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Diversity of Rhizo-Bacteriome of *Crocus sativus* Grown at Various Geographical Locations and Cataloging of Putative PGPRs

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Earlier plant growth promoting rhizo-bacteria (PGPRs) were isolated from the plants, by cultivation based techniques and the interaction was mostly thought to be bilateral. The routine bilateral study, with no information on the associated microbiome, could be one of the reasons for the limited success of PGPRs in the field conditions. Keeping in view the role of PGPRs in rhizo-bacteriome on the growth and production of plant, the present study was aimed at studying the diversity of the rhizo-bacteriome of saffron grown across three geographical locations namely Kashmir, Kishtwar and Bengaluru. Variation in the rhizo-bacteriome of saffron growing across 10 different sites from 3 geographical locations was studied using 16S rDNA amplicon metagenomic sequencing. 16 bacterial phyla, 261 genera and 73 bacterial species were cataloged from all the rhizosphere samples. *Proteobacteria* was a dominant phylum in all the rhizosphere samples. Rhizo-bacteriome of saffron grown in Kishtwar was found to be significantly different from the rhizo-bacteriome of saffron grown in Kashmir and Bengaluru. Interestingly, the rhizo-bacteriome of saffron grown in Bengaluru was very similar to the saffron grown in Kashmir, thereby indicating that the rhizo-bacteriome in saffron is "plant driven" as the corm sown in Bengaluru were from Kashmir. Despite variation in rhizo-bacteriome, core rhizo-bacteriome in saffron was identified that was represented by 53 genera and eight bacterial species belonging to 11 phyla irrespective of their geographical distribution. In addition, 21 PGPRs were reported for the first time from the saffron rhizosphere. The high yielding saffron field Wuyan was found to have the highest number of PGPRs; this indicates that the presence of PGPR is important for yield enhancement than diversity. The two PGPR *Rhizobium leguminosarum* and *Luteibacter rhizovicinus* were reported from all the locations except Kishtwar that had escaped isolation in our previous attempts using cultivation based techniques. It is being proposed instead of going for random isolation and screening for PGPRs from plant rhizosphere, an alternate strategy using metagenomic cataloging of the rhizo-bacteriome community and cultivation of the dominant PGPR should be undertaken. This strategy will help in the selection of dominant PGPRs, specific to the plant in question.

Keywords: PGPR–plant growth-promoting rhizobacteria, *Crocus sativus*, rhizo-bacteriome, core microbiome, 16S rDNA

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INTRODUCTION

Microbiomics is a fast-growing field in which collective genomes of microorganisms, of a given community (a microbiota) are investigated. Literature is replete, with the studies on the microbiomes of environmental samples such as soil and water (Gui-Feng et al., 2020), human gut and skin (Parello, 2020), plant rhizosphere, cormosphere and phyllosphere (Stone et al., 2018; Bhagat et al., 2021; Rüger et al., 2021). The plant microbiome is reported to be critical to host adaptation, productivity and health (Zhao et al., 2019; Trivedi et al., 2020). The plant microbiome acts as a reservoir of microbes, that directly influences the structure and composition of the plant, promotes plant growth, increases stress tolerance, mediates local patterns of nutrient cycling and can also be used as molecular markers (Bakker et al., 2013; Coats and Rumpho, 2014; Trivedi et al., 2020; Bhagat et al., 2021). Earlier microbiome associated with different plants would be studied mostly using cultivation based techniques. However, it is a fact now, that not more than 1% of bacteria can be cultivated by routine cultivation and 99% remained uncultivated (Steen et al., 2019). In order to study microbiomes associated with any niche, cultivation independent technique metagenomics, complements the cultivation based techniques. In metagenomics, genomes of the bacterial communities are extracted collectively and sequenced directly. Metagenomics has revolutionized the study of complex microbial communities, as it overcomes the limitation of cultivation based methods, as far as cataloging of bacteria present and functions performed by them, in any niche is concerned (Boughner and Singh, 2016; Alteio et al., 2020; Taş et al., 2021). Further, the attention has shifted from plant-microbe interaction to plant-microbiome interaction and the role of microbiome in plant growth promotion (Kour et al., 2019; Yadav et al., 2020). The rhizosphere microbiome's contribution to plants has been acknowledged by giving it the title of "plant's second genome" (Ofek-Lalzar et al., 2014; Yin et al., 2020). Plant growth promotion by Plant growth promoting bacteria (PGPBs) has been reported in various plants such as in rice (Chauhan et al., 2019), wheat (Çakmakçı et al., 2017), maize (Zerrouk et al., 2019), tomato (Cordero et al., 2018), soya bean (De Gregorio et al., 2017) and saffron (Ambardar and Vakhlu, 2013; Ambardar, 2014; Ambardar et al., 2014, 2016; Kour et al., 2018; Magotra et al., 2021).

Crocus sativus, commonly known as saffron, is the world's costliest spice with medicinal and cosmetic value (Zhao et al., 2019; Bhooma et al., 2020). 1 kg of saffron costs about 11,000 USD/Kg (Ambardar et al., 2014). The other reasons for the selection of the *Crocus sativus,* as a test organism, in addition to its economical status is, (i) reference microbiome of root and corm during various growth stages has been cataloged previously, (ii) PGPBs have been isolated and evaluated for their efficacy, (iii) and most importantly, it is reported to be a monogenetic herb world over with the uniform genotype, hence variation in rhizo-bacteriome and its effect on yield was the question worth asking. Variation in the quality and yield of saffron growing across different locations has been reported due to various factors such as epigenetic, climate change, soil characteristics and microbes associated with plants. It is a sterile triploid plant ($2n = 3x = 24$) and reproduces vegetatively by underground bulb-like, starch-storing organs, known as corms (Wani et al., 2018; Nemati et al., 2019). The herb has an interesting biannual life cycle that is characterized by three distinct growth stages, dormant stage from July to August, flowering stage from October to November and vegetative stage from January to May. The flowering stage lasts ∼30 days a year. Each flower has three red trumpets like stigmas (2 to 3 cm long) which when dried, are commercialized as a saffron spice (Yasmin and Nehvi, 2013). The bacteriome and mycobiome of saffron rhizosphere were, for the first time, reported by our group (Ambardar et al., 2014, 2016). In addition, PGPBs were isolated and evaluated in the course of the study (Ambardar and Vakhlu, 2013; Ambardar, 2014; Kour et al., 2018; Magotra et al., 2021). Recently, the cormosphere bacteriome of saffron was compared across the geographical locations from India and Morocco and preliminary data suggests that the microbiome is location specific and simultaneously, there is a core microbiome that is common to all locations (Bhagat et al., 2021). Such reports of core and location specific microbiome have also been reported in other plants such as, rice (Eyre et al., 2019); coffee (Fulthorpe et al., 2020); switch grass (Grady et al., 2019); red sage (Chen et al., 2018), wheat (Kuzniar et al., 2020) etc.

Since saffron is reported to be a monogenetic plant and has the same genotype worldwide, the present study was undertaken to explore the variation in the rhizo-bacteriome associated with the plant grown across different geographical locations in India to evaluate the effect of geography on rhizo-bacteriome of the saffron. Rhizo-bacteriome associated with a saffron plant was compared and a core rhizo-bacteriome was identified using 16S rDNA gene targeted metagenomic analysis during the flowering stage in November 2016. In addition, an inventory of putative PGPRs present in the rhizo-bacteriome was made and compared across these locations and a correlation with the yield was attempted.

MATERIALS AND METHODS

Sample Collection

Saffron rhizosphere samples were collected from 10 locations including eight saffron fields from Kashmir district (Wuyan, Patalbagh_upper range, Patalbagh_lower range, Alchibagh, Galandar, Chandhara, Hatiwara, Samboora); one from Berwar saffron field from Kishtwar, Jammu district and one laboratory experiment conducted in Bengaluru (**Figure 1**). The details of all the saffron fields, geographical coordinates (latitude and longitude), fertilizers usage, total production, and total area of cultivation have been tabulated in **Table 1**. Composite sampling was performed from all the fields wherein the *Crocus sativus* rhizosphere samples were collected from the three corners of five fields of each location and all the 15 samples per location were pooled together to form composite samples (Ambardar and Vakhlu, 2013; Ambardar, 2014). Rhizosphere samples were collected by uprooting the plants and vigorously shaking by hand. The soil that remained adheres to the roots after vigorous shaking was taken as rhizosphere soil. The roots were washed in normal

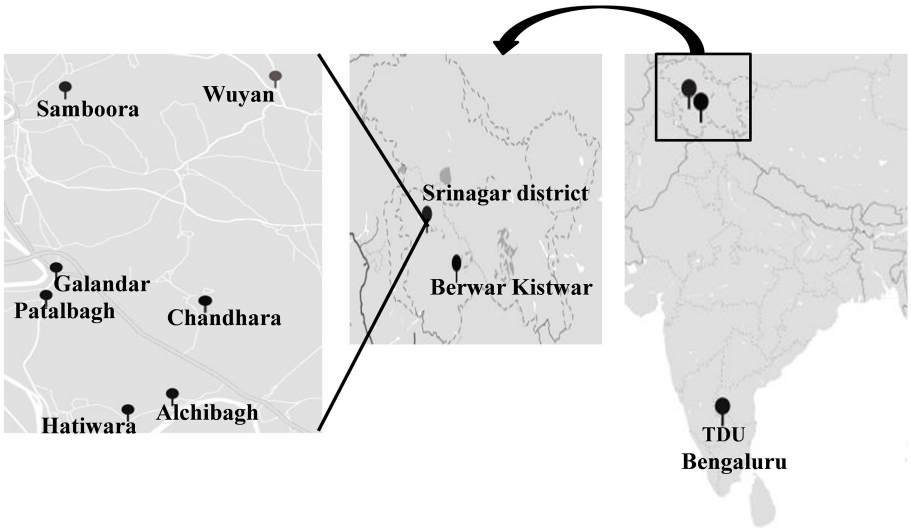

FIGURE 1 | Geographic location of sample collection sites on the map of India. TDU (The University of Trans-Disciplinary Health Sciences and Technology), Bengaluru, (Berwar) Kishtwar and 8 locations with in Srinagar district (Wuyan, Patalbagh_upper range, Patalbagh_lower range, Alchibagh, Galandar, Chandhara, Hatiwara and Samboora).

saline (0.85% NaCl) that was taken further for metagenomic DNA extraction.

A separate experiment was conducted in which saffron corms were collected from Kashmir, Pampore during the dormant stage of the saffron life cycle (July, 2016). These corms were then planted in 10 pots (5 corms/pot) filled with the garden soil at Trans-Disciplinary University, Bengaluru (Karnataka). These pots were kept in open under natural conditions (temperature 20°C) for a period of 3 months (August–November 2016). The rhizosphere samples from 15 plants were collected randomly and pooled together to form a composite sample during the flowering stage similar to field samples. This experiment was performed to address the question whether the root microbiome is soil driven or plant driven. Root microbiome was harvested using same design and method as in case of field samples.

Rhizosphere Metagenomic DNA Extraction

Metagenomic DNA was extracted from rhizosphere samples using a MoBio Power soil DNA extraction kit (MoBio Laboratories Inc. Carlsbad, CA, USA) following the manufacturer's instructions. The DNA quality and quantity were determined by using a Nano Drop device (Thermo Scientific, Wilmington, DE) and electrophoresis on 0.8% agarose gel, with 1 kb plus ladder as molecular weight marker.

16S rDNA Sequencing of the Extracted Rhizosphere Metagenome

A 16S rDNA sequencing library was constructed targeting the V3 and V4 hyper-variable regions of the 16S rDNA gene, according to the 16S rDNA metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA). The PCR was performed with 12 ng template DNA and region-specific primers with Illumina index and sequencing adapters (forward_primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGTCGGCAGCGT CAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; reverse primer: 5′GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAGGTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGGACTACHVGGGTATCTAATCC-3′) using KAPA HiFi Hot Start Ready Mix (KAPA Biosystems, Wilmington, MA, USA). The amplicons were purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, Brea, CA, USA). Subsequently, purified PCR products were visualized after gel electrophoresis and quantified with a Qubit dsDNA HS Assay Kit (Thermo Scientific) on a Qubit 3.0 fluorometer. The PCR products of all the samples were pooled to 4 nM concentration, prior to sequencing, and were analyzed on an Agilent 2,200 Tape Station (Agilent Technologies, Santa Clara, CA, USA) for quantity and quality analysis. The pooled sample (4 nM) was denatured with 0.2 N NaOH, diluted further to 4 pM concentration, and combined with 20% (v/v) denatured 4 pM PhiX as control, following Illumina's guidelines. Samples were sequenced on the MiSeq sequencing platform (Illumina) using a 2 × 250 cycle V3 kit, following standard Illumina sequencing protocols.

Data Analysis

Raw data of 16S rDNA sequencing was analyzed for a quality check using the FastQc tool kit (Brown et al., 2017). The adapter and low quality reads ($Q < 30$ Phred score) were trimmed using the Cutadapt tool of Trimgalore (Jiang et al., 2014). The reads with phred score above 30 were selected for QIIME1 pipeline analysis (version 1.9.1). The QIIME 1 pipeline (Quantitative Insights into Microbial Ecology version 1.9.1) was used to process and filter multiplexed sequence reads. The sequencing reads were grouped into an operational taxonomic unit (OTUs) that were further clustered against GreenGenes_13_8 sequences to

TABLE 1 | Area under cultivation and production data of saffron from various saffron fields under study. Saffron production was maximum in the Wuyan fields and lowest in Kishtwar fields.

Sample id	Description	Coordinates		Area under cultivation in (canals)	Area under cultivation in (Hectare)	Fertilizer used	Production for year 2016 (Kg/hectare/year)	Total Production
		Latitude N	Longitude E					
Sample 1	Kishtwar	33.311591	75.76622	101	5.1		1.26	6.4
Sample 2	Alchibagh	33.964289	74.946839	600	30.36		2	60.72
Sample 3	Galandar	33.988966	74.924863	2,000	101.18		2	202.36
Sample 4	Hatiwara	33.961191	74.938362	2,100	106.23		2	212.46
Sample 5	Patalbagh_upper range	33.983498	74.923041	2,000	101.18	DAP (Diammonium phosphate−5 kg),	2	202.36
Sample 6	Chandhara	33.98243	74.953026	12000	607.03	MOP (Muriate of potash−3 kg),	2	1214.06
Sample 7	Patalbagh_lower range	33.983498	74.923041	200	10.2	Potash, Urea (1.5 Kg) A and Vermicompost	3	30.6
Sample	Samboora	34.024078	74.926665	2500	126.46		2.5	316.15
Sample 9	Wuyan	34.026266	74.966175	10000	505.86		5	2529.3
Sample 10	Bengaluru	13.123444	77.547963	Experiment in pots	Experiment in pots	–	–	–

The details of the fertilizer used was provided by SKAUST, Kashmir, India.

TABLE 2 | 16S rDNA amplicon sequencing reads, total phyla, genera and species cataloged in saffron rhizosphere from various saffron fields under study.

Sample id	Description	16S rDNA amplicon sequencing PE reads	Phyla	Genera	Species
Sample 1	Kishtwar	68,384	16	138	37
Sample 2	Alchibagh	90,658	15	147	39
Sample 3	Galandar	67,052	14	130	31
Sample 4	Hatiwara	88,215	16	140	34
Sample 5	Patalbagh_upper range	51,918	12	122	29
Sample 6	Chandhara	59,826	16	143	28
Sample 7	Patalbagh_lower range	97,484	13	111	31
Sample 8	Samboora	75,753	15	122	34
Sample 9	Wuyan	63,972	15	154	43
Sample 10	Bengaluru	49,388	16	143	30
Total (unique phyla, Genera and species)			16	261	73

ascertain the taxonomical affiliation of bacteria (McDonald et al., 2012). Reads failing to hit the reference were subsequently clustered *de novo* at the 97% similarity level using the UCLUST greedy algorithm. Chimeric sequences were identified by the UCHIME algorithm of USEARCH61 and removed. OTU sequences were aligned using PYNAST. OTU taxonomy was determined using the Green Genes taxonomy database (Caporaso et al., 2010).

Statistical Analysis of Metagenomic Sequence Data

The bacterial community associated with each sample was compared for alpha and beta diversity analysis using Qiime 1 (Sinclair et al., 2015). For alpha diversity analysis, rarefaction curves and diversity indices like Chao1, Shannon, Simpson, phylogenetic diversity were calculated for estimating the richness and evenness in all the samples (Chao, 1987; Gotelli and Colwell, 2011). For rarefaction curves, the operational taxonomy units OTU table was rarified to an even depth of 1,00,000 sequences per sample, to avoid biases generated by the difference in sequencing depth. Rarefaction curves (97% identity) were plotted between the number of observed OTUs (cluster count) and the number of reads of the samples. In order to evaluate β diversity, PCOA plots, venn diagram and heat maps were constructed. In addition, a circos plot was generated for representing the core rhizo-bacteriome across all locations using the tool Circos Version 0.63-9 (Krzywinski et al., 2009).

RESULTS

Ten rhizosphere samples were collected from 8 different saffron fields from Kashmir and 1 from Kishtwar (Jammu

and Kashmir); and 1 from corms grown in pots at Trans-Disciplinary University, Bengaluru (Karnataka). The saffron fields were selected from different geographical locations having variations in total production of saffron as represented in **Table 1**. Wuyan in Kashmir had maximum saffron production and Kishtwar in Jammu had minimum production in 2016, the year of sample collection (**Table 1**). The corms were collected from Kashmir and sown in pots in Bengaluru to test the hypothesis that rhizo-bacteriome in saffron is plant driven, hence lacks production data, as flowering needs special climatic conditions. Rhizosphere samples were analyzed for bacterial diversity using 16S rDNA sequencing and the total number of 16S rDNA sequence reads per sample ranged between 51,918 and 97,484 (**Table 2**). The bacterial communities associated with all the rhizosphere samples were classified up to the phylum, genus and species level (**Table 2**).

Rhizo-Bacterial Diversity

Bacterial diversity associated with all the 10 rhizospheric samples was cataloged into 16 phyla, 261 genera and 73 species (**Table 2**). Out of 16 phyla, *Proteobacteria* was the dominant phyla in all the samples with higher relative abundance in Kashmir (avg 59.1%) followed by Bengaluru (49%) and Kishtwar (31.28%). However, *Firmicutes* and *Planctomycetes* were relatively abundant in Kishtwar (20, 10%) as compared to Kashmir (avg 4.9, 3.12%) and Bengaluru (5, 4%) respectively (**Figure 2**).

Out of total of 261 bacterial genera cataloged, the abundance of 4 bacterial genera namely *Bacillus*, *Lysobacter*, *Rhodoplanes* and *Janthiobacterium* were observed. *Bacillus* was dominant at four locations namely Kishtwar (54%), Hatiwara (24%), Patalbagh_lower range (22%) and Bengaluru (15%), (**Figure 3**). However, *Bacillus* was also found co-dominant with other genera at two locations namely Wuyan [*Bacillus* (16%) and *Rhodoplane* (17%)]; and Galandar [(*Bacillus* (12%) and *Lysobacter* (13%)]. The abundance of *Bacillus* was comparatively less in rest of the four locations i.e., Alchibagh (4%), Chandhara (7%), Samboora (8%), and Patalbagh_upper (11%). *Janthiobacterium* was dominant in Alchibagh (19%). *Lysobacter* was the most abundant bacterial genera in two locations namely Patalbagh_upper range (15%) and Samboora (18%), whereas *Lysobacter* (15%) shared dominance with *Rhodoplanes* (14%) in Chandhara (**Figure 3**).

53 genera out of 261 bacterial genera were common in all the samples, are considered as the core rhizo-bacteriome that has been discussed in detail under core rhizo-bacteriome section. on comparing all the 10 samples, unique genera specific to each location were also identified. The maximum number of unique bacterial genera were identified in Wuyan (19) followed by Alchibagh (10), Galandar and Kishtwar (6 each), Chandhara (4), Patalbagh_lower range and Samboora (2 each), Patalbagh_upper range, Hatiwara and Bengaluru (1 each), (**Figure 4**).

73 bacterial species were present in all the samples, out of which, 3 bacterial species i.e., *Bacillus flexus, Lysobacter brunescens* and *Janthiobacterium lividum* were dominant; but their abundance varied (**Figure 5**). *Bacillus flexus* was abundant in seven locations namely Kishtwar (49%), Patalbagh_lower range (38%), Hatiwara (35%), Bengaluru (31%), Galandar (28%), Wuyan (22%) and Patalbagh_upper range (20%). However, *Janthiobacterium lividum* (19%) was abundant in Alchibagh (**Figure 5**). *Lysobacter brunescens* was abundant in rest of the two locations namely Samboora (14%) and Chandhara (40%). Out of 73 bacterial species, 8 bacterial species were considered as the core rhizo-bacteriome (common in all the samples) that has been discussed in core rhizo-bacteriome section. In addition to core species, each sample has unique bacterial species that were specific to that particular location and absent in all other samples. Kishtwar had four unique bacterial species namely *Bacillus marisflavi, Macrococcus caseolyticus, Myroides odoratimimus* and *Staphylococcus scuiri* whereas two species were unique in Alchibagh namely *Methylotenera mobilis* and *Sphingomonas suberifaciens* and Galandar namely *Paenibacillus barengoltzii* and *Paracoccus marcusii*. Only one unique species was cataloged from Patalbagh_upper range (*Vellonellas dispar*), Wuyan (*Paenibacillus ginsengarvi*) and Samboora (*Roseomonas mucosa*). However, Bengaluru, Hatiwara, Chandhara and Patalbagh_lower range did not have any unique bacterial species (**Figure 6**).

Statistical Analysis of Rhizo-Bacterial Diversity

The bacterial diversity of each sample was also analyzed using the alpha diversity indices i.e., Chao1, Simpson, phylogenetic diversity and Shanon (**Table 3**), rarefaction curves (**Figure 7**), PCOA plots (**Figure 8**) generated by Qiime software. Rarefaction curves (97% identity) of rhizosphere samples did not reach plateau indicating bacterial diversity was well-represented but could increase on repetitive sampling (**Figure 7**). The bacterial community in saffron rhizosphere from Samboora field was diverse as compared to other sample, as depicted by higher number of different species (OTUs) in the rarefaction curve. This result was further complemented by diversity indices such as Chao1, phylogenetic diversity, Shannon and Simpson indexes were also higher in the case of Samboora (**Table 3**). Beta diversity analysis using PCOA plots was done to evaluate the significant variation in bacterial diversity among all the samples. Beta bacterial diversity of Kishtwar was significantly different from Kashmir and Bengaluru, as it does not cluster with the rest of the samples in PCOA plots (**Figure 8**).

Core Rhizo-Bacteriome of Saffron

Despite the variation in the rhizobacterial community associated with saffron plant across different locations, the core rhizo-bacteriome that remains constant in the saffron growing across different geographical locations was identified. Core rhizo-bacteriome in saffron consisted of 11 phyla, out of total of 16 phyla i.e., *Proteobacteria*, *Chloroflexi, Firmicutes, Acidobacteria, Cyanobacteria, Gemmatimonadetes, Bacteroidetes, Nitrosiprae, Planctomycetes, Verrucomicrobia* and *Actionobacteria*. At genera level, it constituted 53 out of 261 genera and eight bacterial species out of 73 bacterial species. The bacterial species that constituted the core comprised of *Bacillus flexus, Bacillus muralis, Edaphobacter modestum, Lysobacter brunescens, Pseudoxanthomonas mexicana, Psychrobacter celer, Roseateles*

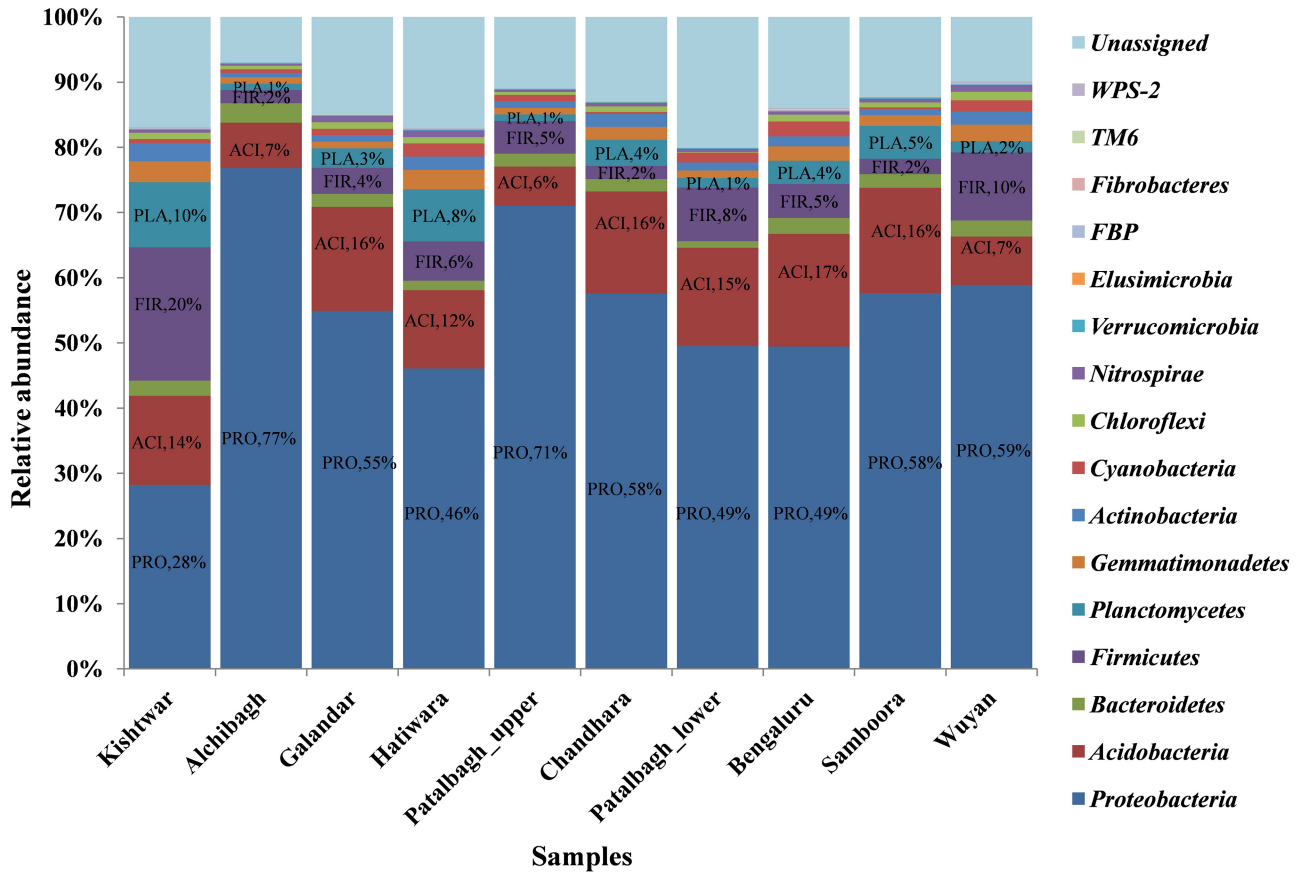

FIGURE 2 | Comparison of rhizo-bacteriome of saffron from different geographical location at phylum level representing dominance of *Proteobacteria* in all 10 samples. The relative abundance of *Proteobacteia* is less in Kishtwar (Jammu) as compared to all the samples from Kashmir and Bengaluru whereas Firmicutes and Planctomyces are more in Kishtwar as compared to others. PRO, *Proteobacteria*; FIR, *Firmicutes*; ACI, *Acidobacteria*; PLA, *Planctomycetes*.

depolymerans and *Variovorax paradoxus*. The core rhizo-bacteriome in saffron is also well-represented in heat maps at genus and species level and with circos plot (**Figures 9–11**).

DISCUSSION

Metagenomic approaches using NGS technologies can be used to explore the taxonomic and functional diversity of bacteria associated with the plants, thereby accelerating the research specific to the effect of plant bacteriome on plant health (Parvathi et al., 2019; Nathan et al., 2020). The present study was aimed at (i) exploring variation in the rhizo-bacteriome of the saffron grown across 10 sites from 3 geographical locations namely Kashmir, Kishtwar and Bengaluru, (ii) identify the core rhizo-bacteriome that persist in saffron irrespective of its geographical locations (if any), and (iii) presence of PGPRs in rhizo-bacteriome and its correlation with yield. The selected fields varied in the saffron production with Wuyan in Kashmir with the highest production i.e., 5 kg/hectare) whereas the fields in Kishtwar, Jammu with lowest production i.e., 2 kg/hectare in the year 2016 (**Table 1**).

Comparative Rhizo-Bacteriome Diversity

Out of 16 bacterial phyla, *Proteobacteria* was the most abundant phylum in all the samples (**Figure 2**). This is in accordance with a previously published report on saffron rhizosphere by our group (Ambardar and Vakhlu, 2013; Ambardar et al., 2014, 2016). *Proteobacteria* have been also reported to be the dominant phyla in the rhizosphere of other plants such as, *Gossypium hirsutum*, *Artemisia argyi*, *Ageratum conyzoides*, *Erigeron annuus*, *Bidens biternata*, *Euphorbia hirta* and *Viola japonica* (Qiao et al., 2017; Lei et al., 2019). *Proteobacteria* are known to regulate nutrients (carbon, nitrogen, sulfur) cycling in the environment that enhance plant growth (Rampelotto et al., 2013; Mukhtar et al., 2020). In our recent publication, *Proteobacteria* was also found to be the most abundant phylum in the cormosphere of saffron from Kashmir and Kishtwar, as well as in Morocco (Bhagat et al., 2021). On comparing all the samples, the rhizo-bacterial diversity of saffron growing in Kishtwar was found distinct from the rest of the samples as the relative abundance of *Proteobacteria* (PRO-31.28%) was comparatively less whereas the relative abundance of *Firmicutes* (FIR-20%) and *Planctomycetes* (PLA-10%) were more as compared to Kashmir (PRO-59.1%, FIR-4.9%, PLA-3.12%) and Bengaluru (PRO-49%, FIR-5%, PLA-4%), (**Figure 2**). This

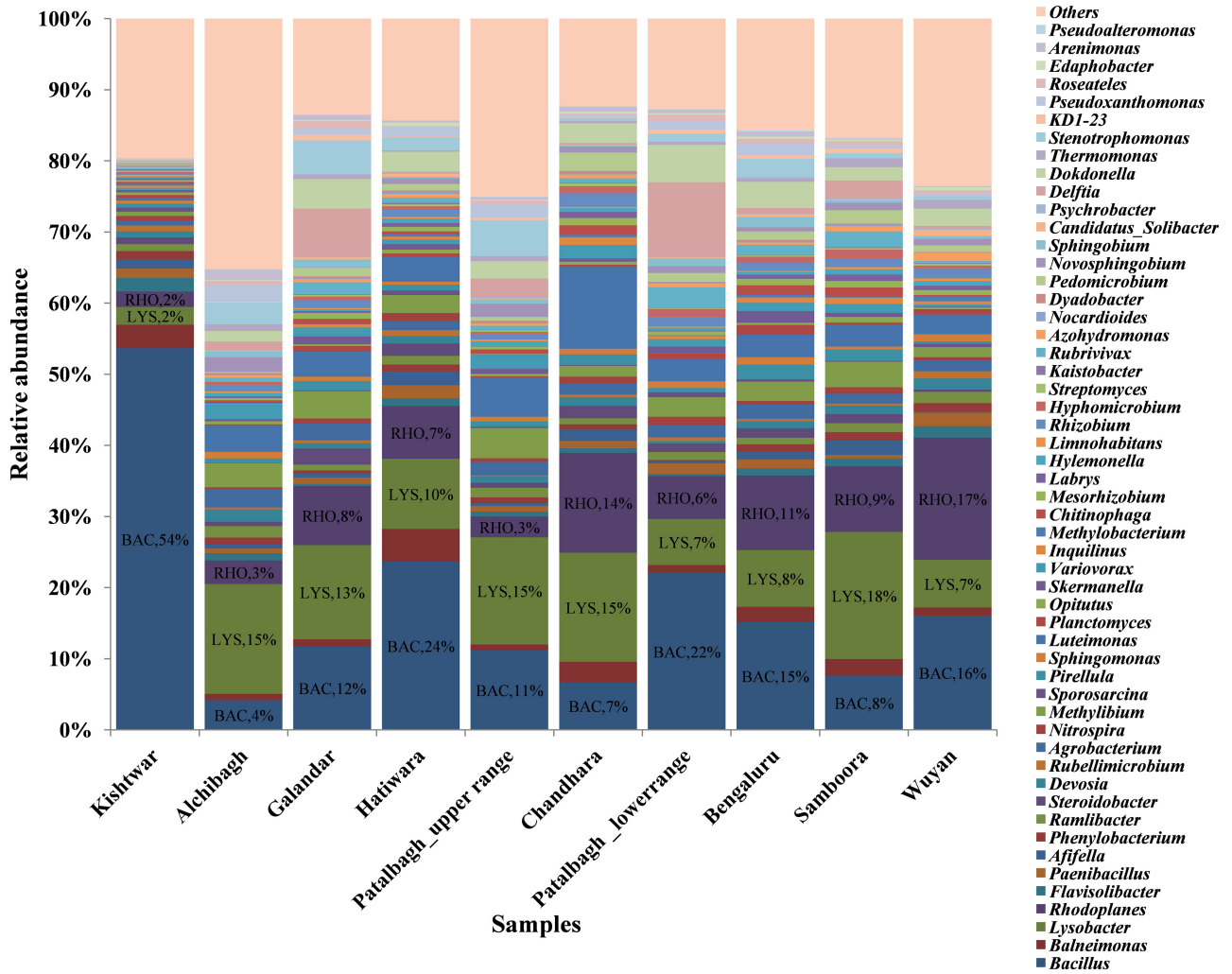

FIGURE 3 | Comparison of bacterial genera in saffron rhizosphere from different geographical locations. The OTU cataloged up to genus level were normalized to 10,000. BAC, *Bacillus;* LYS, *Lysobacter*; RHO, Rhodoplane; JAN, *Janthiobacterium* were dominant genera in these samples. In this figure, Kishtwar sample is distinct from the Kashmir samples. The microbial communities of Kashmir and Bengaluru samples are similar as compared to Kishtwar sample.

was further complemented by beta diversity analysis wherein rhizo-bacterial diversity of saffron grown in Kishtwar did not cluster with the rest of the samples based on the PCOA plots and heat maps at genus and species level (**Figures 8–10**) respectively. Similar to rhizo-bacteriome, cormo-bacteriome of Kishtwar was found significantly different than that of Kashmir and surprisingly, relatively similar to Morocco in our recent report (Bhagat et al., 2021). The significant variation in the rhizo-bacterial diversity of saffron growing in Kishtwar from rest of the samples can be preliminarily attributed to corms (plant) as Kishtwar is closer to Kashmir geographically than Morocco. Though saffron is reported to be a sterile monogenetic triploid plant, with no genetic variation and reproduces vegetatively by corms (Nemati et al., 2019), however, the variation in the yield and quality of saffron has been reported worldwide (Cardone et al., 2021). Climate and soil are thought to be two major abiotic factors (Cardone et al., 2019, 2020) and epigenetic influences have been reported to be one of the biotic factors to affect the quality and yield of saffron (Chen et al., 2021). In the preliminary observation, it seems that Cormo/rhizo-bacteriome has a role to play and is "plant driven" because rhizo-bacteriome of saffron grown in different climates and soil at Bengaluru was similar to that of Kashmir. The reason was the corms that were collected from Kashmir and grown in Bengaluru indicating that rhizo-bacteriome in saffron could be plant driven.

Comparison of rarefaction curve (97% identity) of all the samples indicated that the bacterial community in saffron rhizosphere from Samboora field in Kashmir was more diverse as compared to other samples, as the number of different species (OTUs) was higher here. This result was further complemented by diversity indices like Chao1, phylogenetic diversity, Shannon and Simpson indexes which were also higher in the case of

FIGURE 4 | Radical Venn diagram representing the unique genera identified in 10 different locations. Wuyan-Kashmir has the maximum number (19) of unique genera followed by 10 in Alchibagh, 6 in Gullandar and Kishtwar, 4 in Chandhara, 2 in Samboora and Patalbagh_lower range and 1 genera in Hatiwara, Bengaluru, Patalbagh_upper range.

Samboora (**Table 3**). However, higher bacterial diversity in the rhizosphere of saffron growing in Samboora field does not affect the saffron production positively as saffron yield in Samboora (2.5 kg/hectare) was less than that of Wuyan fields (5 kg/hectare) that had less diversity than Samboora.

The dominance of three bacterial species, *Bacillus flexus*, *Lysobacter brunescens* and *Janthiobacterium lividium* were observed in the rhizo-bacteriome of saffron grown in all the 10 locations (**Figures 3**, **5**). *Bacillus flexus* was dominant in the rhizosphere of saffron grown in seven locations namely Kishtwar, Bengaluru, Wuyan, Galandar, Hatiwara, Patalbagh_upper range and Patalbagh_lower range. *Lysobacter brunescens* was dominant in Chandhara and Samboora; and *Janthobacterium lividium* in Alchibagh (**Figure 5**). Dominant bacterial genera identified in the saffron rhizosphere (*Bacillus*, *Lysobacter*, *Janthobacterium* and *Rhodoplanes)* have been reported from plant rhizospheres such as saffron (Ambardar and Vakhlu, 2013; Ambardar, 2014; Ambardar et al., 2014, 2016), green pepper (Liu et al., 2019), grapevine (Sacca et al., 2019) and oilseed rape (Gkarmiri et al., 2017), etc. However, only *Bacillus flexus* have been reported in *Limoneum sinense* and rice (Roy et al., 2020; Xiong et al., 2020) whereas *Lysobacter brunescens* and *Janthobacterium lividium* have not been reported from any plant so far.

In our previous studies, *Pseudomonas* was found dominant in the saffron rhizospheres by cloning based 16S rDNA metagenomic approach wherein full length 16S rDNA was amplified, TA cloned and sequenced using Sanger sequencing (Ambardar et al., 2014). Surprisingly, in the present study, *Pseudomonas* was only present in the saffron field from Alchibagh and absent in all other samples. The absence of *Pseudomonas* could be attributed to DNA extraction protocol and PCR primers and cloning approaches that were different from the present study. In a previous study, the metagenomic DNA was extracted manually using four protocols and pooled together and full length 16S rDNA primers followed by TA cloning were used to cataloge the diversity (Ambardar et al., 2014), whereas, in the present study, a commercial kit was used for DNA extraction followed by amplification of only V3-V4 region of 16S rDNA.

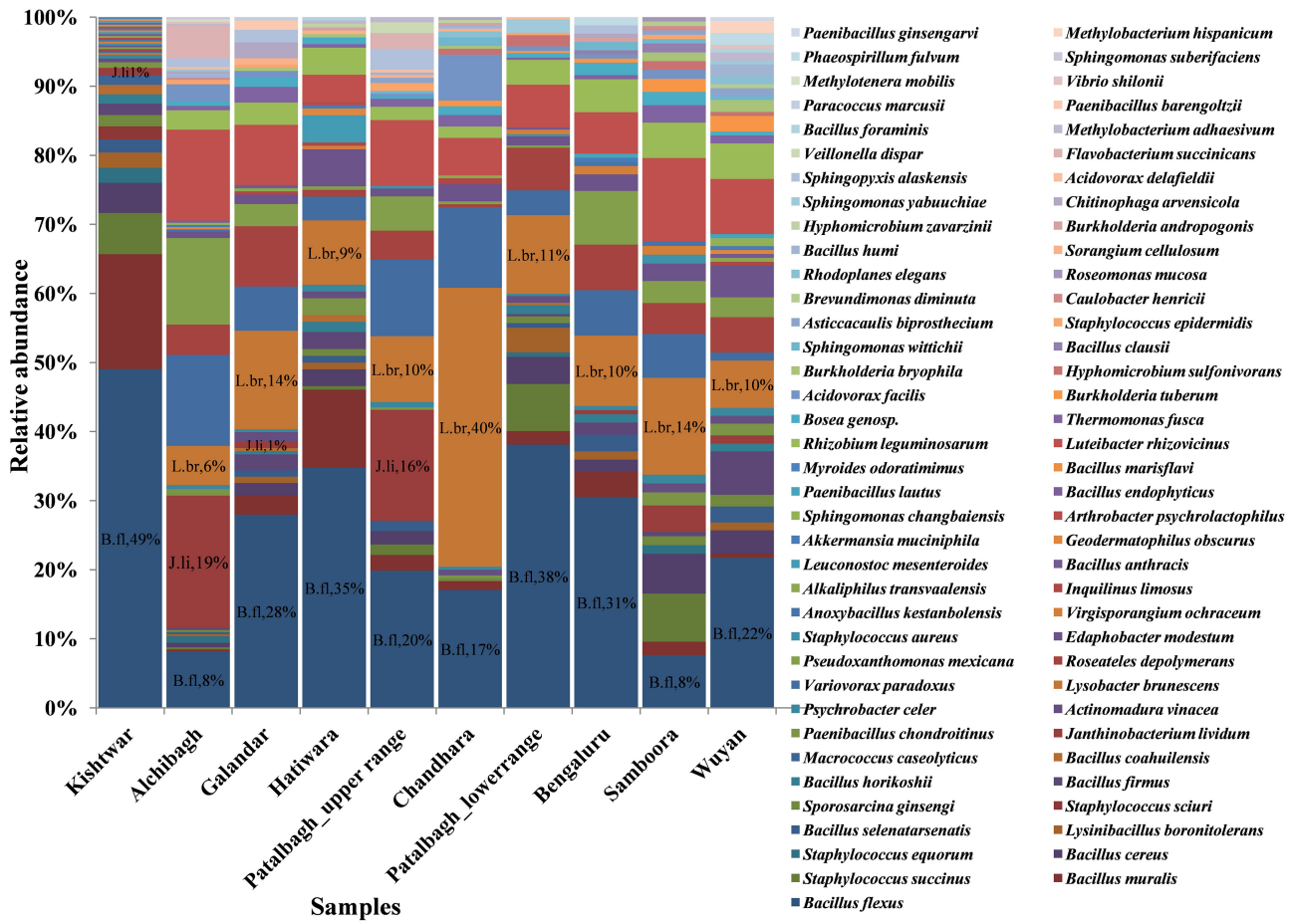

FIGURE 5 | Comparison of bacterial species in saffron rhizosphere from different geographical locations. The OTU cataloged up to species level were normalized to 2,000. A total of 73 species were cataloged from all the samples wherein *Bacillus flexus* (B. fl), *Lysobacter brunescens* (L. br), Janthiobacterium lividum (J. li) were found dominant.

In addition to this, the previous sampling was done in the year 2009 and present sampling was done after about 5 years in 2016, therefore climate change and floods in between in Kashmir can also be probable reasons, though this needs further investigation.

Various bacterial species, being reported in the present study, have been also reported by our group earlier by cultivation based method and were characterized *in-vitro* and *in-vivo* as well (Ambardar and Vakhlu, 2013; Ambardar et al., 2014; Kour et al., 2018; Bhagat et al., 2021). However, in the present study, six bacterial phyla, 212 bacterial genera and 70 bacterial species have been reported from the rhizosphere of saffron for the first time. Though, the rhizo-bacteria cataloged in the present study have been reported from other plants, but not reported from the cormosphere and rhizosphere saffron by our group earlier.

Core Rhizo-Bacteriome of Saffron

Comparative analysis of rhizo-bacteriome of saffron across different sites revealed the presence of various common bacterial phyla, genera and species representing the core rhizo-bacteriome of saffron. Core rhizo-bacteriome in saffron was represented by 53 bacterial genera and eight bacterial species namely *Bacillus flexus, Bacillus muralis, Edaphobacter modestum, Lysobacter brunescens, Pseudoxanthomonas mexicana, Psychrobacter celer, Roseateles depolymerans, Variovorax paradoxus.* These bacteria mostly belonged to 11 bacterial phyla namely *Proteobacteria, Chloroflexi, Firmicutes, Acidobacteria, Cyanobacteria, Gemmatimonadetes, Bacteroidetes, Nitrosiprae, Planctomycetes* and *Actionobacteria.* Core rhizo-bacteriome in saffron has been illustrated in the heat maps at genus & species level and with circos plot (**Figures 9–11**) respectively. Recently, core bacteriome associated with the corms of saffron were compared across different geographical locations i.e., Kashmir, Kishtwar and Morocco. 24 bacteria genera belonging to the phylum *Proteobacteria*, *Actinobacteria*, *Bacteriodetes* and *Verrumicrobia* made the core of cormo-bacteriome in saffron (Bhagat et al., 2021). Core rhizo-bacteriome in saffron represents 20.3% (53 out of 261) of the whole rhizo-bacteriome which was found comparatively less than the core cormo-bacteriome i.e., 32.8% of total cormo-bacteriome, however the number of bacterial genera constituting the core was more

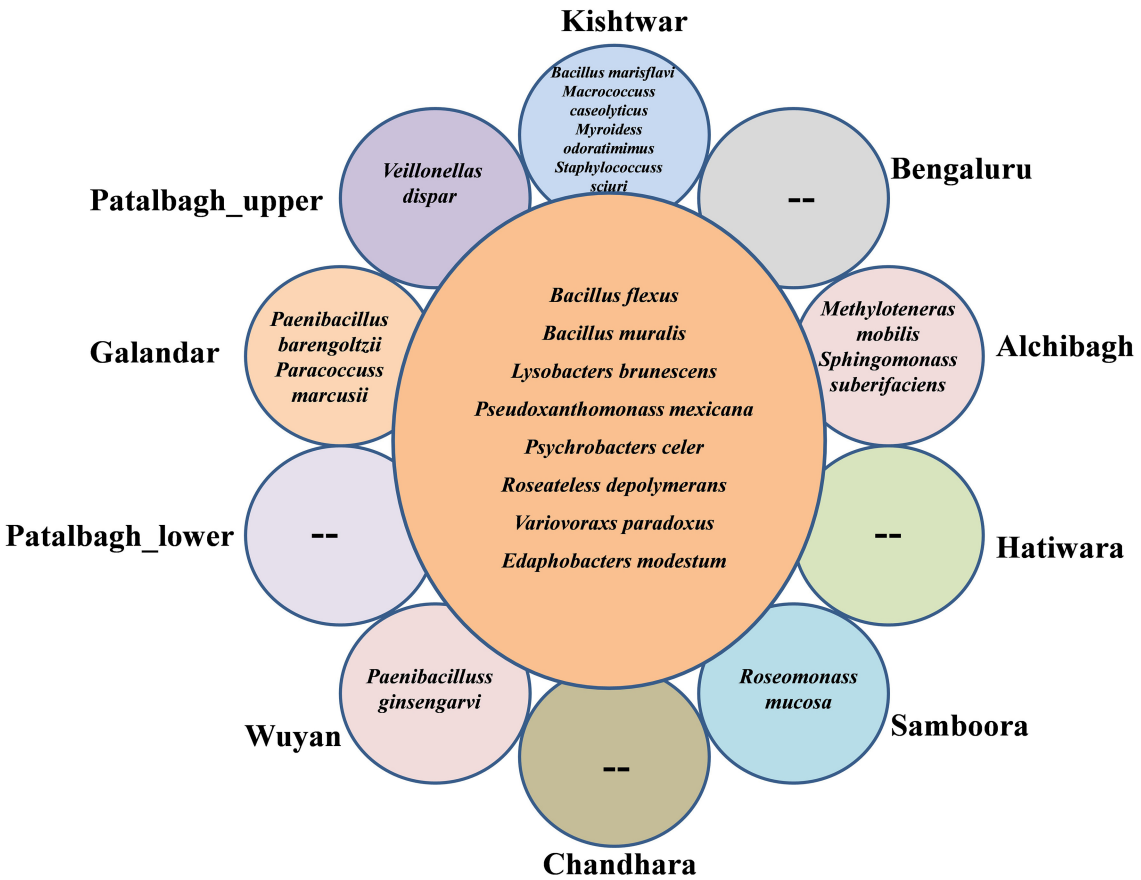

FIGURE 6 | Radical Venn diagram representing the number of unique and common species identified in 10 different locations. 8 common species were identified in 10 samples. Kishtwar had maximum number (4) of unique bacterial species followed by 2 bacterial species each in Alchibagh and Galandar whereas only 1 bacterial species in Patalbagh_upper range, Wuyan and Samboora. Bengaluru, Hatiwara, Chandhara and patalbagh_lower range did not have any unique bacterial species.

in rhizosphere (53 out of 261) as compared to cormosphere (24 out of 73), (Bhagat et al., 2021). Core microbiome of various plants have been studied such as wheat (Kuzniar et al., 2020), tomato (Cordero et al., 2020), *Gymnadenia conopsea* (Lin et al., 2020), coffee (Fulthorpe et al., 2020), switch grass (Grady et al., 2019), *Phaseolus vulgaris* (Pérez-Jaramillo et al., 2019), *Oryza sativa* (Eyre et al., 2019), Vineyards soil (Coller et al., 2019), *Dalbergia spruceana* (Skaltsas et al., 2019), and *Salvia miltiorrhiza* (Chen et al., 2018), etc. Core microbiome of 21 *Salvia miltiorrhiza* seeds represented 54% of the whole microbiome cataloged from seven different geographic origins (Chen et al., 2018). In the case of *Phaseolus vulgaris* rhizosphere, the core microbiome represented 25.9% of the total microbiome in native and agricultural soils (Pérez-Jaramillo et al., 2019). The core microbiome of 30 phylogenetically diverse angiosperm plants constitutes 40% of the whole microbiome (Fitzpatrick et al., 2018). Core rhizo-bacteriome in saffron was 20.3% of the total microbiome, which was comparatively less than *Salvia miltiorrhiza, Phaseolus vulgaris* and Angiosperm plants. In rice seeds, the core microbiome was enriched in *Rhizobium*, *Pantoea*, *Sphingomonas*, *Methylobacterium*, *Xanthomonas*, *Paenibacillus*, *Alternaria*, and *Occultifur* (Wang et al., 2020). Saffron's core rhizo-bacteriome was enriched with *Bacillus*, *Rhizobium*, *Sphingomonas*, *Agrobacterium*, and *Methylobacterium* which was similar to the core microbiome in rice.

TABLE 3 | Diversity indices were the maximum in Samboora representing maximum bacteria diversity and richness in rhizosphere of saffron growing in Samboora as compared to other samples.

Diversity indices →samples	Chao1	Simpson	Shannon	Phylogenetic diversity
Kishtwar	590	0.9698	5.9	15
Alchibagh	534	0.9854	6.3	13
Galandar	621	0.9776	6.1	18
Hatiwara	566	0.7246	3.7	10
Patalbagh_upper range	504	0.9856	6.3	15
Chandhara	628	0.9854	6.3	18
Patalbagh_lower range	467	0.9678	5.8	17
Samboora	2,050	0.9862	6.4	20
Bengaluru	978	0.8566	4.7	15

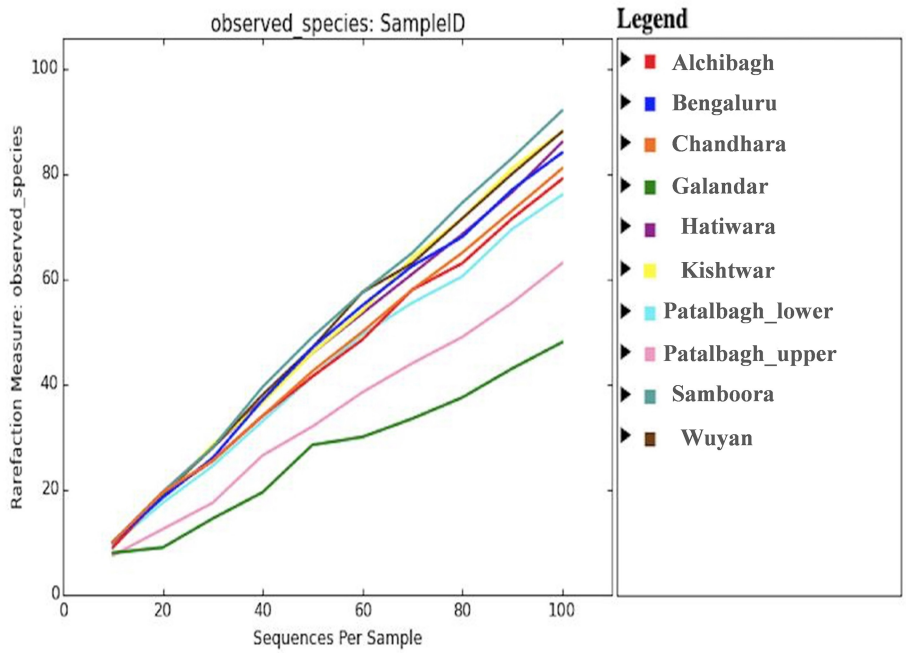

FIGURE 7 | Rarefaction curve representing the bacterial diversity across 10 samples where in Samboora is more diverse and Galandar is least diverse as represented by the number of species observed as per plot.

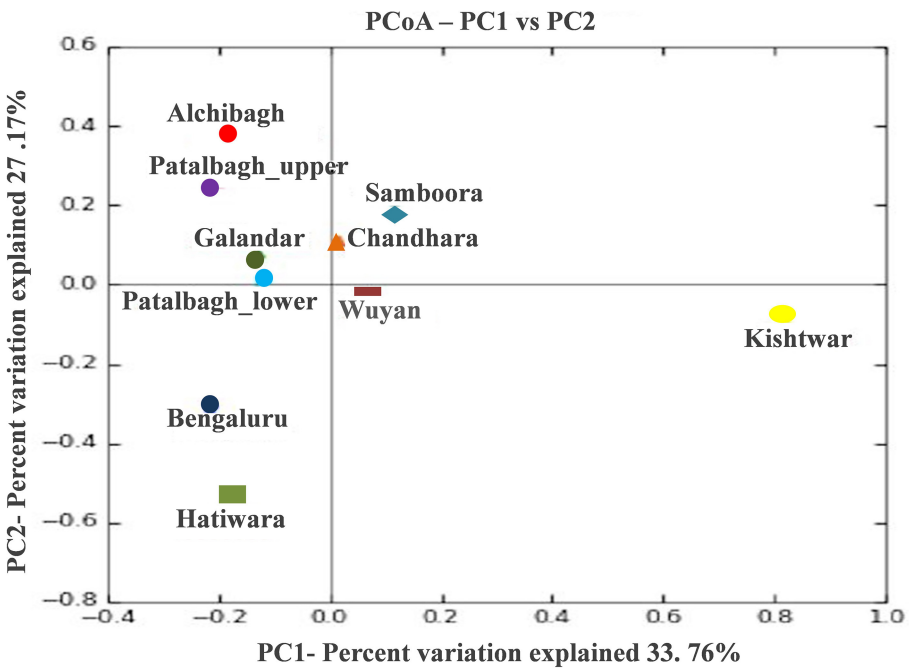

FIGURE 8 | PCOA plots representing the bacterial diversity of Kishtwar is significantly different from rest of sample as it doesn't cluster with rest of the sample.

Out of 53 core rhizosphere bacteria identified in the present study, 19 bacteria were also reported previously from the rhizosphere and cormosphere of saffron grown in Kashmir, by our group using culture dependent and independent approaches (Ambardar and Vakhlu, 2013; Ambardar, 2014; Ambardar et al., 2014, 2016; Kour et al., 2018; Bhagat et al.,

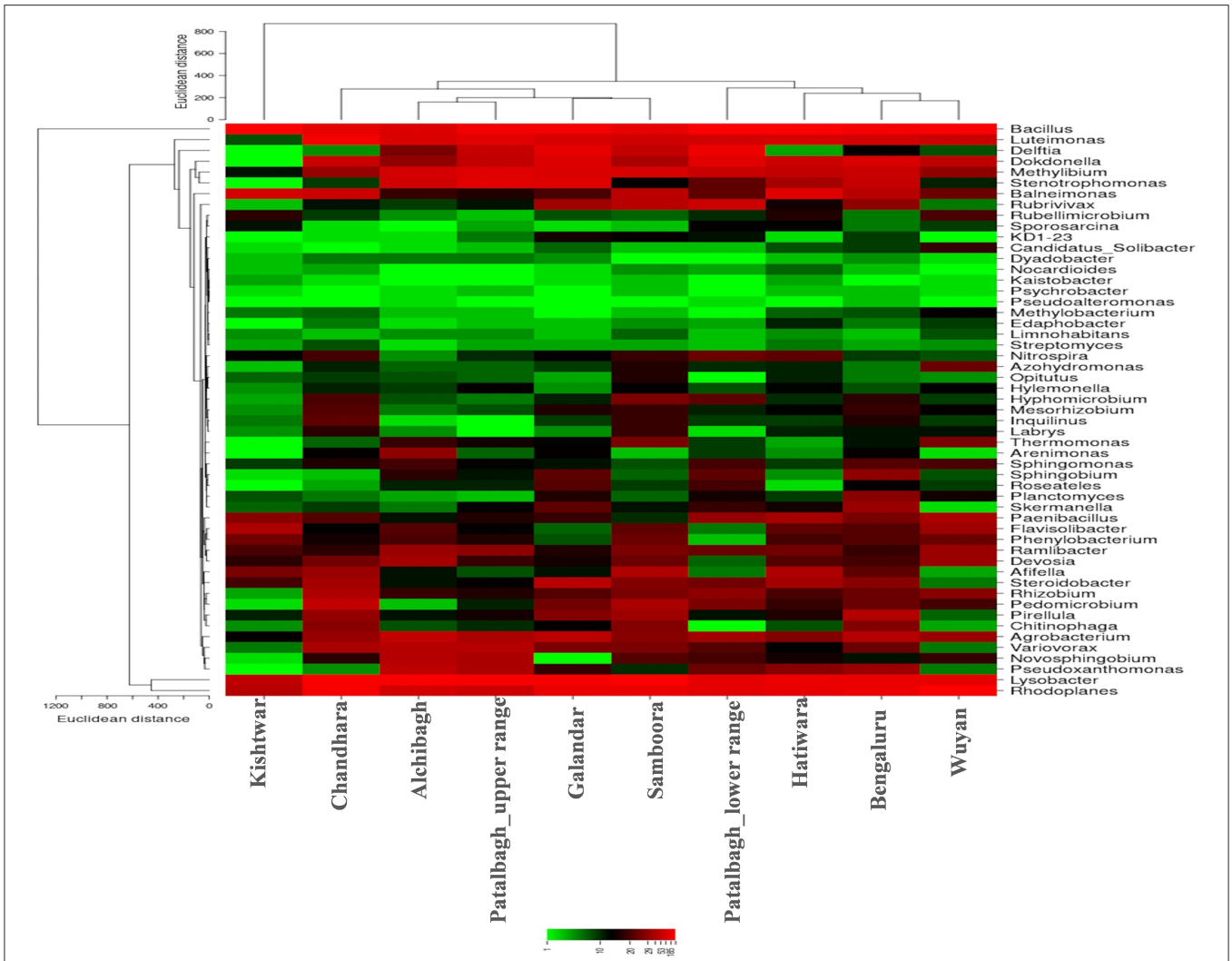

FIGURE 9 | Heat map representing the relative abundance of core genera (53) in the Saffron rhizosphere from 10 different samples. Saffron rhizo-bacteriome in Kishtwar was different than rest of the samples as represented by phylogenetic tree comparing all the samples.

2021). Interestingly, the core rhizo-bacteriome of saffron also harbored some common bacteria as in previously published core cormo-bacteriome. 11 bacterial genera namely *Agrobacterium, Chitinophaga, Dyadobacter, Mesorhizobium, Methylobacterium, Nocardioides, Opitutus, Rhizobium, Sphingomonas, Streptomyces* and *Varivorax* were common in both the underground organs of saffron that is corm (Bhagat et al., 2021) and roots. In addition, *Agrobacterium, Bacillus, Delftia, Rhizobium,* and *Variovorax* have also been reported from rhizosphere and cormosphere of saffron, grown in Morocco using cultivation dependent methods (Chamkhi et al., 2018). Though we could not capture the genera reported in Morocco cormo/rhizosphere by earlier used techniques but with the present technique they were captured and found to be present in Kashmir. However, 34 bacterial genera of the core rhizo-bacteriome of saffron grown in India were reported for the first time in the saffron, in the present study.

Eight core bacterial species identified in the present study was also reported from other plants such as *Bacillus flexus* from rice (Roy et al., 2020), *Bacillus muralis* from peanut (Jiang et al., 2016), *Edaphobacter modestum* from *Magnolia grandiflora* (Stone and Jackson, 2016), *Pseudoxanthomonas mexicana* from maize and peanut rhizosphere (Geng et al., 2018; Youseif, 2018), *Roseateles depolymerans* from ginger (Chen et al., 2014), *Variovorax paradoxus* from mustard roots (Belimov et al., 2005). However, *Lysobacter brunescens* and *Psychrobacter celer* has not been reported from any plant so far.

Plant Growth Promoting Bacteria From Saffron Rhizo-Bacteriome

64.1% of total core rhizo-bacteriome i.e., 34 out of 53 bacteria has been reported as PGPBs from saffron and other plants (**Supplementary Table 1**). While exploring the core rhizo-bacteriome for PGPR, it was found that 21 bacteria have been

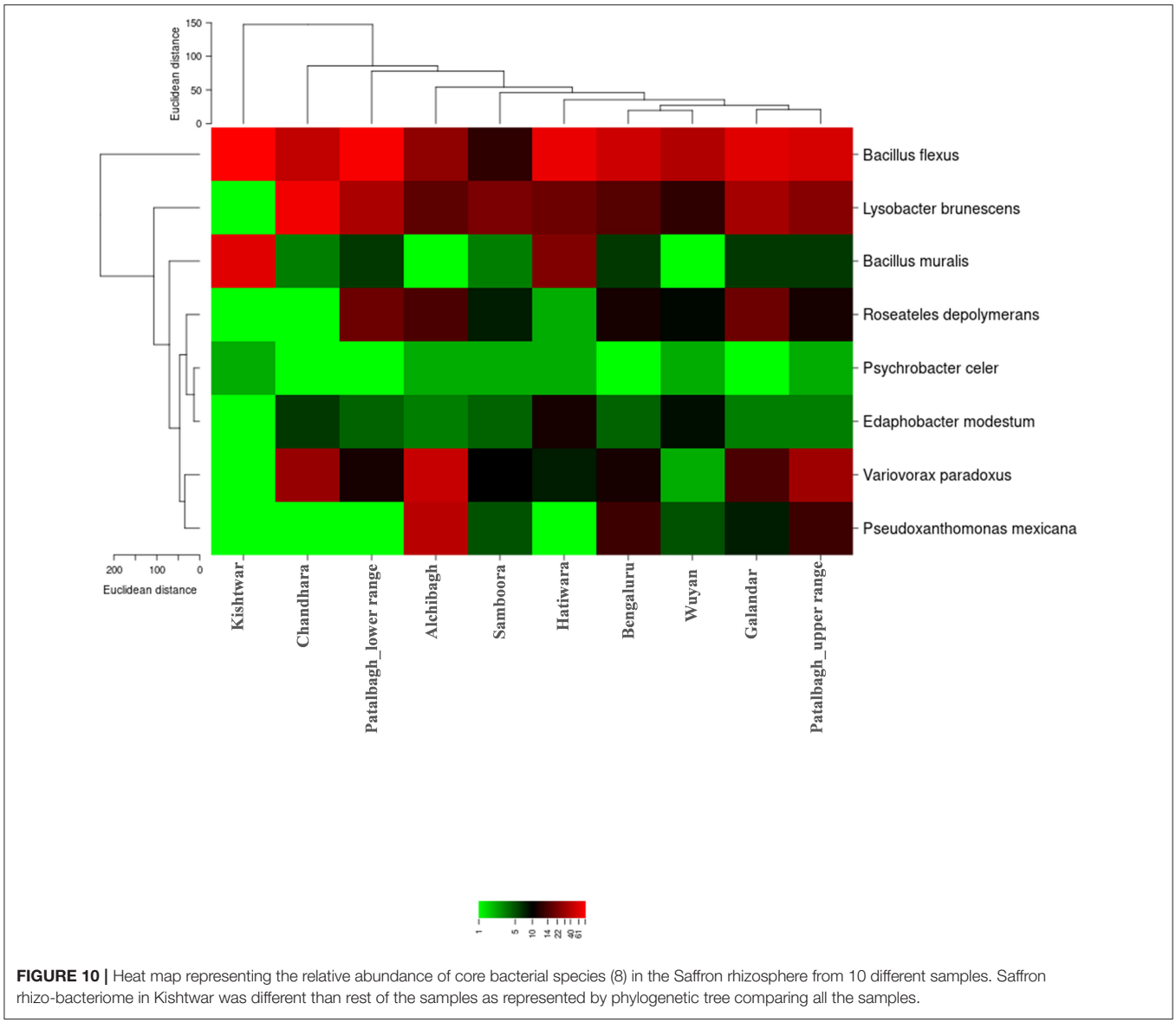

FIGURE 10 | Heat map representing the relative abundance of core bacterial species (8) in the Saffron rhizosphere from 10 different samples. Saffron rhizo-bacteriome in Kishtwar was different than rest of the samples as represented by phylogenetic tree comparing all the samples.

reported as PGPB for the first time from saffron in the present study as previously used methods failed to capture them. The core rhizo-bacteria that are being reported as PGPRs for the first in saffron are *Azospirillum, Agromyces, Agrobacterium, Bradyrhizobium, Burkholderia, Dokdonella, Edaphobacter, Flavobacterium, Flavisolibacter, Frankia, Leuconostoc, Luteimonas, Lysobacter, Phenylobacterium, Pseudoxanthomonas, Mesorhizobium, Nocardia, Novosphingobium, Rhizobium, Rubellimicrobium, Variovorax* etc.

In addition to core rhizo-bacteriome, saffron rhizo-bacteriomes of each location were enriched with some specific bacteria that were unique to that particular location. An inventory of unique PGPBs of all these bacteria across different locations was made based on the previous reports of PGPBs from saffron and other plants. The location specific unique PGPBs details have been summarized in **Supplementary Table 2**. Wuyan had the maximum number of unique genera i.e., 19 out of which 5 are reported as PGPB i.e., *Actinoplanes, Beijerinckia, Desulfotomaculum Dethiobacter* and *Ureibacillus*. In other locations unique number of genera was 4 in Chandhara, 2 in Samboora, 1 in Hatiwara and 1 in patalbagh_upper range but none of them have been reported as PGPB. Kishtwar had six unique genera and three genera namely *Desemzia, Macrococcus* and *Myroides* have been reported as PGPB (**Supplementary Table 2**). In addition, Alchibagh had 10 unique genera and four have been reported as PGPB. Galandar had six unique genera and three PGPB reported, Patalbagh_upper had two and Bengaluru has one unique genera, both locations have one PGPB reported (**Supplementary Table 2**).

In the present study, *Bacillus flexus, Lysobacter brunescens* and *Janthiobacterium lividium* were the dominant bacterial species identified in saffron rhizo-bacteriome. *Bacillus* genus

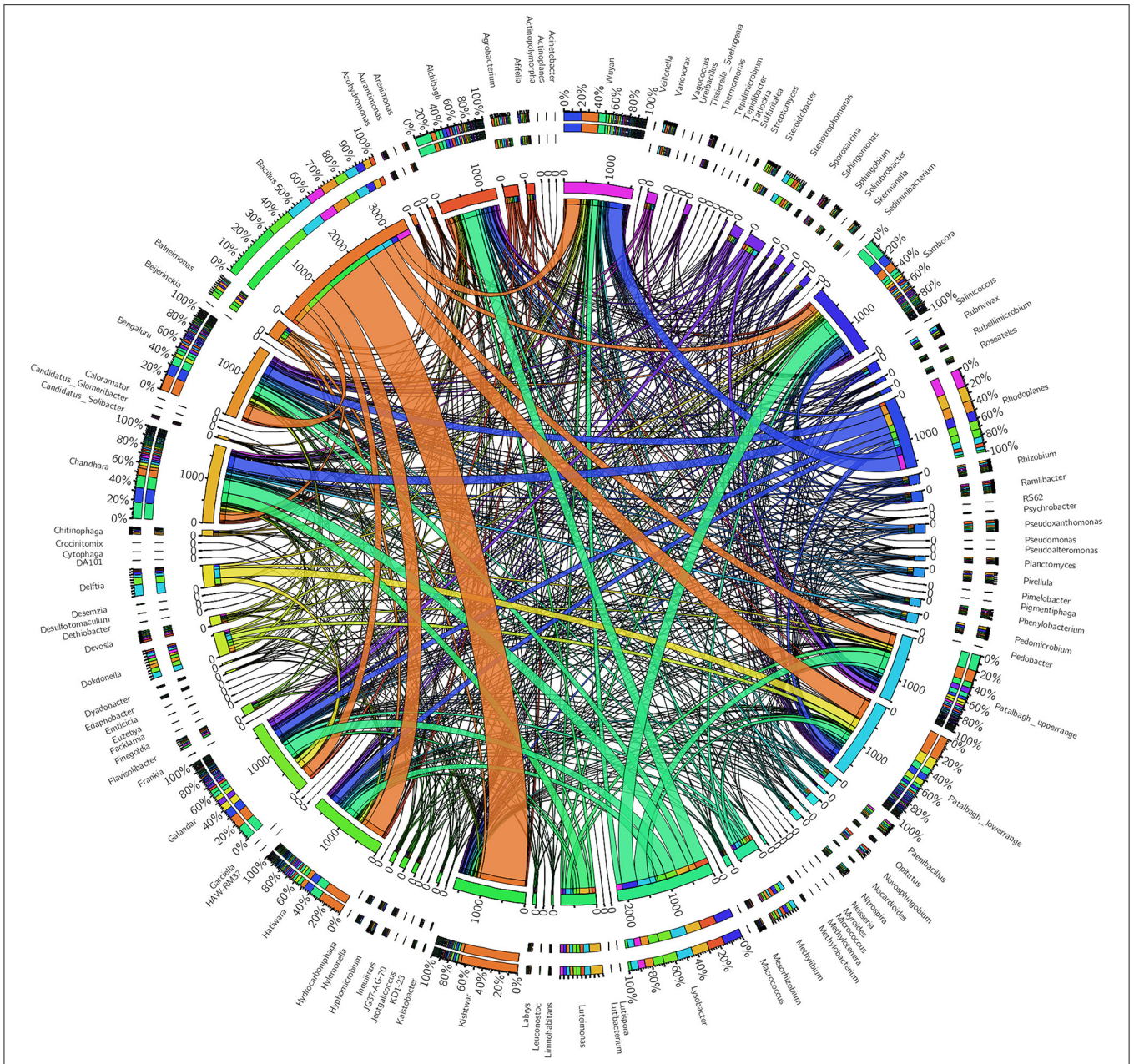

FIGURE 11 | Circos plot representing the core rhizo-bacteriome and unique rhizobacteria in saffron grown across different geographical locations. Central line (ribbons) represents the abundance of each genus and connects genera to samples. The outer three rings are the stacked bars that represent the relative contribution of particular genera in every sample.

dominant in the rhizosphere microbiome is predominantly used as plant growth promoting bacteria, due to their ability to colonize the roots rapidly, competitive colonization potential, production of various phytohormone and conversion of complex nutrients such as phosphorous and nitrogen into simple absorbable forms (Aloo et al., 2019; Enebe and Babalola, 2019; Hashem et al., 2019; Kashyap et al., 2019). *Bacillus flexus* KLBMP 4941 in *Limoneum sinense* (Xiong et al., 2020) and *B. flexus* U8 in rice (Roy et al., 2020) have been reported as PGPR. However, *Lysobacter brunescens* and *Janthobacterium lividium* have not been reported so far from any plant.

In addition to *Bacillus flexus* other bacterial species of core rhizo-bacteriome reported as PGPB are *Bacillus muralis, Pseudoxanthomonas mexicana, Roseateles depolymerans* and *Variovorax paradoxus* (**Supplementary Table 3**). *Bacillus muralis* strain HS4 has been reported as PGPR in peanut (Jiang et al., 2016). *Pseudoxanthomonas Mexicana* has been reported as PGPR from rhizosphere of maize and peanut (Geng et al., 2018; Youseif, 2018). *Roseateles depolymerans* has been isolated as an endophyte

from the seedling stage of ginger and reported to have PGPR properties (Chen et al., 2014) and *Variovorax paradoxus* has been reported as PGPR from Indian mustard roots (Belimov et al., 2005). *Lysobacter brunescens, Edaphobacter modestum* and *Psychrobacter celer* have not been reported as PGPB so far (**Supplementary Table 3**).

Among different locations, Kishtwar had four unique species (*Bacillus marisflavi, Macrococcus caseolyticus, Myroides odoratimimus* and *Staphylococcus scuiri*) and all are reported as PGPB in different plant. In addition, Galandar from Kashmir had only one PGPB (*Paracoccus marcusii*) reported. All other locations have no unique species reported PGPB so far (**Supplementary Table 4**).

Altogether, 110 bacterial genera (out of total 261) and 21 bacterial species (out of total 73), cataloged in present study, have also been reported to be plant growth promoting bacteria from saffron and other plants (**Supplementary Table 5**). Plant growth promoting bacterial species cataloged in saffron are *Acidovorax facilis*, *Bacillus cereus*, *Bacillus firmus*, *Bacillus flexus*, *Bacillus horikoshii*, *Bacillus marisflavi*, *Bacillus muralis*, *Brevundimonas diminuta*, *Burkholderia bryophila*, *Burkholderia tuberum*, *Luteibacter rhizovicinus*, *Macrococcus caseolyticus*, *Myroides odoratimimus*, *Paracoccus marcusii*, *Pseudoxanthomonas mexicana*, *Rhizobium leguminosarum*, *Roseateles depolymerans, Staphylococcuss epidermidis*, *Staphylococcus equorum*, *Staphylococcus sciuri*, *Staphylococcus succinus* and *Variovorax paradoxus.*

On comparison of PGPB from each sample, Wuyan had maximum number of PGPBs (76 out of 154) followed by Galandar (75 out of 132), Alchibagh (73 out of 147), Chandhara (72 out of 143), Hatiwara (73 out of 140), Kishtwar (66 out of 139), Bengaluru (63 out of 143), Samboora (62 out of 124), Patalbagh_lower (61 out of 111) whereas Patalbagh_upper (60 out of 122) with the minimum number of PGPBs (**Supplementary Table 6**). The highest number of PGPB in the Wuyan sample could be correlated to the yield as the production in Wuyan is two times as compared to other fields. In the previous report by our group, PGP *Bacillus amyloliquefaciens* W2 for growth promotion of saffron and corm rot inhibition were isolated from the Wuyan by cultivation based method (Gupta and Vakhlu, 2015). However, in present study a well-reported PGPR *Rhizobium leguminosarum* and *Luteibacter rhizovicinus* was relatively abundant in all the nine locations, including Wuyan and completely absent in Kishtwar. *Rhizobium leguminosarum* possess various PGP characteristics such as phosphorous solubilization, siderophore, IAA, HCN production and nitrogen fixation which enhance the growth of host plants in pea plant, lettuce and carrot (Mishra et al., 2009; Tank and Saraf, 2010; Flores-Félix et al., 2013; Gopalakrishnan et al., 2015). *Luteibacter rhizovicinus* MIMR1 have been reported as plant growth promoting bacteria that promotes root development in barley (Guglielmetti et al., 2013). Absence of *Rhizobium leguminosarum* and *Luteibacter rhizovicinus* may be attributed to the low production status of Kishtwar as compared to Kashmir, but the real confirmation will come only after their evaluation *in-vivo*. The presence of unique location specific PGPB at places other than Wuyan, with a lower yield than Wuyan indicates that the potential of PGP bacteria varies and maybe all the PGPB are not as effective as that of Wuyan fields. The PGPB cataloge in the present and previous studies in saffron by metagenomic approaches will be captured by culture based technique and evaluated for PGP potential in the future.

CONCLUSION

The present study investigated the variation in rhizo-bacteriome of saffron grown across different locations i.e., Kashmir, Kishtwar and Bengaluru in India. The rhizo-bacteriome of saffron grown in Kishtwar was found significantly different from the saffron grown in Kashmir and Bengaluru. Interestingly, rhizo-bacteriome in saffron seems to be "plant driven" though in our earlier report we have suggested the bacteriome of corm is location specific. This need to be further confirmed as for cormo-bacteriome corms were directly taken from the fields and analyzed whereas in the present study, corms from Kashmir were grown in the pots with garden soil from Bengaluru and analyzed. Despite growing in non-native soil, the similarity of Bengaluru rhizo-bacteriome to Kashmir rhizo-bacteriome indicates that it is plant driven. This needs to be studied extensively further by analyzing rhizo-bacteriome of saffron roots developed from corms from Kishtwar and Kashmir, cultivated in Bengaluru in non-native soil. Further, the total PGPBs and unique PGPBs were highest in the Wuyan field that has maximum production indicating, a correlation between number of PGPBs and production. However, unique and common PGPB were cataloged across geographical locations in the present study, in the future they will be cultivated by media engineering (for those that are difficult to cultivate) and evaluated *in-vivo* to estimate their actual efficacy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA687631.

AUTHOR CONTRIBUTIONS

SA, JV, and MG have designed the experiments. SA conducted the experiments, analyzed the sequencing data, and experiment regarding growth of saffron plants in Bengaluru. SA and NB wrote the manuscript, analyzed the taxonomy data. MG conceptualized and supervised the study. JV guided in collection of samples from Kashmir and Kishtwar. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.644230/full#supplementary-material

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