Climate and Soil Properties Influence Species Diversity of Soil *Bacillus* Community in India

Girish R Nair and Suresh SS Raja

Department of Microbiology, Bharathidasan University Constituent College, Perambalur, India.

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ABSTRACT: *Bacillus* is an important genus as it is a source for antibiotics, enzymes, and probiotics. Therefore, several studies are targeted on this genus in order to understand its diversity abundance in different soil environments. In present study, we investigated the diversity of *Bacillus* at species level using culturable approach in soils collected at different climatic zones of India and identified 20 prominent members of genus *Bacillus* species that are able to grow in different media types under same culture conditions. Results also showed that the species diversity of *Bacillus* changes according to the soil microenvironment under the influence of different climatic conditions. As a pilot study using culturable approach, we made an attempt to investigate the shift in *Bacillus* species diversity present in the Indian soils experiencing a climatic gradient over a large geographic area.

KEYWORDS: Soil, climate, *Bacillus*, EzTaxon, phylogenetic, analysis

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CORRESPONDING AUTHOR: Suresh SS Raja, Department of Microbiology, Bharathidasan University Constituent College, Kurumbalur, Perambalur 621107, Tamil Nadu, India. Emails: sudalaimuthuraja@yahoo.co.in

Introduction

The genus *Bacillus* is a widely distributed genus having 347 species and 7 subspecies known till date.[1,2] Members of this genus belong to phylum Firmicutes and are rod-shaped, which stain gram positive. They have ability to form spores that are resistant to extreme heat, bactericidal agents, and chemical disinfectants.[3] Majority of the species belonging to this genus have no pathogenic potential expect a few like *Bacillus anthracis* which causes anthrax, *Bacillus cereus* responsible for food poisoning, and *Bacillus thuringiensis* which produces insecticidal toxin, while others have only few reports on pathogenicity against human and animals.[4–8] Many species of *Bacillus* have wide angle applications in the field of medicine and agriculture, which involve production of biocontrol agents such as antibiotics from species like *Bacillus licheniformis* or *Bacillus subtilis* that produce bacitracin, *Bacillus polymyxa*, which produces polymyxin, and *Bacillus brevis* which produces gramicidin.[9–14] Several *Bacillus* spp. are also used as probiotic supplements in animal feed because of their ability to withstand high temperature and low pH.[15,16] They are also good source for production of enzymes like amylase, protease, cellulase, lipase, and so on, which find variety of industrial applications.[17–23] Therefore, it is imperative to understand the structure and dynamics of soil *Bacillus* communities.

Several studies have reported the role of climate as a major abiotic factor in shaping the bacterial communities present in the soil by controlling the rate of soil formation and its chemical composition.[24–27] India possesses a large variety of climates ranging from extremely hot desert regions to high altitude locations with severely cold conditions similar to northern Europe. Climate in India can be divided into six zones according to Köppen classification system as tropical wet climatic zone (TWCZ), tropical wet and dry climatic zone, arid climatic zone (ACZ), semi-arid climatic zone ACZ, humid subtropical climatic zone (HSCZ), and mountainous climatic zone (MCZ).[28,29] This climatic diversity experienced by Indian soils makes it an interesting investigation site for understanding the community profile of genus *Bacillus* present in different climatic zones.

Most of the recent work in soil microbial ecology which focuses on cataloging the diversity of soil bacteria and documenting how soil bacterial communities are affected by specific environmental changes or disturbances is gaining more importance[30,31] and similar trend of studies could very well be targeted on soil *Bacillus* community of India facing a climatic contrast across the subcontinent. Therefore, as a preliminary study, we investigated the diversity of *Bacillus* species in soil types present at four different climatic zones of India namely, TWCZ, ACZ, HSCZ, and MCZ using culturable approach. These climatic zones were selected on the basis of climatic contrast between them. From this study, we intend to know the prominent members of soil *Bacillus* community in different climatic locations and how species diversity of *Bacillus* varies across the geographical and climatic scale when grown in different media types under same culture conditions.

Materials and Methods

Study sites and sample collection

The sampling was performed at different locations from India falling under four climatic zones according to Köppen classification system (Supplementary Figure 1). Soil was collected in the pre-monsoon season from February 2017 to March 2017 from three different spots at each climatic zone starting from the southern part of India and moving along the northern part of India, the study sites cover four states of India each representing a different climatic zone namely, Kerala (TWCZ), Rajasthan (ACZ), Chandigarh (HSCZ), and Himachal Pradesh (MCZ)

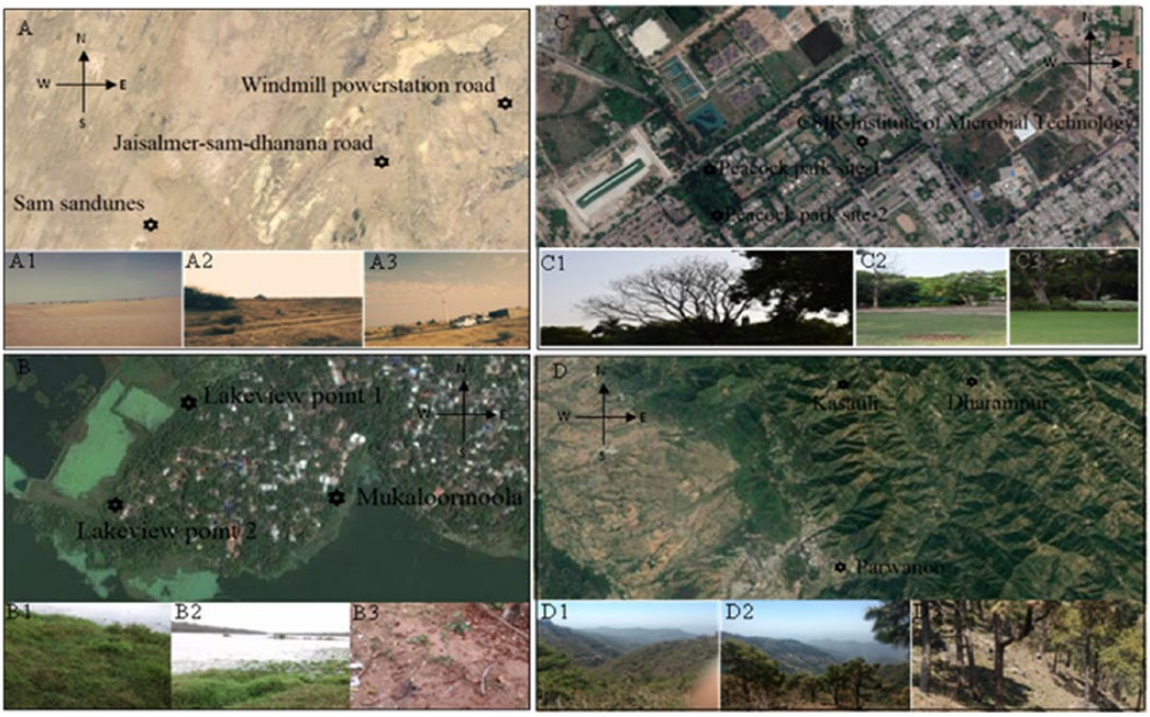


Figure 1. Location of selected soil sampling sites from different climatic zones of India. A. Map of western Rajasthan (arid climatic zone) showing sampling sites marked with 6-point star (★); A1, A2, A3 photographic images of sampling area in western Rajasthan. B. Map of southern Kerala (tropical wet climatic zone) showing sampling sites marked with 6-point star (★); B1, B2, B3 photographic images of sampling area in southern Kerala. C Map of Chandigarh city (humid subtropical climatic zone) showing sampling sites marked with 6-point star (★); C1, C2, C3 photographic images of sampling area in Chandigarh. D Map of Himachal Pradesh (mountainous climatic zone) showing sampling sites marked with 6-point star (★); D1, D2, D3 photographic images of sampling area in Himachal Pradesh. *Source: Adapted and modified from Google maps.*

(Figure 1). All samples were collected from areas having no anthropogenic activity or animal influence and low density of vegetation. At each sampling spot, quadrates of 3.5 × 3.5 m were selected and the surface soil was removed at a depth of 15-20 cm. Thereafter, soil was collected using a sterile shovel and transferred into sterile sampling bags (Nasco: Hi-Media, India) and transported to laboratory in ice. Later, the samples from each climatic location were sieved through sterile 2 mm mesh, pooled together to make a composite sample and processed immediately.

Geochemical parameters of soil

A total of 10 geochemical parameters of soil were assessed, including pH, moisture content, organic carbon, nitrogen, potassium, calcium, magnesium, phosphorous, iron, and boron. The pH was measured using pH electrode (Shimadzu, Japan) in a saturated colloidal solution of deionized water and moisture content was calculated using oven dry method. Total phosphorus was measured colorimetrically and total nitrogen was analyzed by the micro Kjeldahl method.[32] Determination of iron, boron, calcium, potassium, and magnesium was carried out using Spectrometer (Zeenit 700; Analytik, Jena, Germany).[33] Total organic carbon was determined using partial oxidation method.[34]

Plating and isolation of bacterial strains

The samples were immediately processed by serial dilution and plating technique using normal saline (0.45%) by plating on to general purpose media like TSA (Tryptic Soy Agar; Hi-Media), NA (Nutrient Agar; Hi-Media), minimal media involving TSA 1:10 and 1:100 dilutions (TSA; Hi-Media) and selective media involving AIA (Actinomycetes Isolation Agar; Hi-Media), SMA (Streptomyces Agar; Hi-Media) followed by incubation at 30°C for a week subsequently monitoring bacterial growth at 24 h, 48 h, 72 h, and so on. Quantitative analysis for bacterial growth in all the samples was examined using colony counting and determination of colony forming units (CFU) per milliliter. Qualitative analysis of colonies involved identification of bacterial species and their enumeration from all the samples. The colony morphotypes were selected using four parameters: colony size, form, color, and texture. A phenotypic variant was considered when it differed in at least one of the referred morphological parameters and further selected for identification.[35]

Strain identification and phylogenetic analysis

All the strains were identified using through genomic DNA isolation and 16S rRNA gene sequencing. Genomic DNA was extracted using Nucleospin nucleic acid kit followed by PCR amplification using eubacterial universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492 R (5′-CGG TTA CCT TGT TAC GAC TT-3′).[36] Furthermore, the purified PCR product was amplified using 1100R (5′-GGG TTG CGC TCG TTG-3′) primer subjected to Sanger sequencing.[37,38] The 16S rRNA gene

Table 1. Physiochemical properties of soil samples collected at different climatic zones.

SOIL PARAMETERS	TWCZ	ACZ	HSCZ	MCZ
Moisture content	13.06%	0.07%	0.3%	7.7%
pH	3.94	8.05	8.13	5.44
Organic carbon	103.07 mg/kg	93.10 mg/kg	94.60 mg/kg	112.27 mg/kg
Nitrogen	14.11%	17.00%	11.03%	10.90%
Potassium	2.9%	1.86%	2.9%	1.88%
Calcium	4.30%	6.07%	1.8%	8.01%
Magnesium	1.22%	2.43%	0.4%	1.22%
Phosphorous	0.05%	0.08%	0.07%	0.12%
Iron	1.09%	1.26%	1.04%	1.13%
Boron	10.04 ppm	15.12 ppm	12.80 ppm	11.20 ppm

Abbreviations: ACZ, arid climatic zone; HSCZ, humid subtropical climatic zone; MCZ, mountainous climatic zone; TWCZ, tropical wet climatic zone.

sequences obtained after sequencing were searched for similar sequences from 16S rRNA gene database of EzTaxon server[39] (Supplementary Table 1). To determine the phylogenetic relationship of the identified isolates, their 16S rRNA gene sequences were aligned using Mega version 7.0[40] and phylogenetic trees were constructed using the neighbor joining as well as maximum likelihood and maximum parsimony algorithms.[41] Bootstrap analysis of 1000 bootstrap replications was performed to assess the confidence limits of the branching.[42]

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the *Bacillus* isolates were deposited in GenBank database under the accession numbers MF680623, MF680624, MF680626 to MF680629, MF680632 to MF680635, MF680637, MF680638, MF680640 to MF680646, MF680649 to MF680652, MF680654 to MF680665, MF680667, MF680670 to MF680671.

Results

Chemical analysis of soil samples

The soil samples collected at each climatic zone were pooled together to make a composite sample and the highest values of moisture were recorded in soils collected from TWCZ followed by MCZ, HSCZ, and lowest in ACZ. The pH (3.94) was lowest in TWCZ soil which reflects its acidic nature whereas the pH in soils collected from ACZ (8.05) and HSCZ (8.13) was alkaline. The soil from MCZ was mildly acidic having a pH value of 5.44. There was a much less difference in the values of organic carbon, phosphorous and iron in all soil samples. The highest values of organic carbon and phosphorous were recorded in soils collected at MCZ, whereas highest values of iron were recorded in ACZ. Greater differences were observed in values of calcium and magnesium, the value of calcium was highest in MCZ and lowest in HSCZ. The value of magnesium showed higher levels in ACZ and lower levels in HSCZ. The value of boron was highest in ACZ and lowest in TWCZ. The percentage of potassium had similar range in ACZ (1.86%) and MCZ (1.88%) together. Similarly, it was observed as 2.9% for both TWCZ and HSCZ (Table 1).

Culturable diversity of Bacillus species

The viable bacterial population was measured using plating and CFU determination on five different media namely, TSA, NA, SMA, and AIA. Of all these media, the highest CFU (2.58×10^4 CFU/mL) was recorded in TSA in sample TWCZ. The phylum Firmicutes dominated among other identified phyla Actinobacteria and Proteobacteria which could be correlated with the depth of soil collected and presence of rhizospheric soils in the sampling sites. In the phylum Firmicutes, 20 different *Bacillus* species were identified from all four climatic zones, in which maximum diversity and number were obtained in ACZ followed by TWCZ. The other two climatic zones HSCZ and MCZ had similar abundance and diversity. Although all the soils had the presence different species of *Bacillus*, one species was found to be shared among HSCZ, MCZ, and ACZ. No species was found to be common among four soils (Figure 2) There were a lot of species that were found to be unique and confined to only a particular climatic zone like in TWCZ, which showed the presence of *Bacillus aryabhattai, Bacillus mesonae, Bacillus wiedmannii*, that were absent in all other climatic zones. Similarly, ACZ showed the presence of *B. anthracis, Bacillus filamentosus, Bacillus altitudinis*, and *Bacillus firmus* followed by HSCZ which showed presence of *Bacillus pseudomycoides, Bacillus funiculus, Bacillus luciferensis*, and *Bacillus marisflavi*. The climatic zone MCZ had presence of *Bacillus tequilensis* and *Bacillus muralis* (Figure 3). These results indicate that although genus *Bacillus* is ubiquitously

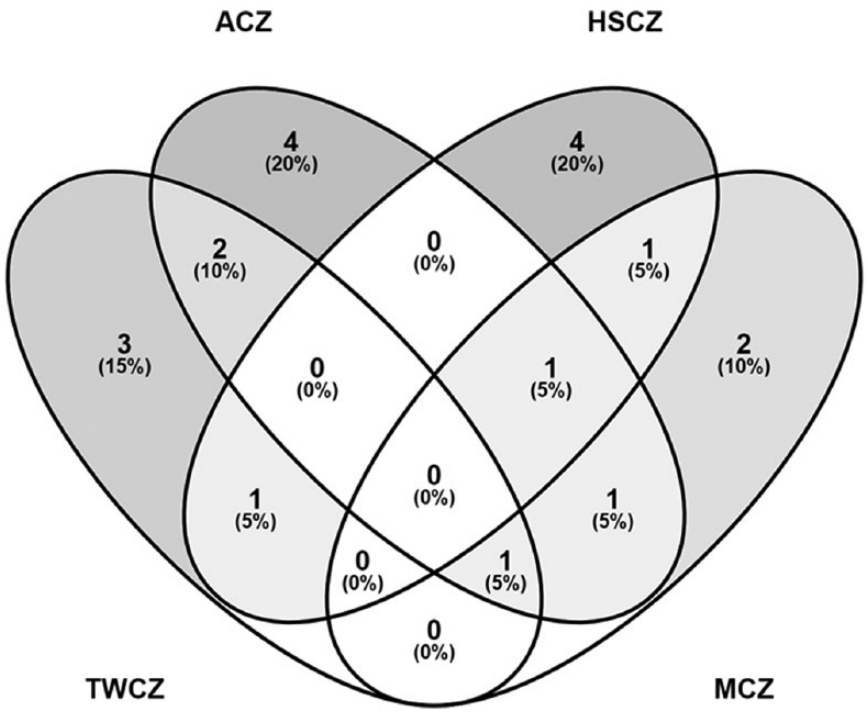


Figure 2. Venn diagram showing the different *Bacillus* species shared among tropical wet climatic zone (TWCZ), humid-sub tropical climatic zone (HSCZ), arid climatic zone (ACZ), and mountainous climatic zone (MCZ) of India. ACZ indicates arid climatic zone; HSCZ, humid subtropical climatic zone; MCZ, mountainous climatic zone; TWCZ, tropical wet climatic zone.

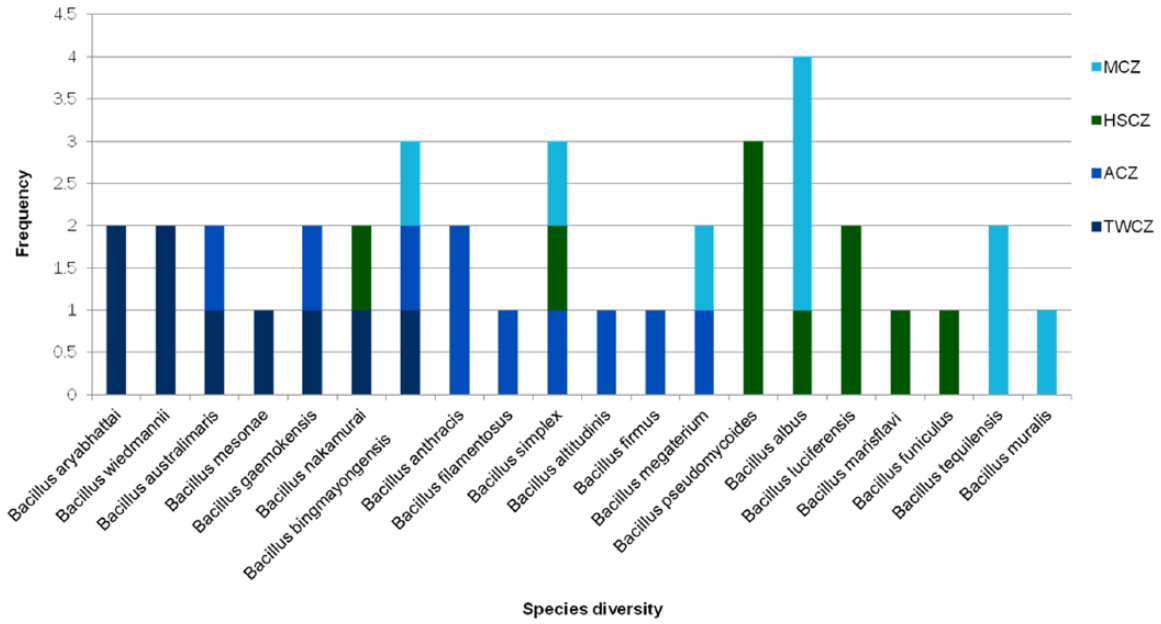
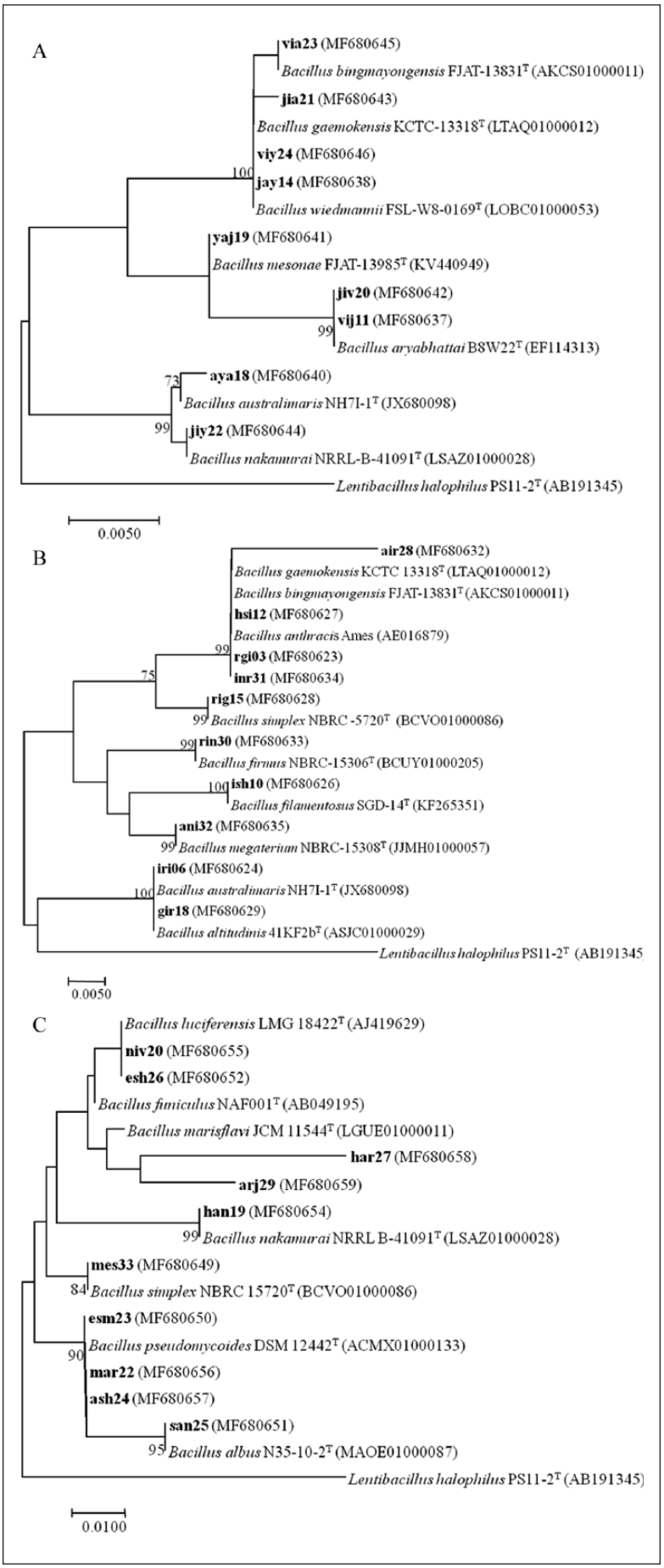


Figure 3. Distribution of *Bacillus* species in different climatic zones of India as inferred from culturing and 16S rRNA gene sequencing. ACZ indicates arid climatic zone; HSCZ, humid subtropical climatic zone; MCZ, mountainous climatic zone; TWCZ, tropical wet climatic zone.

found in different soil habitats, there is a difference in species level distribution due to influence of climate as one of the factors affecting soil microenvironment. The phylogenetic relationship between the isolated 16S rDNA from the four climatic zones was compared with the representative species. All of the isolates were affiliated with 20 different species of *Bacill*us (Figure 4A to D).

Discussion

There are number factors that mold the microbiota surviving in the soil, one of which is the climate and considered as a major abiotic factor controlling the other sub-factors that shape the *Bacillus* community native to the bottom soil present at the different climatic conditions, unaffected by the anthropogenic activities. The climate change and global warming are


A
via23 (MF680645)
Bacillus bingmayongensis FJAT-13831T (AKCS01000011)
jia21 (MF680643)
Bacillus gaemokensis KCTC-13318T (LTAQ01000012)
viy24 (MF680646)
100
jay14 (MF680638)
Bacillus wiedmannii FSL-W8-0169T (LOBC01000053)
yaj19 (MF680641)
Bacillus mesonae FJAT-13985T (KV440949)
jiv20 (MF680642)
vij11 (MF680637)
99
Bacillus aryabhattai B8W22T (EF114313)
73
aya18 (MF680640)
Bacillus australimaris NH7I-1T (JX680098)
99
jiy22 (MF680644)
Bacillus nakamurai NRRL-B-41091T (LSAZ01000028)
Lentibacillus halophilus PS11-2T (AB191345)
0.0050
B
air28 (MF680632)
Bacillus gaemokensis KCTC 13318T (LTAQ01000012)
Bacillus bingmayongensis FJAT-13831T (AKCS01000011)
hsi12 (MF680627)
Bacillus anthracis Ames (AE016879)
99
rgi03 (MF680623)
75
inr31 (MF680634)
rig15 (MF680628)
99
Bacillus simplex NBRC -5720T (BCVO01000086)
99
rin30 (MF680633)
Bacillus firmus NBRC-15306T (BCUY01000205)
100
ish10 (MF680626)
Bacillus filamentosus SGD-14T (KF265351)
ani32 (MF680635)
99
Bacillus megaterium NBRC-15308T (JJMH01000057)
iri06 (MF680624)
100
Bacillus australimaris NH7I-1T (JX680098)
gir18 (MF680629)
Bacillus altitudinis 41KF2bT (ASJC01000029)
Lentibacillus halophilus PS11-2T (AB191345
0.0050
C
Bacillus luciferensis LMG 18422T (AJ419629)
niv20 (MF680655)
esh26 (MF680652)
Bacillus fumiculus NAF001T (AB049195)
Bacillus marisflavi JCM 11544T (LGUE01000011)
har27 (MF680658)
arj29 (MF680659)
han19 (MF680654)
99
Bacillus nakamurai NRRL B-41091T (LSAZ01000028)
mes33 (MF680649)
84
Bacillus simplex NBRC 15720T (BCVO01000086)
esm23 (MF680650)
90
Bacillus pseudomycoides DSM 12442T (ACMX01000133)
mar22 (MF680656)
ash24 (MF680657)
san25 (MF680651)
95
Bacillus albus N35-10-2T (MAOE01000087)
Lentibacillus halophilus PS11-2T (AB191345)
0.0100

(Continued)

Figure 4. (Continued)

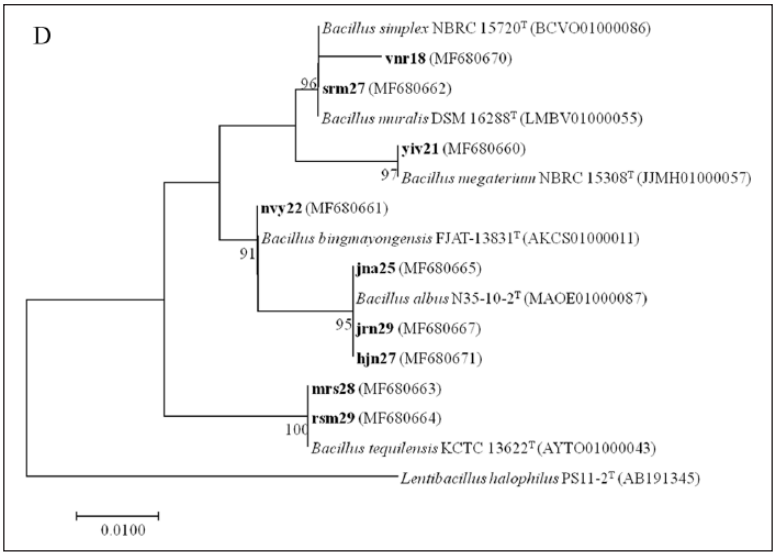


Figure 4. A. Phylogenetic tree based on 16S rDNA sequences showing relationship between different members of phylum Firmicutes isolated from TWCZ. Boot-Strap values (1000 replicates) of >70% are given at the nodes. *Lentibacillus halophilus* strain PSII-2^T was used as outgroup. The strains isolated from the study are shown in bold and the accession numbers in brackets. Scale bar represents the number of substitutions per nucleotide position. Bar, 0.0050 substitutions per nucleotide position. B. Phylogenetic tree based on 16S rDNA sequences showing relationship between different members of phylum Firmicutes isolated from ACZ. Boot-Strap values (1000 replicates) of >70% are given at the nodes. *L. halophilus* strain PSII-2^T was used as outgroup. The strains isolated from the study are shown in bold and the accession numbers in brackets. Scale bar represents the number of substitutions per nucleotide position. Bar, 0.0050 substitutions per nucleotide position. C. Phylogenetic tree based on 16S rDNA sequences showing relationship between different members of phylum Firmicutes isolated from HSCZ. Boot-Strap values (1000 replicates) of >70% are given at the nodes. *L. halophilus* strain PSII-2^T was used as outgroup. The strains isolated from the study are shown in bold and the accession numbers in brackets. Scale bar represents the number of substitutions per nucleotide position. Bar, 0.0100 substitutions per nucleotide position. D. Phylogenetic tree based on 16S rDNA sequences showing relationship between different members of phylum Firmicutes isolated from MCZ. Boot-Strap values (1000 replicates) of >70% are given at the nodes. *L. halophilus* strain PSII-2^T was used as outgroup. The strains isolated from the study are shown in bold and the accession numbers in brackets. Scale bar represents the number of substitutions per nucleotide position. Bar, 0.0100 substitutions per nucleotide position.

causing paradigm shift in the population of soil microbial communities; therefore, microbial surveys of different terrestrial habitats are increasingly becoming significant among microbial society. The culturable dependant approach is gaining equal importance compared with culture independent approach in investigating the composition of soil microbiota on a climatic and geographical scale as evident from a study, where seasonal variation in the bacterial community profile of alpine forest soils was studied. Similarly, another study assessed the response of yeast diversity to microclimatic environmental factors in soil biotypes using culturable approach. Prior to this, researches have compared the microbial community structure of four black soils along a climatic gradient in northeast china and also showed the effect of pedoclimate, geomorphology, and land-use in changing the turnover of soil bacterial communities of France.

The Indian subcontinent has a varied climatic exposure having a tremendous effect on the terrestrial environments that could modulate the soil physiochemical properties in-turn affecting the bacterial community profile of a particular habitat. This makes lot avenues for exploring the microbial community diversity in Indian soils on a climatic and geographical scale which are given much less attention. Several studies have evaluated the culturable microbial diversity of Trans-Himalayas of Himachal Pradesh Similarly, the diversity of culturable *Bacilli* was investigated in six wheat cultivating agro-ecological zones of India namely, northern hills, north western plains, north eastern plains, central, peninsular, and southern. Likewise another study explored the diversity of niche specific *Bacilli* from extreme environments. Although these studies investigated the diversity of Bacillus community, they slightly differ from the present study as this study examines the diversity native soil *Bacillus* over a large climatic and geographical scale in unexplored and non-anthropogenic sites using different growth media and culture setting.

The change in the pattern of diversity in the soil *Bacillus* community is much less studied on a geographical scale in India and this study has made an attempt to investigate that part of *Bacillus* community native to the soil, which has developed over the years under the influence climate and geography of that area. The findings proved that although the genus *Bacillus* is ubiquitously found in all major soil types present at different climatic zones, their species level diversity varies to a great extent, which suggest that climate has a strong influence in shaping the *Bacillus* species structure thriving in different soils. Moreover, it was found that *Bacillus* is able to grow in

general-purpose media, minimal media (nutritionally poor), and selective media that are used to traditionally culture bacteria from soil. In the soil parameters pH, moisture content, magnesium and calcium showed observable difference considering others parameters among the climatic zones. pH had a slighter influence on the survival of members of genus *Bacillus*. Moisture content was less in ACZ; yet, it was more diverse than other climatic zones, which shows little influence of moisture content in survival of different *Bacillus* species. HSCZ soil showed low levels of magnesium and calcium which could be correlated to the survival and presence of some unique species like *Bacillus pseudomycoides, Bacillus licheniformis, Bacillus lucifer-ensis*, and *Bacillus marisflavi*, which were not found in other climatic zones. Remarkably, this study shows the difference in the diversity of *Bacillus* species inspite of similar culture conditions from each climatic location of India, which was never reported in the literature and all these species were industrially important members that have potential enzyme production as well as probiotic capabilities.

Author Contributions

Conceptualization and Study design: Suresh S S Raja (SSR), Manuscript preparation, data analysis, review and editing: Girish R Nair (RGN).

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