Molecular Biology Reports (2021) 48:2389–2397
https://doi.org/10.1007/s11033-021-06272-6

ORIGINAL ARTICLE

High throughput sequencing study of foliose lichen-associated bacterial communities from India

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Received: 29 July 2020 / Accepted: 6 March 2021 / Published online: 18 March 2021

Abstract

Lichens comprise highly diverse and complex microbial communities, the majority consisting of mycobiont, photobiont, Basidiomycetes yeast and bacteriobiont (internal bacterial communities). In this study, bacterial diversity of foliose lichen was reported. Next generation sequence (NGS) such as Illumina Sequencing (150*2) of 16S rRNA (V3 and V6 region) was used to delineate the bacterial communities associated with five foliose lichen samples. Bacterial sequences obtained from lichen samples suggested that, they harboured bacterial community with variable relative abundances. Among all bacterial communities, *Alphaproteobacteria* were dominant in all the tested lichen samples. The principal coordinate analysis, Venn and bar chart showed significant microbial changes between the different useful bacterial lineages across the lichens. The relative abundance of dominant and rare bacterial species found were varied, diverse, distinct and unique in each lichen. The *Proteobacteria* 48.19%, *Actinobacteria* 25.70%, *Bacteroidetes* 8.53%, *Acidobacteria* 9.36% and *Chloroflexi* 0.83% were predominant in all tested lichens. The present empirical study enhances the confirmed knowledge of bacterial diversity inevitably associated with lichens and is the first report on lichenized bacterial diversity and perhaps their potential possible role in lichen symbiosis in addition to phycobiont and mycobiont.

Keywords Alpha diversity · Beta diversity · Microbial diversity · Principal coordinate analysis (PCoA) · Rarefaction curves

Introduction

Lichens are complex symbiotic associations of mycobiont and photoautotrophs (algae and/or cyanobacteria). Appropriate photoautotrophs are an essential factor for the fungus to develop the organized characteristic phenotypes [1–4]. Sometimes, this symbiotic integrity is scarcely affected by fungal parasites [5]. Even though, lichens possess a distinct variety of secondary metabolites, which exhibit antifungal and antibacterial activity [2, 6].

The lichen microecology consisting of fungi, algae, bacteria and yeast along with this other eukaryotes such as of large myxomycete plasmodial amoeba, heterotrophic nanoflagellates, naked/testate amoeba and several microfaunal communities such as rhizopods, ciliates, flagellates, nematodes, rotifers, tardigrades and mites that were also reported [36–41]. The presence of eukaryotes has been confirmed with the modern pyrosequencing of 18S rRNA studies and existence of Blastocladiomycota and Chytridiomycota fungal isolates for first time [37]. The lichen thallus is therefore defined as a bio-network of prokaryotes and eukaryotes; that is, a miniature ecosystem [36, 40] in which bacterial contribution in this bio network needs to be elucidated.

The microbial community composition difference in lichens mainly depends on the availability of fixed carbon and nitrogen, as well as secondary metabolites produced by mycobionts with antimicrobial activity which induce elective pressure on lichenized bacteria [30]. The secondary metabolites of lichens may not adversely affect the growth of bacterial communities, but the sensitive bacteria would not survive or exist. Reports on relationship and effect of secondary metabolites or lichen chemistry on bacterial communities colonizing lichens are scarce. The present study designed to predict the effect of secondary metabolites on lichenised bacteria.

Lichens possess a huge diversity of bacterial species such as *Alphaproteobacteria* in biofilm-like manner that are found on fungal hyphae has been confirmed through advanced

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FISH-CLSM studies [7]. In addition, some opportunistic bacteria from surrounding soil were also found in lichen and culture-dependent methods have been typically employed to isolate and characterize the heterotrophic bacteria present in lichens [1, 8, 9]. Culture-dependent methods are of limited value to determine the actual bacterial diversity residing in lichen thallus [1], hence culture-independent methods like internal transcribed RFLP, pyrosequencing, FISH-CLSM, T-RFLP with pyrosequencing studies and next generation sequencing (NGS) methods have been used to explore both the cultivable and non-cultivable bacterial diversity present in the lichen ecosystem [1, 10, 11].

The utilization of cutting edge NGS technology has been found to be the most pertinent tool for thorough examination compared to conventional Sanger sequencing and 454 pyrosequencing [12, 13]. Advancement in Illumina sequencing technology adequately provides an excellent view of microbial composition and provided detailed insights of both dominant and rare microbial communities [14, 15]. A detailed study on 16S rRNA gene segment analysis revealed that hypervariable regions exhibit considerable sequence diversity among bacterial spp. No single region can differentiate all bacteria; therefore, a combination of genome segments are more useful to reveal the diversity up to species level. V2 and V3 regions are useful to study large bacterial diversity up to the genus level; and the V6 region is noteworthy to study the diversified bacterial population differentiation. V4, V5, V7 and V8 were less useful to target to genus or species specific probes [35]. Therefore in the present study, the bacterial diversity inseparably associated with foliose lichen thalli was studied using Illumina NGS technology (16S rRNA hypervariable regions [V3 and V6]). The main objective is to determine the lichen-associated bacterial communities that are either symbiotic partners of lichen species or normal residents; and also dominant and rare species relative abundance and their variation in various thalli of lichen samples. Illumina reads have processed, and only high-quality sequences were selected as OTUs (Operational Taxonomic Units) for taxonomic grouping of bacteria in the lichen samples. Based on the apparent OTUs abundance, bacterial diversity of lichen was summarized through the use of Principal coordinate analysis (PCoA) plots, Heatmaps, Stacked bar charts, Krona Charts and Pie Charts were assessed.

Materials and methods

Site of sampling and DNA extraction

Five different foliose lichen samples were collected from historical rock at different regions of Chitradurga Fort located in Karnataka, India (14.2152°N; 76.3953°E). Lichen samples were collected using sterile blade and forceps and placed in sterile polythene bags; and the samples were labelled as CL-01, CL-02, CL03, CL-04 and CL-05. All the samples were transported to the laboratory in an ice box within five hours and stored at −80 °C for further processing. The lichens were identified based on their morphology, anatomy and chemistry, using micro and macrolichen keys [42, 43].

Lichen samples were washed with ultra-pure laboratory grade (Milli-Q) water to remove the dirt and debris. Lichen tissues were frozen in liquid nitrogen, ~ 2 cm^2 central thalli were cut and homogenized by the standard bead-beat method using the Tommy Micro Smash MS_100 (Tommy Medico Limited, Japan). The standard CTAB method with proteinase k digestion was used to extract the DNA from the lichen samples [10].

Amplicon library construction

Amplicon library development was carried out as per the guidelines on the Nextera XT Index kit V2 (Illumina, USA) with primers 341F, 5^1-CCTACGGGAGGCAGCAG-3^1 and 518R, 5^1-ATTACCGCGGCTGCTGG-3^1. Then, amplicons were verified on 1.2% agarose gel. Libraries were thereafter normalized, pooled and sequenced [12].

Preprocessing

The Illumina paired end reads (150*2) were demultiplexed using the bcl2fastq tool. The paired-end reads were quality checked using FastQC2. Raw reads with primer and high-quality bases were selected and stitched with the FastQ-join tool [16, 17], and the merged sequences were clustered for the use of the UCLUST method [18]. Preprocessing contained the information corresponding to the raw reads, then a wide variety of identified rRNA sequences and the OTUs were picked from each sample.

Total sequences (both V3 and V6 region) of 43, 82, 008 pair reads were derived from 5 lichen samples with good reads; like 70% of the bases with Phred value greater than 20 was retained, and the rest were removed. OTUs (out of 22,382,008 sequences only 17,170 good quality OTUs) were picked from each sample for further analysis to reveal the quantitative insights of bacterial composition in selected lichen samples.

Statistical data analysis

The taxonomy of the preprocessed cluster was assigned based on ≥ 97% sequence similarity with the curated chimera free16S rRNA database (Greengenes5 v 13.8) [19]. Based on the abundance of sequences, taxonomic assignment from phylum to species level was compiled and assembled. Alpha

diversity was expressed by using the Shannon-Weiner index, Simpson index and Chao1 index. The Shannon-Weiner index Chao 1 index represent species richness in samples. While, the Simpson index is used to measure the degree of concentration of species; where 0 represents infinite diversity as well as 1 represents no diversity. Further, beta diversity was expressed using two indexes (weighted unifrac method and unweighted unifrac method), which were plotted with R package NMF. Heatmap and rarefaction curves were plotted using MG-RAST. Later, the data was clustered row and column-wise with R package NMF [10, 20, 44]. Quantitative insights of bacteria in lichen samples were expressed with Krona charts [21]. Further, stacked bar plots and pie-charts were generated by QIIME pipeline; Venn diagrams were plotted by interactive Venn online version [22].

Lichens 16S rRNA V3 and V6 region illumina sequence accession number

Paired end Illumina sequence of the five lichenized bacterial samples (CL-01 to CL-05) were submitted to the NCBI Sequence Read Archive (SRA).

Results

Identification of lichen samples

Lichen samples were identified as CL-01- *Pyxine sorediata*, CL-02- *Parmotrema tinctorum*, CL-03- *Pyxine reticulata*, CL-04- *Parmotrema praesorediosum*, CL-05- *Pyxine cocoes*.

Generation of illumina reads and selection of high-quality reads

Total 1,995,354 16S rRNA V3 region paired-end reads generated were generated; among them, only 10,141 OTU's (Operational taxonomic units) were picked for analysis, and in the case of 16S rRNA V6 region, out of 2386, 654 reads, only 7029 OTU's were picked for further taxonomic analysis (Supplementary Tables S1 and S2).

The alpha and beta diversity of bacteria associated with lichens

Rarefaction curves of all the five lichen samples (both 16S rRNA V3 and V6 region) were observed to reach a plateau; thereby confirming that sampling as well as sequencing coverage was stable and good (Fig. 1a, b). An analysis of the alpha-diversity index of 16S rRNA V3, V6 region of the bacteria in the lichen samples revealed that *Parmotrema tinctorum* has maximum bacterial diversity index, followed by P*yxine sorediata*, *Pyxine reticulata*, P*armotrema praesorediosum* and *Pyxine cocoes*. Alpha diversity of lichens V3 region's Shannon-Weiner index showed *Parmotrema tinctorum* had more diversified bacterial species, followed by *Pyxine sorediata* and *Pyxine reticulata*; while CL-04-*Parmotrema praesorediosum* and *Pyxine cocoes* showed an average similar diversified bacterial species. However, Alpha diversity of lichens V6 region's Shannon-Weiner index showed *Pyxine sorediata* has more diversified bacterial species followed by *Parmotrema tinctorum* and *Pyxine reticulata,* while *Parmotrema praesorediosum* and *Pyxine cocoes* showed an average similar diversified bacterial species that were harbored within them. Further, Simpson and Chao 1 indexes showed more or less equal distribution of bacterial species in all tested 5 lichen samples (Supplementary Tables S3 and S4).

Beta diversity was measured by the weighted and unweighted unifrac methods. In the 16S rRNA V3 region weighted unifrac study, significantly high bacterial diversity was observed in tested lichen samples as appeared in the first axis (PC1) of the PCoA. In that analysis, the *Pyxine reticulata* was observed in PC3, while the rest were found in PC1 (Supplementary Fig. S1). A PCoA plot of the V3 region obtained with the unweighted unifrac method showed that, besides *Parmotrema tinctorum*, the other 4 samples were observed in PC1 with substantial variation. However, only *Parmotrema tinctorum* observed in PC3 was less dissimilar with other lichen samples (Supplementary Fig. S2).

Further, the 16S rRNA V6 regions weighted unifrac method analysis showed that all the 5 lichen species were found in PC1; however, there was distinct variation in bacterial diversity among the samples observed (Supplementary Fig. S3) In another case, the 16S rRNA V6 region unweighted unifrac PCoA analysis showed CL- 04 in PC2; and the remaining samples were found in PC1, thus indicating the dissimilarity of bacterial diversity of lichen samples (Supplementary 4).

Taxonomic assignments of bacterial communities at phylum level

Taxonomic assignment of 16S rRNA V3 and V6 hypervariable region sequences were performed with QIIME and MG-RAST pipelines. Phylum level analysis of 16S rRNA V3 region of all 5 lichen samples showed a varied distribution of *Proteobacteria, Bacteroidetes, Acidobacteria, Actinobacteria, Gemmatimonadetes, TM7, Chloroflexi, Cyanobacteria.*

All the lichen samples were dominated with *Proteobacteria, Actinobacteria, Acidobacteria, Cyanobacteria* and *Bacteroidetes* phyla. The 16S rRNA V3 region study showed that an average frequency of dominant phyla among all five lichen samples followed by increasing patterns: *Proteobacteria* 48.19% > *Actinobacteria* 25.70% >*Bacteroidetes* 8.53%

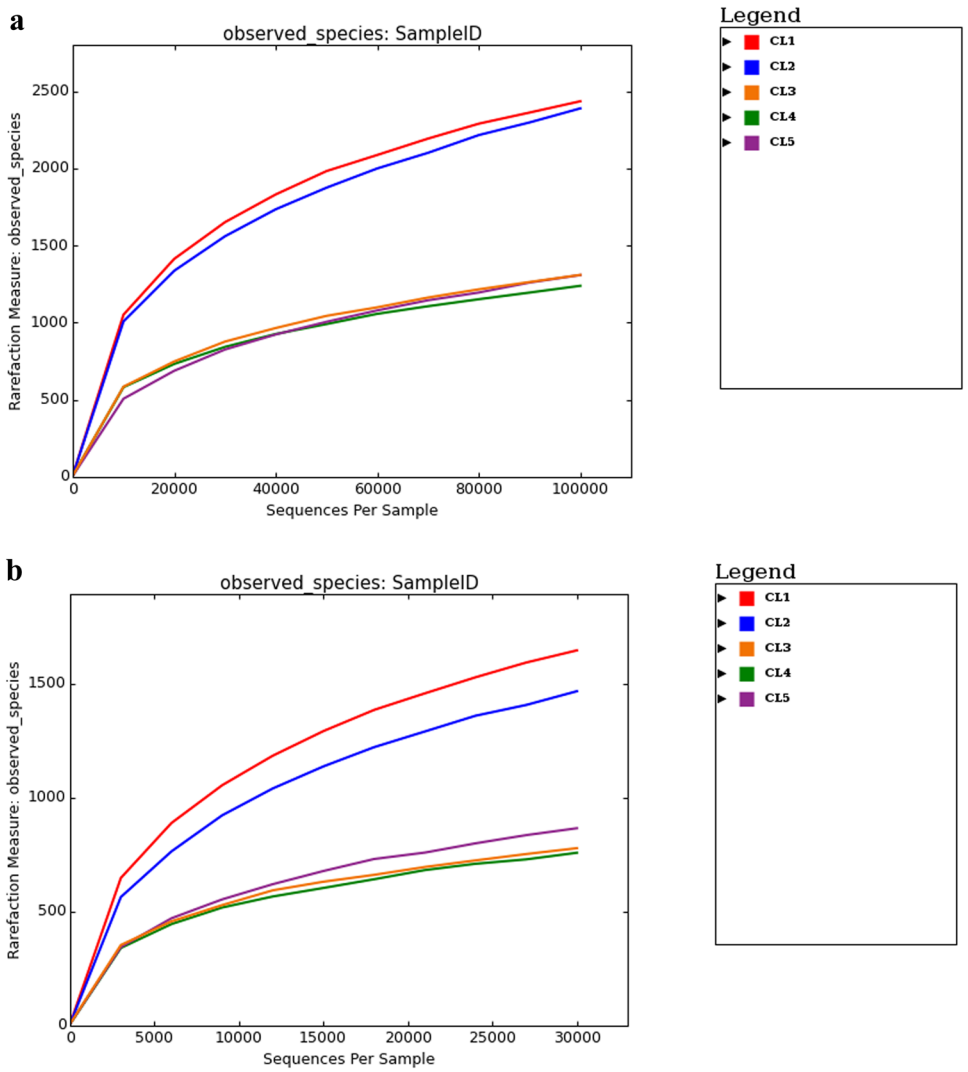

Fig. 1 **a** Rarefaction plot of 5 samples for V3 region. **b** Rarefaction plot of 5 samples for V6 region

>*Acidobacteria* 9.36% > *Chloroflexi* 0.83%. Relative abundance of bacteria in lichens was expressed in stacked bar plots and heat maps, and the bacterial phyla were more or less similar in five lichens (Fig. 2a and b).

Phylum level analysis of 16S rRNA V6 region of lichens showed the presence of bacterial taxa *Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria, Verrucomicrobia, Cyanobacteria, FBP, Planctomycetes, Firmicutes, Gemmatimonadetes, TM7, Thermi, Armatimonadetes, Nitrospirae*; but their frequency differed. The average frequency of dominant phyla found in five lichens was as follows: *Proteobacteria* 32.54%, *Actinobacteria* 36.44%, *Bacteroidetes* 20.16%, *Acidobacteria* 5.27% and *Verrucomicrobia* 1.32%. This result has also been complemented by stacked bar plot and heat map analysis (Fig. 3a and b).

Lichen-associated bacterial diversity up to species level was visualized by a krona chart, which allows exploration of relative abundances and confidences within the complex hierarchies of metagenome classifications for V3 region (Supplementary Figs. S5–S9) and for V6 (Supplementary Figs. S10-S14).

The percent distribution of predominate 20 bacterial species across the 5 lichens at phylum level was represented by Pie charts. The data of both V3 region (Supplementary Figs. S15-S19) and V6 region (Supplementary Figs. S20–S24) confirmed the predominance of *Actinobacteria, Acidobacteria,* phyla., were equally distributed among the 5 lichens. Furthermore, a metagenomic dataset has been made available under MG-RAST ID mgm4731953. 3 (CL1), mgm4731955. 3 and mgm4731956. 3 (CL2), mgm4731958. 3 (CL3), mgm4731960. 3 (CL4) and mgm4731961. 3 (CL5). Paired end Illumina sequences of all the five lichens (CL-01 to CL-05) were submitted to the NCBI Sequence Read Archive (SRA) under accession number SRS2063867.

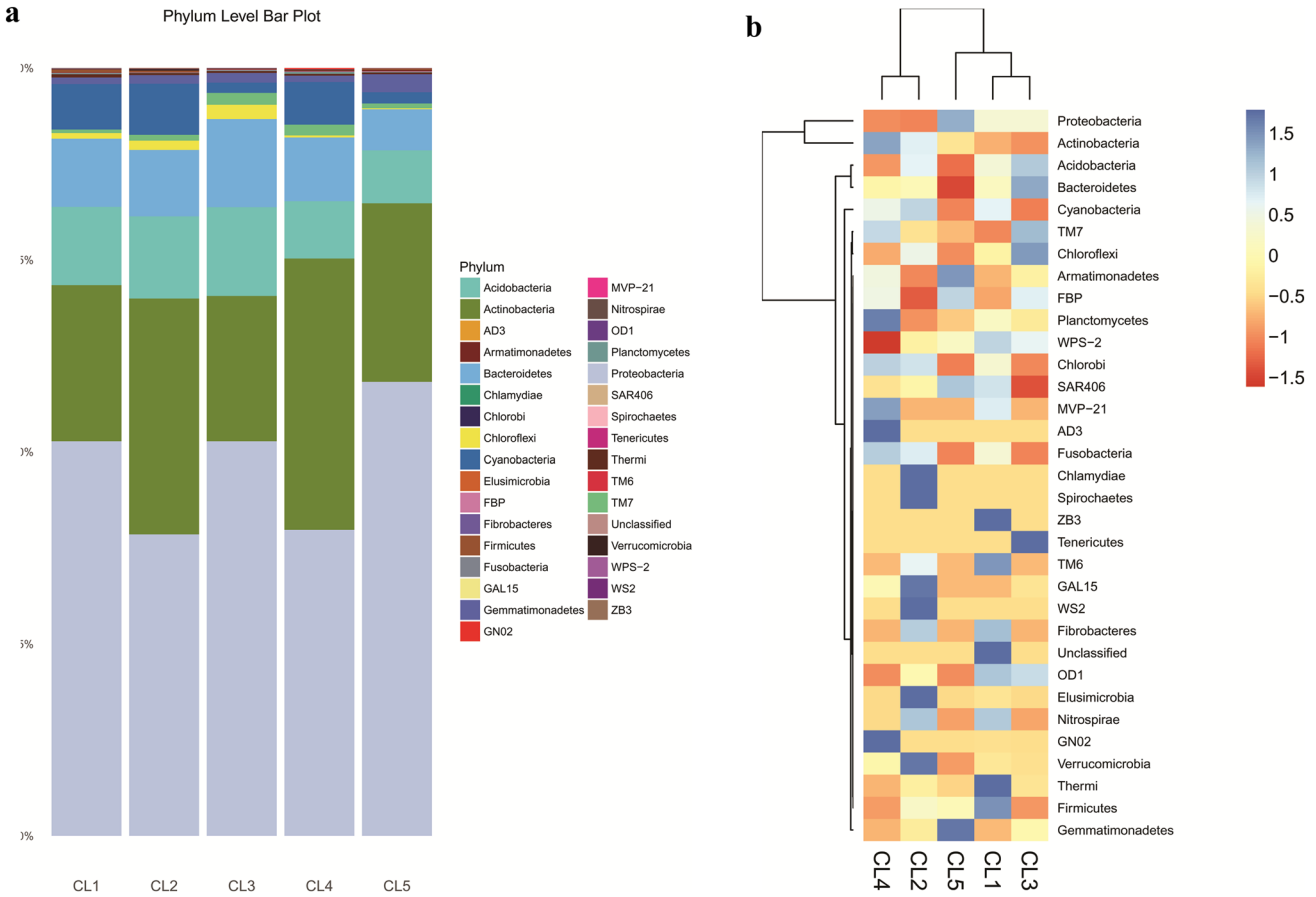

Fig. 2 **a** Stacked barplot for V3 samples at Phylum level. **b** Heatmap has been generated at phylum level across 5 samples (V3) based on the relative abundance values

Distribution of higher taxonomic level bacterial communities

The relative distribution of lichen-associated bacteria was assessed at the class and genus level. The presence of various classes of bacteria, such as *Alphaproteobacteria, Actinobacteria, Solibacteres, Thermomicrobia* and *Deltaproteobacteria* was recorded in the 16S rRNA V3 region analysis at the class level. Further, genus level analysis showed the presence of *Sphingomonas, Flavisolibacter, Methylobacterium, Amycolatopsis, Hymenobacter, Rhizobium, Bartonella* and others. The class level analysis of the 16S rRNA V6 region showed the presence of *Alphaproteobacteria, Actinobacteria, Saprospirae, Cytophagia, Thermoleophilia, Chloracidobacteria, Acidobacteria* and others. The dominant genera, such as *Proteobacteria* were present in all lichen samples.

Abundant and rare species diversity in lichens

Abundant and rare species existed in the bacterial population of lichens. Rare species frequency was < 0.01% of the total population, and the rest of the bacterial populations were considered as abundant. Further, Venn diagrams showed the number of dominant and rare bacterial species shared among five lichen samples of both the V3 and V6 hypervariable regions (Fig. 4a and b). It was observed that some of the rare species were shared commonly among the samples; while most of the species were unique to a particular sample. However, in case of abundant species, most of the species were shared among all the samples and only a few were unique.

The 16S rRNA V3 region analysis confirmed the abundant unclassified species present in lichens, which belonged to the genera *Sphingomonas, Flavisolibacter, Methylobacterium, Amycolatopsis, Hymenobacter, Bartonella* etc. Further, the V6 region analysis confirmed that rare species of different genus *Kaistobacter, Sphingomonas, Flavisolibacter, Pseudonocardia, Methylobacterium, Hymenobacter, Spirosoma* and several different unclassified species.

In the present study, rare bacterial species frequency was found to be less in number when compared to other bacterial lineages present within the same lichen thallus; while they were found in different abundance among the lichen samples. The rare species present in the lichens by study of the V3 region were *Providencia, Yersinia, Rheinheimera,*

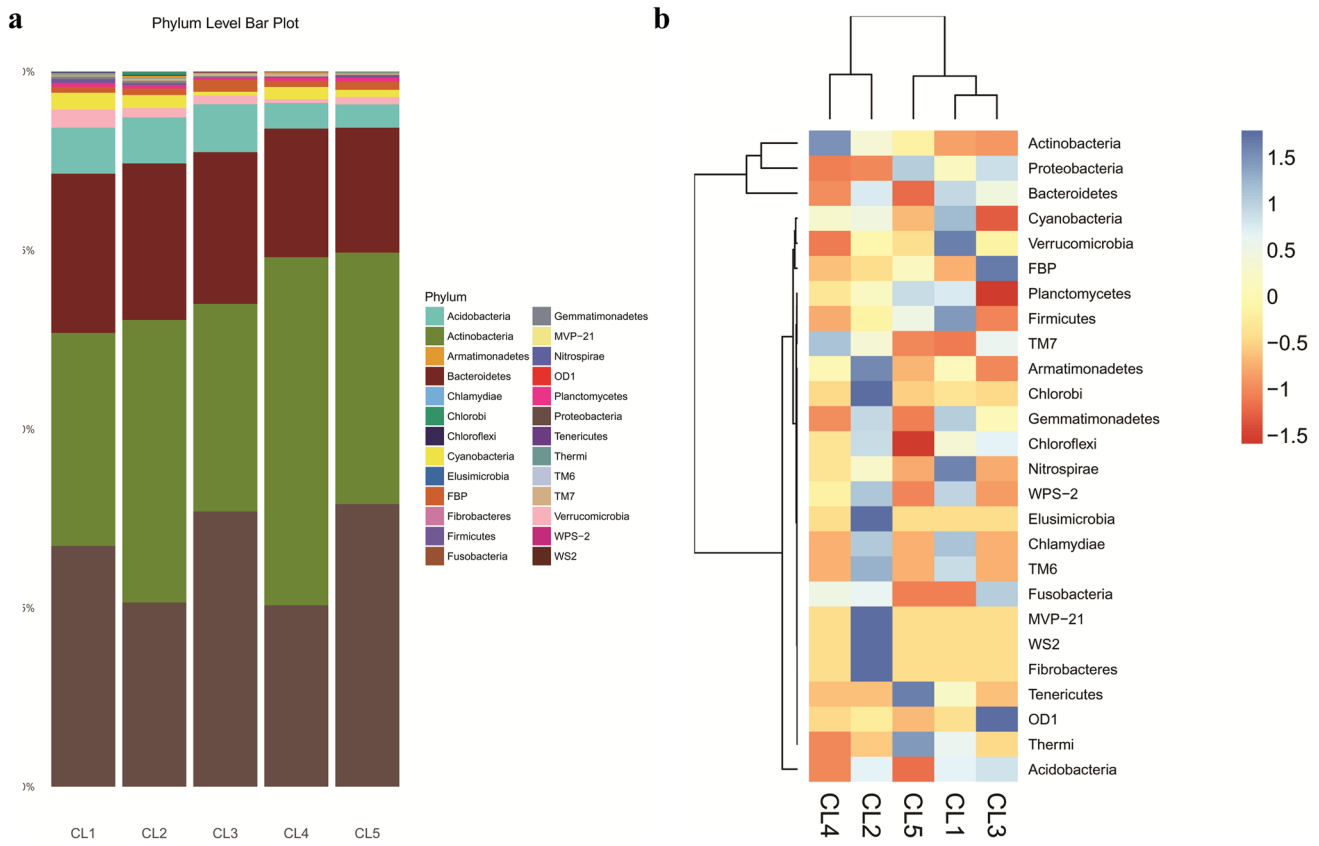

Fig. 3 **a** Stacked barplot for V6 samples at Phylum level. **b** Heatmap has been generated at phylum level across 5 samples (V6) based on the relative abundance values

and several unclassified species of different genera. In the meantime, a V6 region study showed that *Roseomonas aerilata, Hyphomicrobium zavarzinii, Brevundimonas diminuta, Lysinibacillus boronitolerans* and several unclassified species of different genera were prominent in lichens.

Discussion

Lichens are the common colonizers of bare rocks and contribute to weathering by producing various secondary metabolites; however, the information on other lichen symbionts is scarce [23]. Lichen symbioses are conventionally referred to as an obligate association of photobiont and mycobiont; while more recent studies have revealed that less explored bacterial partners also significantly contribute to the integrity and robustness of lichen symbioses [1, 15]. Bacterial diversity associated with the lichen was hitherto explored by several researchers by using conventional and advanced molecular studies [1, 7, 24].

Culture-dependent methods were used previously to estimate the bacteria in lichen [24], however, a complete delineation of overall bacterial lineage associated within lichens were obscure due to limitations of culturing some of the lichen-associated bacteria. The use of 454 bar-coded pyrosequencing technique address the presence of lichen associated bacteria [10]. Presently, Illumina sequencing has surpassed the utility of 454 in microbial diversity studies due to less sequencing costs, and its high, deep coverage of taxa [12]. Therefore, in the present study, we employed high throughput Illumina paired end sequence (150*2) for sequencing of 16S rRNA hypervariable regions (V3 and V6) of five foliose lichen samples. The OTUs picked from the 16S rRNA V3 region and the V6 region showed that the saturated rarefaction curves, and alpha diversity index, ensured the reliability of OTUs.

Alpha diversity measures the diversity within the sample; the Shannon index measures richness and evenness of species, and are less sensitive to the experimental parameters of PCR and sequencing platforms [10, 25]. In earlier published work, lichenized bacterial diversity studied by 454 pyrosequencing of variable region of V4 and V5 were record in the Shannon values ranged from 4.22 to 4.86 [5], while in the present study, the rarefaction curve plot showed the annotated species richness in V3 and V6 metagenome samples with Shannon index of V3 region in much higher range of

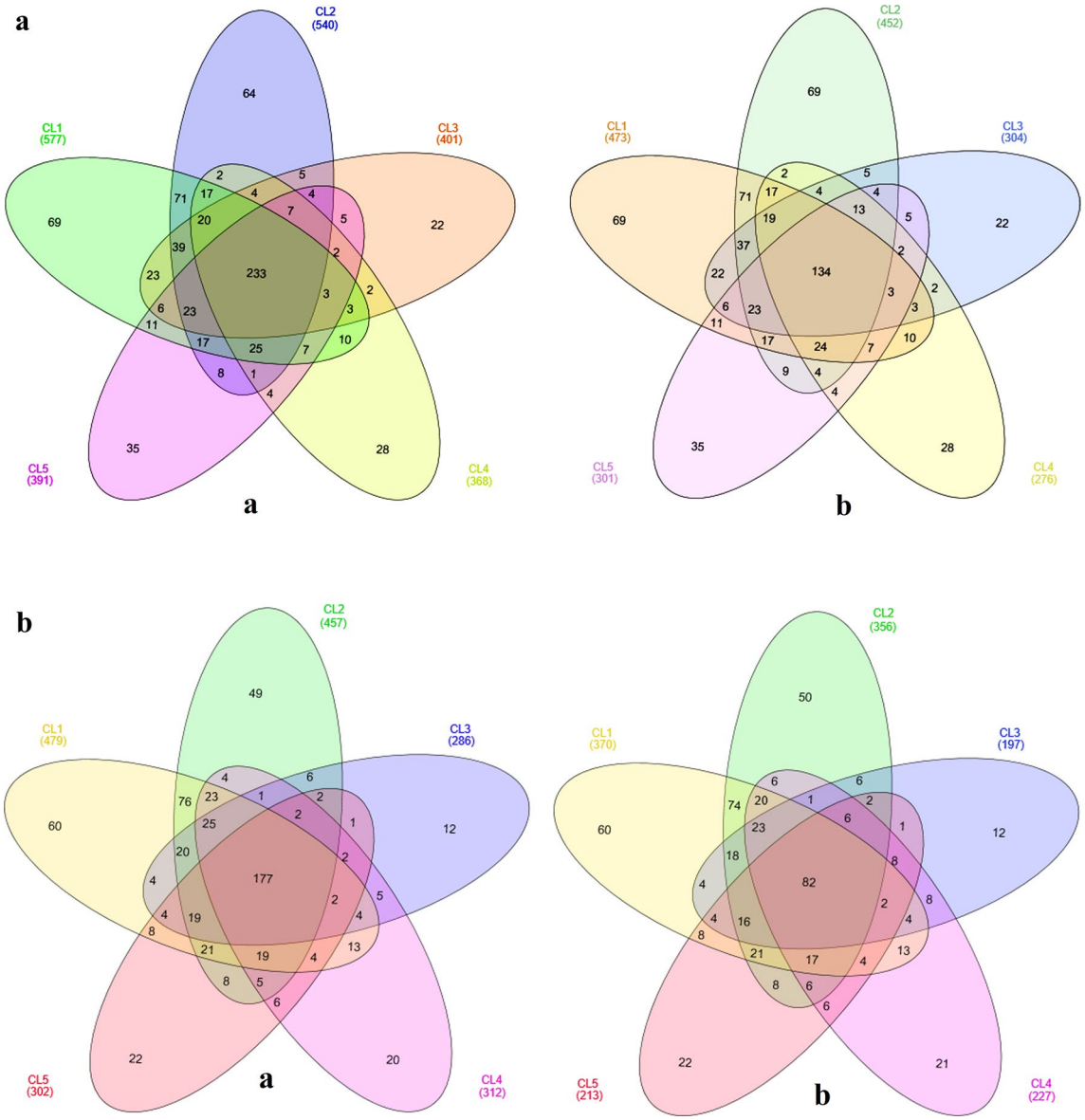

Fig. 4 **a** All bacterial species and rare species analysis of 16S rRNA V3 region. **b** All bacterial species and rare species analysis of 16S rRNA V6 region

6.101 to 7.71 and in V6 region 6.04 to 8.267 respectively. Though the sequencing platforms are changed, the diversity expression via Shannon index due to the sensitivity of the sequencing platforms was observed. This has confirmed that the employment of NGS compared to the less-favourable 454 pyrosequencing in lichen-associated bacterial study, is one of the best approaches as per earlier reports. Further, all rarefaction curves of the Shannon index approached the plateau phase indicating that these values were saturated and a reasonable number of individuals were sampled (Fig. 1a and b; Supplementary Table S1 and S2).

Beta diversity provides a measure of the distance or dissimilarity between each sample based on the relative abundance profiles of bacterial taxa [26]. In the present study, lichen sample CL2 showed only a slight variation in the relative bacterial abundance when compared to the remainder of the four lichens as per weighted unifrac analysis. In the case of unweighted unifrac analysis, CL3 showed different relative abundance of bacteria and as visualized by Principal Coordinate Analysis.

Earlier studies, used 454 pyrosequencing and NGS platforms to generate the amplicons, QIIME pipeline and MG-RAST server were used for taxonomic assignment, phylogeny construction and functional annotations of the sequences were also obtained from lichens [10, 15, 27]. In the present study, both QIIME and MG-RAST pipelines were used for

taxonomic assignment and phylogeny construction. Further, the 16S rRNA hypervariable V3 and V6 region analyses of all five lichens showed that members of the phylum *Proteobacteria* and *Actinobacteria* (Average 38% and 32%, respectively) were dominant genera along with *Cyanobacteria, Chloroflexi, Thermi, Firmicutes,* and *Gemmanodates*. These findings were in agreement with the results of previous researchers where *Proteobacteria,* and *Actinobacteria* members were dominant [15]; although, geoclimatic conditions were varied. However, slight variation in the relative abundance of bacteria was also noticed, perhaps due to the lichen thalli conditions (e.g., water activity, nutrient supply, and the photobiont and mycobiont present), physiological activities, secondary metabolites in thalli and environmental parameters. The present study indicated that the lichens sheltered diverse bacterial species, which were in both dominant and rare fractions; thus indicating that perhaps they play a major role in lichen survivability and longevity.

In diverse taxonomic groups, the *Alphaproteobacteria* group is reported as dominant among lichen-associated bacteria [28], with *Rhizobiaceae, Beijerinckiaceae, Xanthobacteriaceae* and *Phylobacteriaceae* were found to be less abundant in lichen thalli [15, 23]. The number of *Gammaproteobacteria* such as *Enterobacter* sp. and *Providencia rettgeri*, that exhibited multiple plant growth promoting traits, were also reported [9]. In the present study, class and genus level analysis confirmed that *Alphaproteobacteria* class was dominant followed by *Actinobacteria*. At the genus level analysis confirmed the presence of *Sphingomonas, Methylobacterium, Beijerinckia,* and several unclassified genus members, while *Gammaproteobacteria* and *Deltaproteobacteria* members in lichens occurred with less abundance (Fig. 2a and b). *Actinobacteria* and *Proteobacteria* phyla were found to be predominant followed by *Acidobacteria* and their relative abundance was expressed in stacked bar-plots (Figs. 3 and 4). *Acidobacteria* is one of the most abundant bacterial phyla in lichen thalli [1, 11, 29, 30], and it was observed that 32% *Acidobacteria* comprising an overall bacterial diversity in the living and decaying portion of thalli [31]. It is interesting to note that, in the present study, physiologically active lichen samples recorded a rich abundance of *Acidobacteria*.

Cyanobacteria is one of the pioneer photobionts present in lichens, which is helpful in photosynthesis and nitrogen fixation [11, 32]. A high abundance of cyanobacterial presence (47 database hits) was reported by the Illumina sequencing. Recently, five different types of cyanobionts were reported from the *Peltigera* lichens with the help of ribosomal markers [33]. In the present study, *Nostoc, Leptolyngbya, Pleurocapsa*, and other unclassified cyanobacterial genera were recorded, which are secondary photobionts in these 5 lichen samples; and as such, perhaps helpful in nitrogen fixation.

In addition to these, *Firmicutes* were previously reported from lichens [1, 10, 30] and are widely considered as producers of antibiotics and enzyme inhibitors [34]. A number of *Firmicutes* were recorded in the present study but their relative abundances were very less compared to *Proteobacteria* and *Actinobacteria*. A few rare species with less frequency were shared in all lichens; and most of them were unique to a sample. These rare species, and along with the dominant members of the phyla *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi,* they could play a functional role in lichen thalli to establish a successful symbiosis. This is the first time report that focused on bacterial diversity of lichens using 16S rRNA hypervariable regions of V3 and V6; thus, this study extends the current knowledge of bacterial diversity associated with lichens.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06272-6.

Authors Contribution CTS conducted the work and prepared the manuscript. DG Research supervisor, designed the work plan, executed and edited the MS.

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