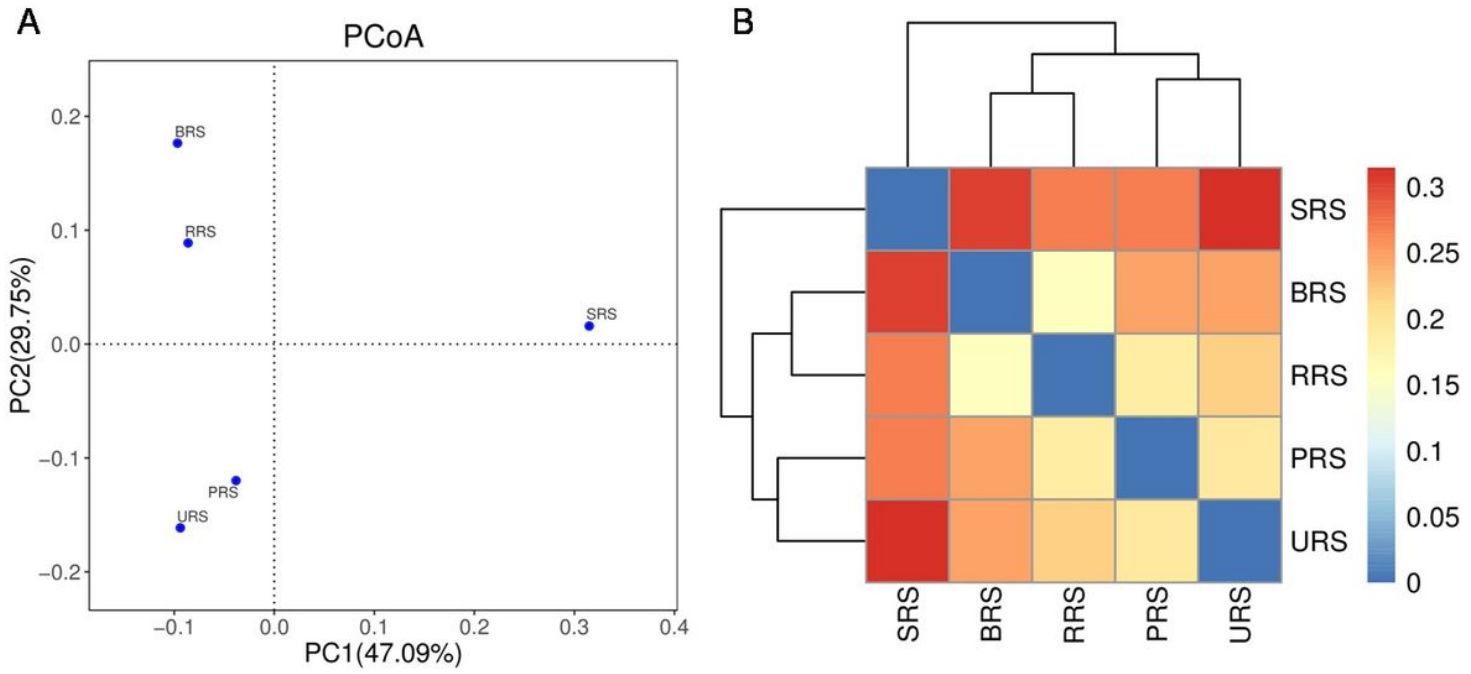

Figure 6

Beta diversity analysis to estimate the dissimilarity and similarity of 16S bacterial communities and composition among different samples. (a) Principal coordinated analysis (PCoA) derived from dissimilarity matrix of weighted UniFrac distance. (b) Multi-sample differential matrix heat map weighted UniFrac based cluster analysis of bacterial community composition among different samples. S. officinarum L. cv Badila (BRS), S. barberi Jesw. cv pansahi (PRS), S. robustum (RRS), S. spontaneum (SRS), and S. sinense Roxb. cv. Uba (URS)

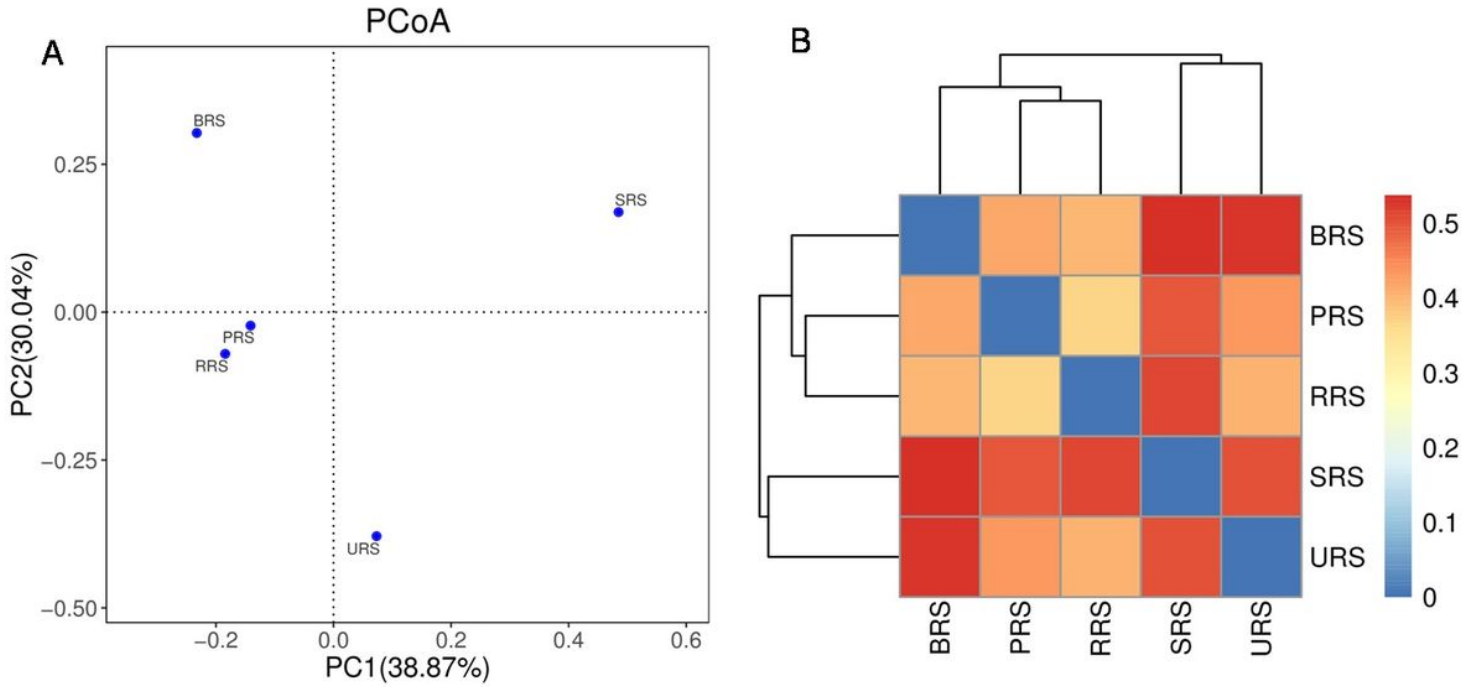

Figure 7

Beta diversity analysis to estimate the dissimilarity and similarity of nifH bacterial communities and composition among different samples. (a) Principal coordinated analysis (PCoA) derived from dissimilarity matrix of weighted UniFrac distance. (b) Multi-sample differential matrix heat map weighted UniFrac based cluster analysis of bacterial community composition among different samples. S. officinarum L. cv Badila (BRS), S. barberi Jesw. cv pansahi (PRS), S. robustum (RRS), S. spontaneum (SRS), and S. sinense Roxb. cv. Uba (URS)

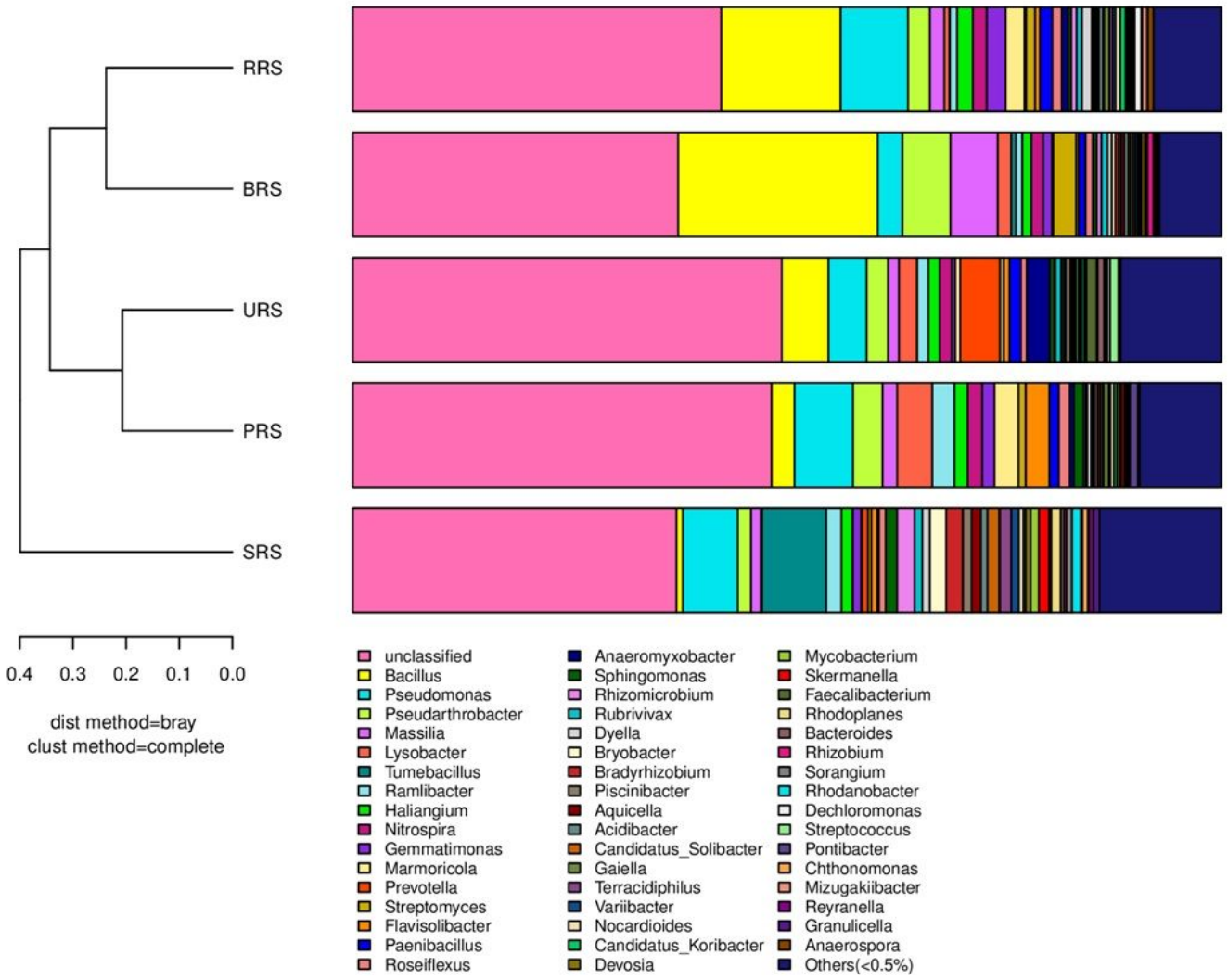

Figure 8

A combination of horizontal multi-sample similarity trees and histograms of 16S genus. On the left is hierarchical clustering between samples based on community composition (bray-curtis algorithm), and on the right is a column chart of the sample's community structure. S. officinarum L. cv Badila (BRS), S. barberi Jesw. cv pansahi (PRS), S. robustum (RRS), S. spontaneum (SRS), and S. sinense Roxb. cv. Uba (URS).

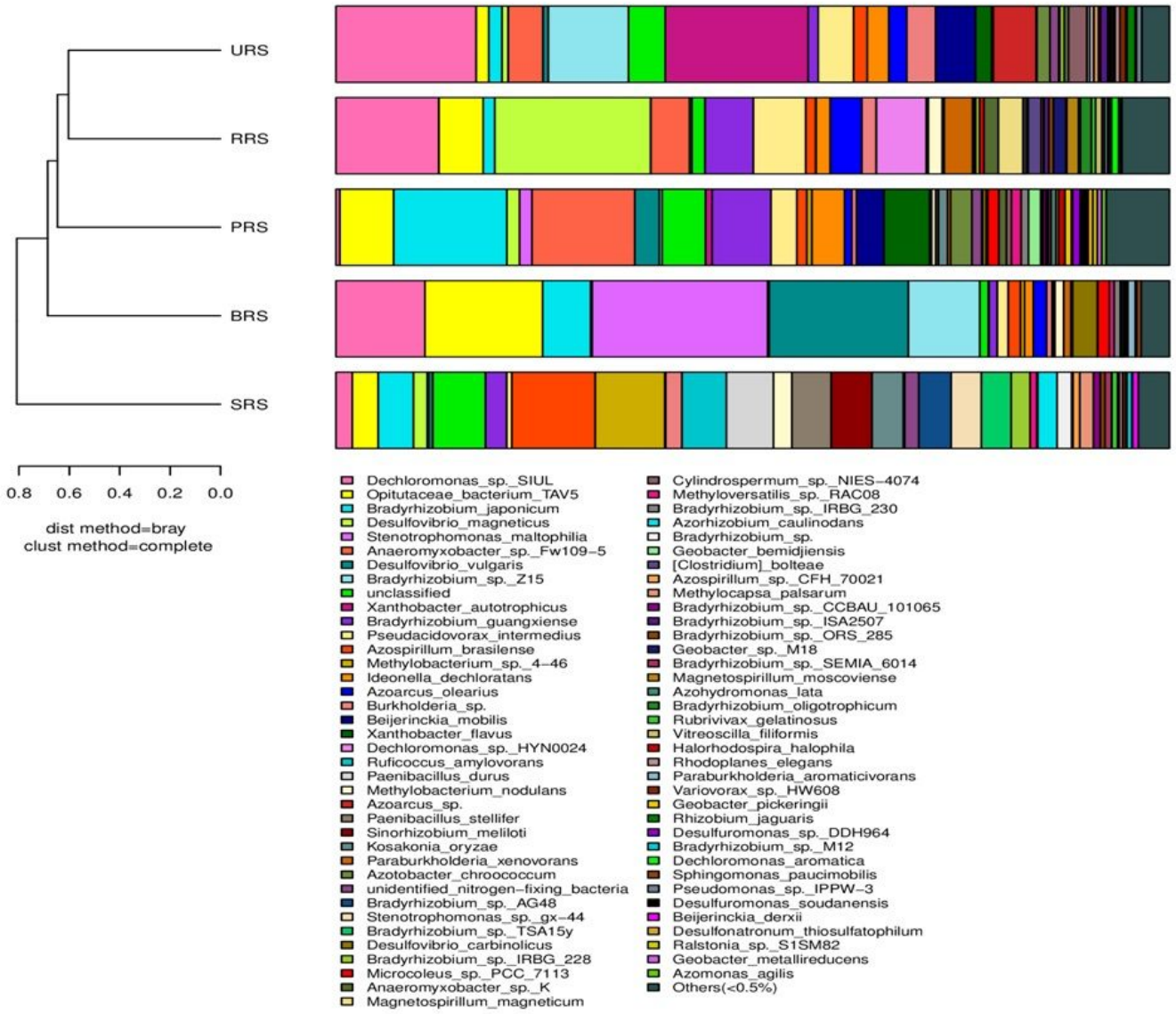

Figure 9

A combination of horizontal multi-sample similarity trees and histograms of nifH genus. On the left is hierarchical clustering between samples based on community composition (bray-curtis algorithm), and on the right is a column chart of the sample's community structure. S. officinarum L. cv Badila (BRS), S. barberi Jesw. cv pansahi (PRS), S. robustum (RRS), S. spontaneum (SRS), and S. sinense Roxb. cv. Uba (URS).

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