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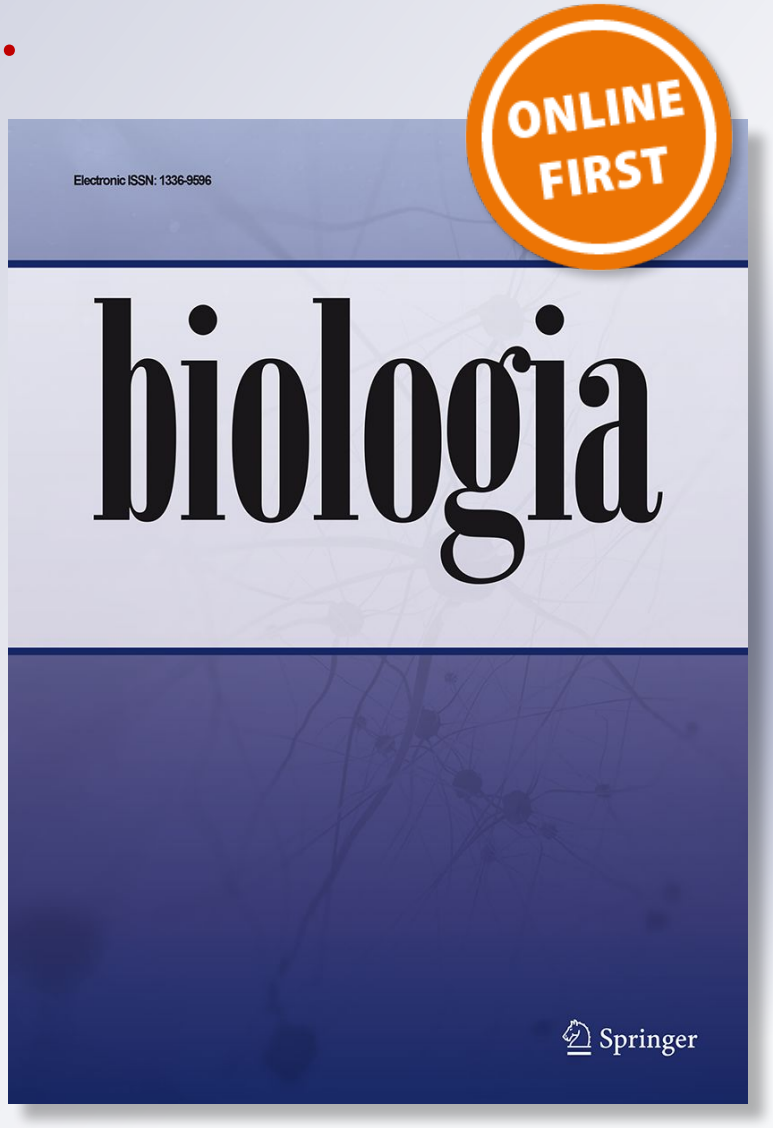
Biologia
Botany, Zoology and Cellular and Molecular Biology

ISSN 0006-3088

Biologia
DOI 10.2478/s11756-019-00259-2



Springer

Biologia
https://doi.org/10.2478/s11756-019-00259-2

ORIGINAL ARTICLE

Seasonal variations in culturable archaea and their plant growth promoting attributes to predict their role in establishment of vegetation in Rann of Kutch

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Received: 2 November 2018 / Accepted: 17 April 2019

Abstract

Archaea are unique microorganisms that are present in ecological niches of high temperature, pH and high salinity. Archaea may be present freely or associated with plant rhizosphere. The plant-microbe interactions may be implicit to plants adaptation to abiotic stress of hypersalinity. With an aim to look for population dynamics of archaea at different seasons of the year in hypersaline environments of Rann of Kutch, the rhizospheric, non-rhizospheric, water and sediment samples were collected during autumn, winter and summer. Sampling sites were selected on the basis of topography and vegetation which included barren land, salt pan and rhizosphere of monocot and dicot plants. Soil pH and salinity (mS cm^{-1}) varied from 7.4–10.15 and 1.19–106.7 respectively. A total of 157 halophilic archaea were isolated using seven different selective media. The isolated archaeal were screened for abiotic stress and it has been found they show the wide range of in the tolerance to temperatures (25–65 °C), NaCl concentrations (0.86–5.48 M), water stresses (upto −0.75Mpa) and pH (4–10). The profiling of archaeal community using 16S rRNA gene sequencing and phylogenetic analysis revealed that all archaeal isolates belonged to a family halobacteriaceae of phylum euryarchaeota. Based on 16S rRNA gene sequencing the cultures were identified and belonged to twenty eight distinct species of 16 genera namely *Haladaptatus, Haloarcula, Halobacterium, Halococcus, Haloferax, Halogeometricum, Halolamina, Halopenitus, Halorubrum, Halosarcina, Halostagnicola, Haloterrigena, Natrialba, Natrinema, Natronoarchaeum* and *Natronomonas*. In the present study, seasonal and niche-specific archaea were reported and characterized from hypersaline environments. The haloarchaea with multifunctional plant growth promoting attributes, prevalent in the hypersaline environments must be colonizing the rhizosphere of plants and contributing to the growth and sustenance of plants.

Keywords Archaeal biodiversity · Haloarchaea · Hypersaline · Population dynamics · Rann of Kutch

Introduction

Hypersaline environment is one of the most extreme habitats with respect to the salt concentration. The salt concentrations in these environments range from 15% to saturation, with pH values from slightly acidic to alkaline (pH 6–11). The most dominant microbes in such systems are members of the halophilic archaeal of phylum Euryarchaeota (Thomas et al. 2014), no halophilic representatives have yet been identified within the phylum Crenarchaeota (Oren 2008). Haloarchaea are most abundance in hypersaline environments on the Earth such as solar salterns, hypersaline lakes, the Dead Sea, hypersaline microbial mats and underground salt deposits (Bodaker et al.

Electronic supplementary material The online version of this article (https://doi.org/10.2478/s11756-019-00259-2) contains supplementary material, which is available to authorized users.

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Published online: 03 May 2019

2010; Oren 2015; Youssef et al. 2012). Archaea exist in a broad range of habitats, and as a major part of global ecosystems, may contribute up to 20% of Earth's biomass (DeLong and Pace 2001). Hypersaline habitats are an extreme environment which is dominated by haloarchaea that required a minimum of 9% (*w*/*v*) (1.5 M) NaCl for growth (Oren 2008). In general, haloarchaeal strains require high salt concentration for growth and cell integrity. Most of species in Halobacteriaceae are true extreme halophiles according to Kushner (1978), However, *Halobacteriaceae* contains some species which can grow in low salinity for instance, *Haloferax sulfurifontis* (Elshahed et al. 2004), *Haladaptatus paucihalophilus* (Savage et al. 2007) and *Halosarcina pallida* (Savage et al. 2008).

Archaea have been reported as ubiquitous and present in a wide range of environments from hypersaline habitats (Budakoglu et al. 2014; Dang et al. 2010; Yadav et al. 2017; Saxena et al. 2015); cold environment (Dong and Chen 2012); thermal springs (De León et al. 2013) and tropical and temperate biome (Tripathi et al. 2015). Archaea are a common component of different extreme environments. Many species of haloarchaea of halobacteriaceae family have been isolated from hypersaline environments including *Halococcus* sp. (Kocur and Hodgkiss 1973); *Haloferax volcanii* (Mullakhanbhai and Larsen 1975); *Haloarcula argentinensis* (Ihara et al. 1997); *Natrinema* sp. (McGenity et al. 1998); *Natronorubrum bangense*, *Natronorubrum tibetense (*Xu et al. 1999*); Haloterrigena* sp.*, Haloterrigena thermotolerans* (Montalvo-Rodríguez et al. 2000); *Haloferax* sp. (Gutierrez et al. 2002); *Haloferax alexandrinus* (Asker and Ohta 2002); *Halococcus hamelinensis* (Goh et al. 2006); *Haloferax larsenii* (Xu et al. 2007); *Natronoarchaeum mannanilyticum* (Shimane et al. 2010); *Halostagnicola kamekurae* (Nagaoka et al. 2010); *Halolamina pelagica* (Cui et al. 2011); *Haloplanus salinus* (Qiu et al. 2013); *Halolamina salina* (Zhang et al. 2013); *Halobacterium rubrum* (Han and Cui 2014) *Halorubrum rutilum* (Yin et al. 2015) and *Halolamina pelagica* (Gaba et al. 2017).

Rann of Kutch represents a unique ecosystem characterised by saline and marshy tracts, low rain fall and sparse vegetation. It also comprises of 'Banni' grassland. The seasonal variations are more pronounced, in winters the temperatures are as low as 0 °C whereas during summers the temperatures can peak up to 49.5 °C. The flora, fauna and microflora of the Kutch have evolved to adapt to these extremes. In the salt crystallizers of the Little Rann of Kutch, Gujarat, India the gradual increase in concentration of salt in the salt pans, encourages different groups of organisms to thrive at different salt concentrations; the ones which cannot survive at higher concentrations die out. The Rann of Kutch is primarily inhabited by prokaryotes, mainly haloarchaea, capable of thriving in these conditions. These groups of microorganisms are relatively unexplored due to the prevalence of extreme conditions and inaccessibility due to marshy nature of the ecosystems and inherent difficulties in culturing these groups of organisms.

Microorganisms, particularly eubacteria and fungi are known to play an important role in biogeochemical cycling and making available important nutrients like nitrogen (N), Phosphorus (P) and potassium (K) to the plants through fixation, solubilisation or mobilization of nutrients. However the role of archaea, that inhabits extreme environments, comprise more than 20% of the world' biomass and are among the most primitive and ancient life forms on earth, in biogeochemical cycling and in sustenance of vegetation in saline environments have not been studied. We hypothesized that archaea prevalent in these extreme environments must be colonizing the rhizosphere of plants and contributing to the growth and sustenance of plants through various plant growth promoting activities. Few reports are available for archaea as plant growth promoting, which includes phosphorus solubilization by haloarchaea (Yadav et al. 2015d; Yadav et al. 2017), nitrogen fixation by methanogens (Leigh 2000), siderophores production (Dave et al. 2006) and IAA production (White 1987). To give credentials to our hypothesis, the population dynamics of archaea in different seasons was studied as a prelude to select strains that are not only halotolerant but can survive under different moisture and temperature regimes prevalent in Kutch.

Materials and methods

Sampling site, sample collection and Physico-chemical characteristics of samples

Survey of hypersaline regions of Rann of Kutch, Gujarat was conducted three times in different seasons: September, 2012 (autumn); January 2013 (winter) and June 2013 (summer). The surface of Rann of Kutch exhibits wide and deep cracks with veneer of salt. Overall, Aridisol and Entisol orders dominate soil of the Kutch peninsula. The soil of the mainland areas is characterized by varying depth and textures. The soils of this region are moderately calcareous, alkaline, and loamy in texture and are excessively drained. For sampling, four regions designated as R1, R2, R3 and R4 were selected based on vegetation, moisture, salinity and distance (R_1: 23°30′21″ N: 69°39′69″E, R_2: 23°48′20″N: 69°43′75″E, R_3: 23°57′69″ N: 69°43′95″E and R_4: 23°49′67″N: 69°31′43″E). The monocot vegetation in the selected hypersaline regions were mainly Banni grasses which included species of *Dicanthium*, *Sporobolous* and *Cenchrus*. Among dicots, *Suaeda nudiflora* was the predominant flora with few species of cucurbits also growing in certain regions. All the four selected regions received scant rainfall of less than 70 mm in whole year and varied significantly in salinity levels. The region R4 lies below mean sea level and act as basin. During rainy season due to percolation of water from adjoining Indus River in Pakistan

and other local rivers, the ground water rises above ground and accumulates at least 1 to 2 ft above ground. This water dries up as temperature rises and salt crust gets deposited as a result of evaporation. A total of thirty samples, three from each location, were collected from ten different locations of Rann of Kutch (details provided in Table 1). The samples were collected in sterilized bottles/polythene bags labelled, transported on ice and stored at 4 °C until analysis.

The pH and electrical conductivity of the samples was recorded at the sampling site. Soil samples were analyzed for soil organic carbon and total nitrogen. The contents of exchangeable cations (Ca^{2+}, K^{+}, Mg^{2+} and Na^{+}) were determined using atomic absorption spectrophotometer after extraction with 1 M ammonium acetate (pH 7.0) and available phosphorus was as described in earlier studies (Verma et al. 2016). Principal component analysis (PCA) was performed for different soil properties as described earlier (Yadav et al. 2015b).

Isolation, enumeration and characterization of haloarchaea

The population of culturable haloarchaea was isolated using the standard serial dilution plating technique. Seven different complex medium, minimum growth medium, and modified growth medium known as halophilic media such as DSMZ-97, DSMZ-823, DSMZ-1184, Halophilic medium, chemically defined medium, complex media and OS media, which were used with NaCl concentration ranging from 10, 15, 20 to 25% (*w*/*v*). In another isolation method, soil aliquots (~0.5 g) were spread directly onto surface of different medium plates at sampling sites. Serially diluted water samples were filtered through 0.22 μm membrane filters and isolation was done using imprinting method. Irrespective of the plating method used, the plates were incubated at 30, 37 and 45 °C in different light conditions (Dark, bright and diffused light) for 10–25 days. For slow growing archaea, plates were incubated upto for 45–60 days. Brightly coloured colonies were selected and purified by repeated streaking, to obtain isolated colonies using respective medium plates. The pure cultures were maintained at 4 °C as slant and glycerol stock (20%) at −80 °C for further use. The effect of salinity, temperature, water stress and pH on archaeal isolates was studied by observing their growth on respective halophilic growth medium as method described earlier (Yadav et al. 2015e). The isolated archaeal strains were screened for plant growth promoting attributes using standard protocols.

PCR amplification of 16S rRNA gene and phylogenetic analysis

The genomic DNA of purified archaea was isolated using standard methods as described earlier (Verma et al. 2015). Amplification of 16S rRNA gene was done by using archaeal specific primers 27F (5′-TTCCGGTTGATCCYGCCGGA-3′) and 958R (5′-YCCGGCGTTGAMTCCAATT-3′). Amplification of haloarchaeal 16S rRNA gene was done by polymerase chain reaction (PEQ STAR Thermocyclers, PEQLAB Biotechnology, USA). The PCR amplification was carried out in a 100 μL volume by mixing 50–90 ng DNA template with the polymerase reaction buffer (10X); 100 μM (each) dATP, dCTP, dTTP and dGTP; primers 27F and 958R (100 ng each) and 1.0 U Taq polymerase. The amplification conditions were as follows: initial denaturation of 5 min at 95 °C, followed by 25 cycles of 1 min at 95 °C, 1 min at 50 °C and 2 min at 72 °C, and a final extension period of 10 min at 72 °C. The PCR amplified 16S rRNA genes were purified by QIA quick PCR product purification kit (Qiagen).

Table 1 Details of sampling sites, pH and electrical conductivity of samples collected from Rann of Kutch

Sample Code	Sampling sites	Autumn		Winter		Summer	
		pH	EC*	pH	EC	pH	EC
R1SN	*Suaeda nudiflora*	7.6	24.2	8.2	32.3	8.1	43.1
R1BG	*Cressa critica*	9.2	5.42	9.1	3.5	9.9	2.25
R2OP	Rhizospheric soil	7.9	54.2	7.9	32.3	8.8	14.12
R2SO	Non-rhizospheric soil	8.3	58.5	7.6	106.7	9.5	40.2
R3SP	*Sporobolus*	8.7	1.29	8.2	88.5	9.1	50.3
R3AB	*Abutilon*	8.4	1.19	8.4	1.81	8.8	30.0
R3CS	*Cenchrus setigerus*	7.8	2.69	8.3	2.21	8.9	32.0
R3SC	Non-rhizospheric soil	8.2	62.2	8.1	59.3	9.2	67.18
R4WR	White Rann water	8.9	ND	8.8	ND	9.3	58.7
R4SC	White Rann sediments	9.1	ND	8.8	109.3	9.3	95.3

Regions: R_1: 23°30′21″N: 69°39′69″E; R_2: 23°48′20″N: 69°43′75″E; R_3: 23°57′69″N: 69°43′95″E and R_4: 23°49′67″N: 69°31′43″E; *Electrical conductivity; EC (mS cm^{-1})

PCR products of partial 16S rRNA gene were sequenced with fluorescent terminators (Big Dye, Applied Biosystems) and run in 3130xl Applied Biosystems ABI prism automated DNA sequencer at SCI Genome Chennai, India. 16S rRNA gene sequences were analysed using codon code aligner v.4.0.4. The 16S rRNA gene sequences were aligned to those of closely related archaeal species available at GenBank database using BLASTn program. Archaeal isolates were identified based on percentage of sequence similarity (≥97%) with that of a prototype strain sequence in the GenBank. The phylogenetic tree was constructed on the aligned datasets using the Maximum likelihood (ML) method implemented in the program MEGA 4.0.2 (Tamura et al. 2007). Bootstrap analysis was performed as described by Felsenstein (1985) on 1000 random samples taken from the multiple alignments. In order to compare the archaeal diversity within the three seasons and at four different regions, the 16S rRNA gene sequences were used to analyze diversity index. The Shannon index (H), Evenness (J) and the Simpson's index (D) were calculated as described earlier (Yadav et al. 2015a). Using 16S rRNA gene sequences, the rarefaction curves were generated to compare the relative diversity and coverage of each sample. Principal component analysis (PCA) was used to determine the statistical correlation between population diversity of three seasons as described earlier (Yadav et al. 2015c).

Accession numbers

The sequences obtained in this study were submitted to the GenBank database at NCBI, and accession numbers assigned were KF650663–691 and KJ875291–352.

Result

Physico-chemical characteristics of samples

Physico-chemical characteristics of the soil, sediment and water varied considerably amongst the samples and seasons. The values of pH were highly variable from 7.6 to 9.2, 7.6–9.1 and 8.1–9.9 in autumn, winter and summer respectively. The pH values of all samples collected in summer season were higher than samples of autumn and winter (Table 1). The electrical conductivity is presented in Table 1. Electrical conductivity is highest in samples collected from Region 4 of Rann of Kutch during all seasons. Correlation analysis proved existence of significant relationship between the different parameters and sampling sites. The first two factorial axes (F1 to F2) of biplot represent 73.20 to 20.8%, 47.89 to 31.02% and 79.22 to 13.17% for autumn, winter and summer respectively (Supplementary Fig. S1).

Enumeration and characterization of archaea

The population of archaea was enumerated in different samples collected from Rann of Kutch, Gujarat, India (Supplementary Table S1). Significant variations were observed among the culturable putative archaeal population of each sample on seven different media. The abundance of archaea in the samples did not showed significant variations among time of sampling and the CFU values varied from 6.0×10^4 to 2.65×10^5, 7.0×10^4 to 2.45×10^5 and 5.7×10^4 to 2.33×10^5 CFU g^{-1} sediment or mL^{-1} water during autumn, winter and summer season respectively (Supplementary Table S1). During all the three sampling times, highest population was recorded in the rhizosphere of *Suaeda nudiflora* on halophilic medium while lowest was recorded in the rhizosphere of Banni grass on DSMZ-97 medium. The pure colonies obtained from each sample on different media were isolated based on colony morphology and cultural characteristics. A total of 253 distinct putative archaeal colonies were obtained from 10 samples in each season at four different regions of Rann of Kutch, Gujarat, India. Among 253 putative archaea, only 157 isolates showed amplification with archaea specific primers for 16S rRNA gene and were selected for further studies. The remaining isolates showed amplification with 16S rRNA gene universal primers (pA and pH) for eubacteria and the sequencing of the amplified gene confirmed them to be bacteria mainly belonging to Firmicutes (*Bacillus vallismortis, Bacillus halodurans*, *Bacillus mojavensis*, *Halobacillus* sp., *Halobacillus dabanensis*, *Virgibacillus* sp., *Virgibacillus halodenitrificans*, *Oceanobacillus manasiensis*) (Yadav et al. 2015e) and Proteobacteria (*Marinobacter alkaliphilus, Halomonas venusta, Halomonas* sp., *Nitrincola lacisaponensis, Ochrobactrum* sp.). The halophilic bacteria were dominant on plats incubated for pro-longed durations.

All the 157 archaeal isolates were characterised for pigmentation and their tolerance to temperature, pH, salt and water stress and the results are presented for 28 representative archaeal strains (Table 2). All twenty eight strains showed variations in their ability to grow at different pH ranging from 4 to 10; NaCl concentrations ranging from 0.86–5.48 M; temperature ranging from 25 to 65 °C and water stress ranging from 0 to −0.75 Mpa (Table 2). Strain IARI-ABB4 could not grew beyond 0.86 M NaCl concentration, while four strains IARI-MAAB1, IARI-CFAB1, IARI-CFAB4 and IARI-SGAB2 could tolerate upto 5.48 M NaCl concentration. Among twenty eight strains, two strains IARI-TWAK7 and IARI-WRS9 showed growth in a narrow range of NaCl concentrations, that is 3.42–5.13 M and 3.42–4.28 M respectively. Strains, IARI-CDK2, IARI-CSK1, and IARI-WRAB4 could grow in the range of pH 6–10 while eleven strains showed a narrow pH range of 6–8. Majority of archaeal strains could grow in water stress condition (−0.50 Mpa). Five strains, IARI-SNS3, IARI-SNS2, IARI-WRAK9, IARI-WRAK7 and IARI-SGAB3 could tolerate 60 °C while one

Table 2 Identification and characterization of halophilic archaea

Strain number	Nearest GenBank match	Similarity	Salinity	Temp	pH	Drought (Mpa)	Pigmentation
IARI-ABB4	*Haladaptatus paucihalophilus*	100	0.86–5.13	25–50	6–8	−0.50	Pink
IARI-SOAB1	*Haloarcula argentinensis*	99	1.71–4.28	30–55	6–9	−0.25	Orange- red
IARI-DWAK3	*Haloarcula* sp.	99	2.57–5.13	30–55	6–8	−0.25	Red
IARI-WRAK3	*Haloarcula tradensis*	100	2.57–5.13	30–55	6–8	−0.25	Red
IARI-SNS3	*Halobacterium* sp.	98	2.57–4.28	25–60	5–9	−0.75	Red
IARI-SNS2	*Halococcus hamelinensis*	99	1.71–5.13	30–60	4–8	−0.50	Orange- pink
IARI-BGAK2	*Halococcus* sp.	99	1.71–4.28	25–55	5–8	−0.50	Red
IARI-MAAB1	*Haloferax alexandrinus*	98	1.71–5.48	25–55	5–8	−0.25	Red
IARI-CFAB1	*Haloferax larsenii*	98	1.20–5.48	30–55	6–9	−0.25	Orange -red
IARI-MAAK4	*Haloferax* sp.	100	1.20–4.28	30–55	6–9	−0.25	Red
IARI-CFAB4	*Haloferax volcanii*	99	1.20–5.48	30–45	6–9	−0.25	Orange -red
IARI-WRAK9	*Halogeometricum borinquense*	99	1.20–4.28	30–60	6–9	–	Pink
IARI-WRAK7	*Halogeometricum rufum*	99	1.71–5.13	25–60	5–9	–	Red
IARI-CSK1	*Halolamina pelagica*	98	1.20–5.13	25–50	6–10	−0.75	Red
IARI-CDK2	*Halolamina* sp.	99	1.71–5.13	25–50	6–10	−0.50	Red
IARI-MAAB3	*Halopenitus persicus*	100	1.71–4.28	25–50	6–8	–	Pink
IARI-WRAB4	*Halorubrum* sp.	99	2.57–5.13	30–55	6–10	−0.25	Red-orange
IARI-WRAB3	*Halosarcina* sp.	99	2.57–5.13	25–50	6–8	−0.50	Red
IARI-TWAK7	*Halostagnicola kamekurae*	98	3.42–5.13	25–55	7–10	–	Pink
IARI-SGAB3	*Haloterrigena hispanica*	99	2.57–4.28	30–60	6–9	−0.75	Red
IARI-SOAB2	*Haloterrigena* sp.	99	1.71–4.28	30–55	6–8	−0.25	Red
IARI-SNAB1	*Haloterrigena thermotolerans*	100	2.57–4.28	37–65	6–8	−0.75	Red
IARI-SGAB2	*Natrialba* sp.	99	2.57–5.48	25–55	7–10	−0.75	Red
IARI-WRAK5	*Natrinema altunense*	100	2.57–4.28	30–55	6–8	–	Orange -red
IARI-WRAK8	*Natrinema pallidum*	100	2.57–4.28	30–50	6–8	–	Orange
IARI-WRS9	*Natrinema* sp.	99	3.42–4.28	30–55	6–8	−0.75	Red
IARI-SSAB3	*Natronoarchaeum mannanilyticum*	100	2.57–5.13	25–55	6–9	–	Light Red
IARI-MAAB4	*Natronomonas pharaonis*	100	2.57–5.13	30–55	6–8	−0.25	Red

strain IARI-SNAB1 could grow beyond 65 °C. The results showed that all the isolates formed pigmented colonies (red, pink and orange) (Table 2).

16S rRNA gene sequencing and phylogenetic diversity

Sequencing of 16S rRNA gene was carried out for all the 157 archaeal isolates and the sequenced data were analysed by BLAST. The nearest match from the NCBI GenBank database for each of the 157 isolates has been reported. 16S rRNA gene based phylogenetic analysis performed on a total of 28 representative sequences from all three seasons and different regions revealed that the sequences were affiliated with twenty eight distinct species of 16 genera namely *Haladaptatus, Haloarcula, Halobacterium, Halococcus, Haloferax, Halogeometricum, Halolamina, Halopenitus, Halorubrum, Halosarcina, Halostagnicola, Haloterrigena, Natrialba, Natrinema, Natronoarchaeum,* and *Natronomonas* (Fig. 1). On phylogenetic analysis the 28 halophilic archaea were distributed into five groups (I-V) as shown in Fig. 1. Overall, among all isolates members of genera *Halococcus* (27%) were most dominant followed by *Haloferax* (18%), *Halolamina*, *Haloterrigena* (10% each), *Haloarcula* (9%), *Halobacterium* (8%), *Natrinema* (5%), *Halogeometricum*, *Halostagnicola* (3% each), *Halosarcina*, *Natrialba* (2% each) and *Haladaptatus*, *Natronoarchaeum*, *Halopenitus*, *Halorubrum*, *Natronomonas* (1% each) (Fig. 2).

Seasonal variations in archaeal community

Seasonal variations were observed in the community structure of archaea isolated from different regions of Rann of Kutch. The 46 archaeal isolates in autumn seasons were phylogenetically grouped into nine genera: *Haladaptatus*, *Haloarcula, Halobacterium*, *Halococcus, Haloferax, Halolamina Halosarcina, Haloterrigena* and *Natrinema*. Among them,

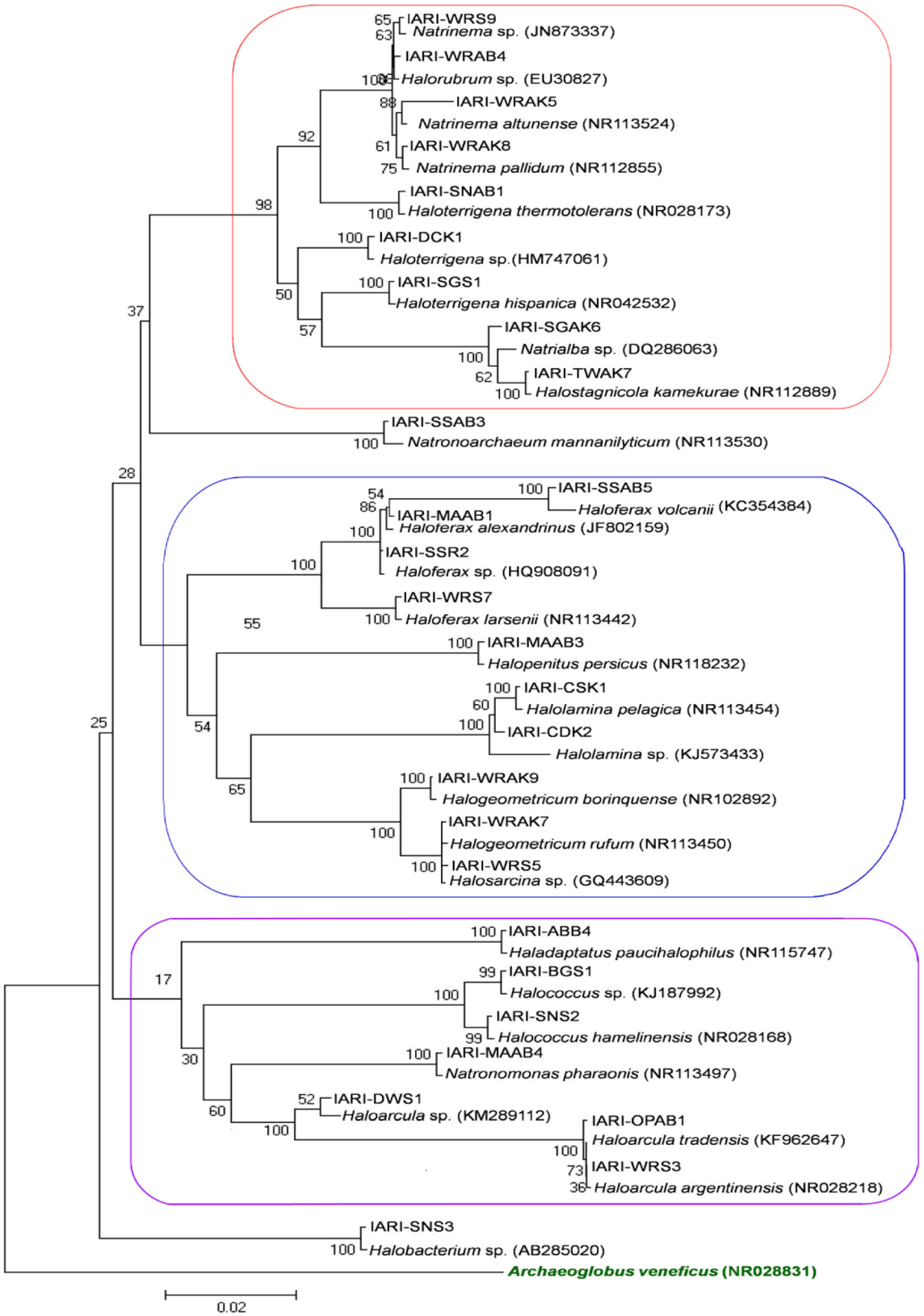

Fig. 1 Phylogenetic tree showing the relationships among 28 archaea, 16S rRNA gene sequences with reference sequences obtained through BLAST analysis. The sequence alignment was performed using the CLUSTAL W program and trees were constructed using Maximum likelihood (ML) with algorithm using MEGA4 software (Tamura et al. 2007). The tree was rooted using *Archaeoglobus veneficus* (NR028831), as the out group

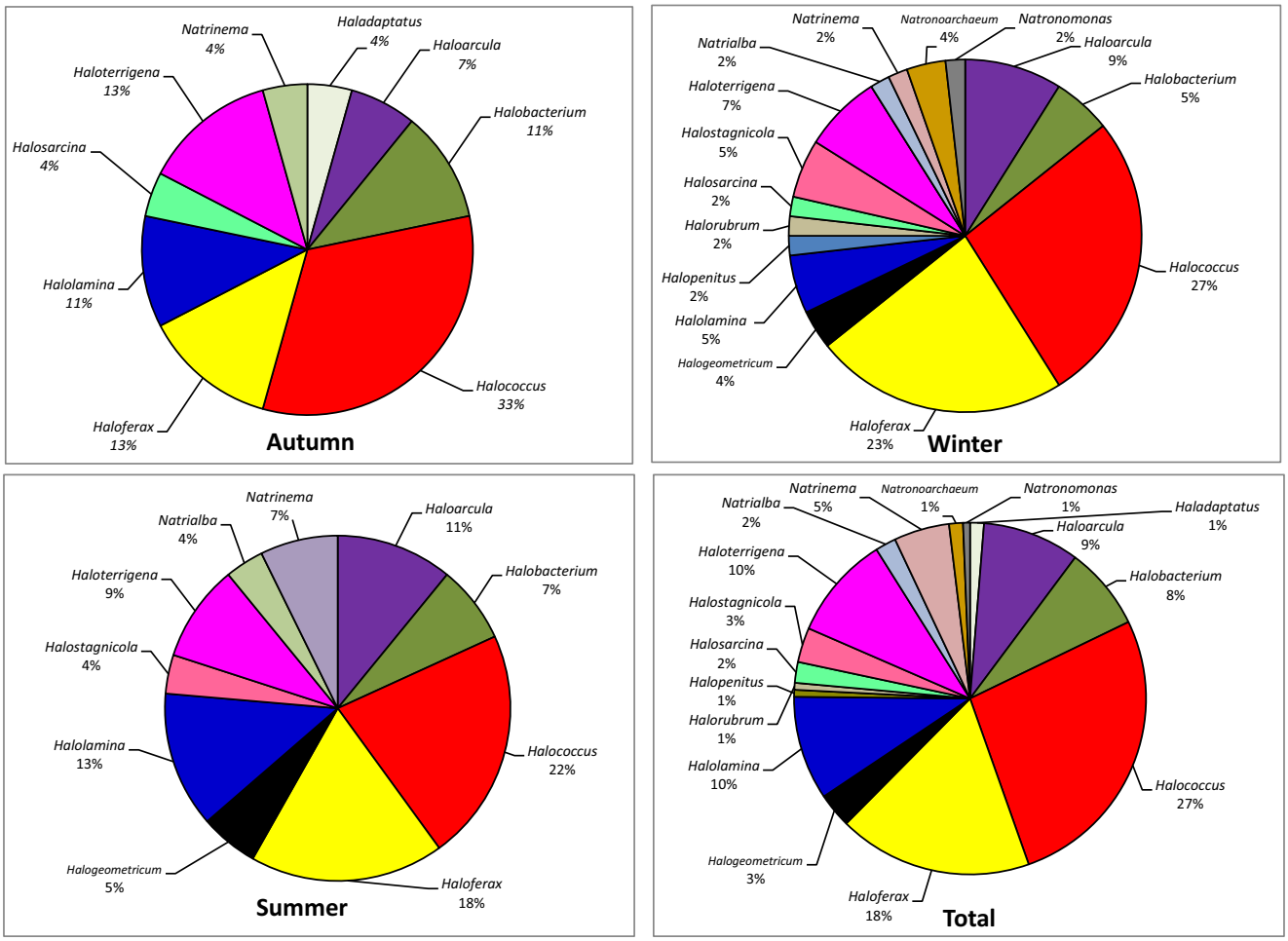

Fig. 2 Relative abundance of different archaeal genera isolated from the four investigated regions in three seasons. The pie charts show the relative proportions of archaeal genera calculated as percentage of the total number of isolates retrieved from each regions

Halococcus was most dominant while *Haladaptatus* was least dominant (Fig. 2). In winter season, a total 56 archaeal isolates were identified as 23 distinct species of 15 genera with highest diversity of *Halococcus* (27%) followed by *Haloferax, Haloarcula, Halobacterium, Halogeometricum, Halolamina, Halopenitus, Halorubrum, Halosarcina, Halostagnicola, Haloterrigena, Natrialba, Natrinema, Natronoarchaeum* and *Natronomonas* (Fig. 2). The partial 16S rRNA gene sequences belonging to 55 isolates in summer season grouped into 10 genera: *Haloarcula*, *Halobacterium*, *Halococcus*, *Haloferax*, *Halogeometricum*, *Halolamina, Halostagnicola, Haloterrigena, Natrialba* and *Natrinema* (Fig. 2). Again *Halococcus* sp. was predominant at all the sites (27%).

Sequencing of the 157 archaeal isolates from the ten sites of four regions could be categorised into 22, 38 and 31 clusters (Supplementary Table S2). The calculated indices of diversity showed that the phylogenetic diversity was highest among the haloarchaea cultured in winter season, while the lowest diversity was found in autumn season (Supplementary Table S2). Shannon's diversity index recorded highest and lowest value for winter and autumn season respectively (Supplementary Table S2; Fig. 3a). These observations are also supported by archaeal diversity parameters, such as Simpson's index, Chao-1, Evenness and Shannon Entropy and individual rarefaction curves (Supplementary Table S2; Fig. 3a). Principal component analysis was used to investigate relationships between archaeal diversity. The first two dimensions of PCA (PCA1 and PCA2) explained 79.24% of the total variation, with component 1 accounting for 46.20% and component 2 for 33.04% of the variance (Fig. 3b). Archaeal genera reported as common and season-specific are represented in Venn diagram (Fig. 3c).

Archaeal community structures at different regions

The community structure of archaea also varied at different sampling sites. The distribution of 157 archaeal isolates was 33 each from R1 and R2, 50 from R3 and 41 from R4 (Supplementary Table S2). Again *Halococcus* was the

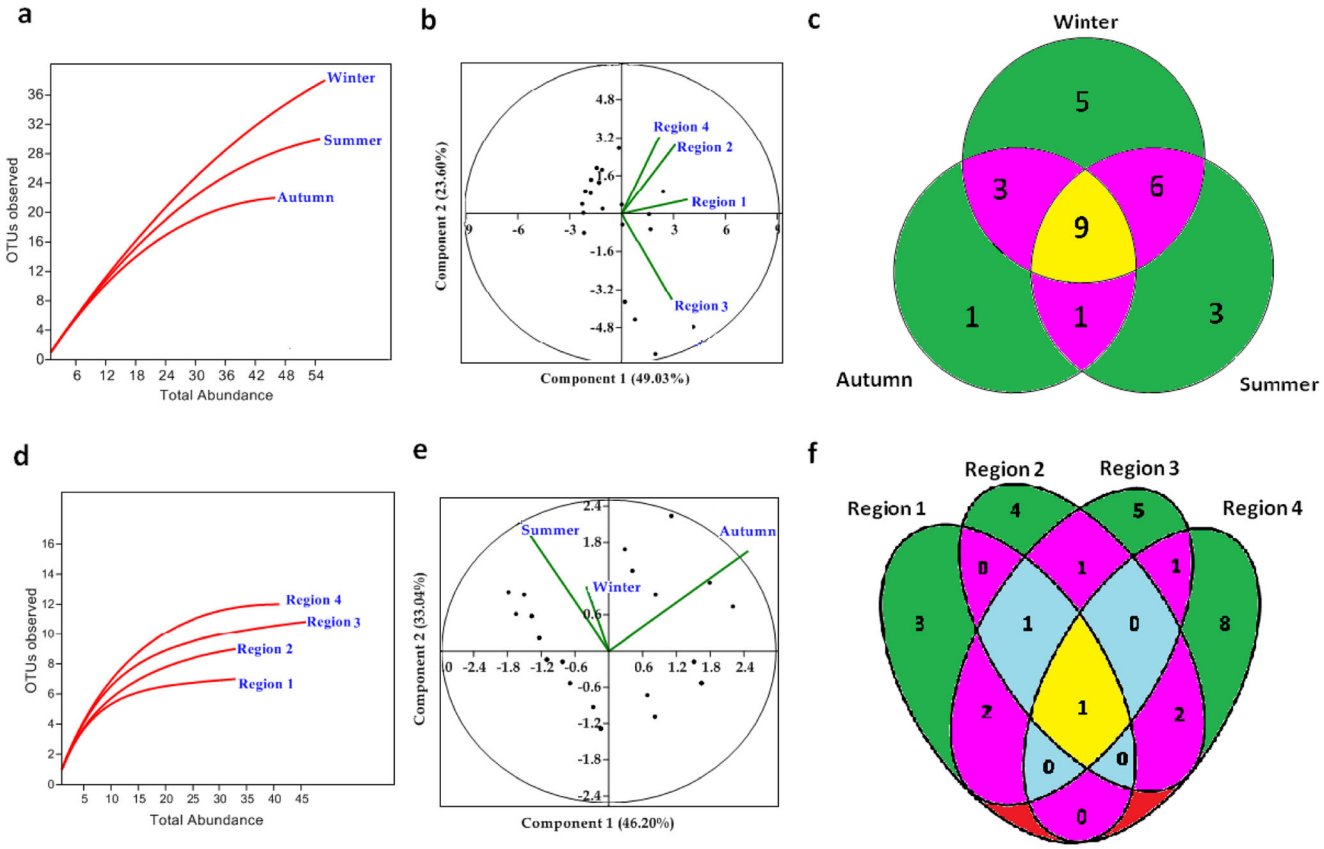

Fig. 3 **a.** Rarefaction curves of observed OTUs in the samples from three seasons**, b**. Principal component analysis of the diversity indices (H) of the16S rRNA gene profiles of archaeal isolates from three seasons, **c.** Venn diagram showing the numbers of shared and niche-specific archaeal isolates in different seasons and regions of Rann of Kutch

predominant genera from regions R2 and R4 while *Haloterrigena* and *Haloferax* dominated in regions R1 and R3. The diversity indices showed that the archaeal diversity was highest diverse among the haloarchaea cultured in region 4 followed by region 3 (Supplementary Table S2). Shannon's diversity index recorded highest (H = 2.34) and lowest value (H = 1.80) for region 4 and region 1 respectively (Supplementary Table S2) and these observations are also supported by Chao-1, Evenness and Shannon Entropy and individual rarefaction curves (Fig. 3d). PCA was used to investigate relationships between archaeal diversity in different regions and the first two dimensions of PCA (PCA1 and PCA2) explained 74.63% of the total variation, with component 1 accounting for 49.03% and component 2 for 23.60% of the variance (Fig. 3e). Archaeal genera reported as common and niche-specific are represented in Venn diagram (Fig. 3f).

Archaeal community comparison using PCA biplot

The relative abundances of genera among four different regions were used in a principal component analysis (PCA) to create a PCA biplot (Fig. 4). The distances of genus in the biplot with respect to regions are indicative of the respective maximal abundance of genera; e.g., *Haloferax* abundance is highest in the region 3. Genus in biplot between two regions said to be nearly equal abundance such *Halococcus* between region 2 and region 1. The results indicate shared genera between region 2 and region 3 such as *Halococcus* and *Haloterrigena*. The results also highlight the uniqueness of the region 4, mainly due to the high abundance of the genus *Haladaptatus* and *Natrinema* (Fig. 4).

Discussion

Haloarchaea are known to inhabit hypersaline environments such as solar salterns, hypersaline lakes, the dead sea, hypersaline microbial mats and underground salt deposits. We are carrying out studies at Rann of Kutch to look for role of archaea in sustenance of vegetation under extreme saline conditions further accentuated by drought and nutrient deficiency. In this context, seasonal variations in population dynamics of archaea inhabiting barren

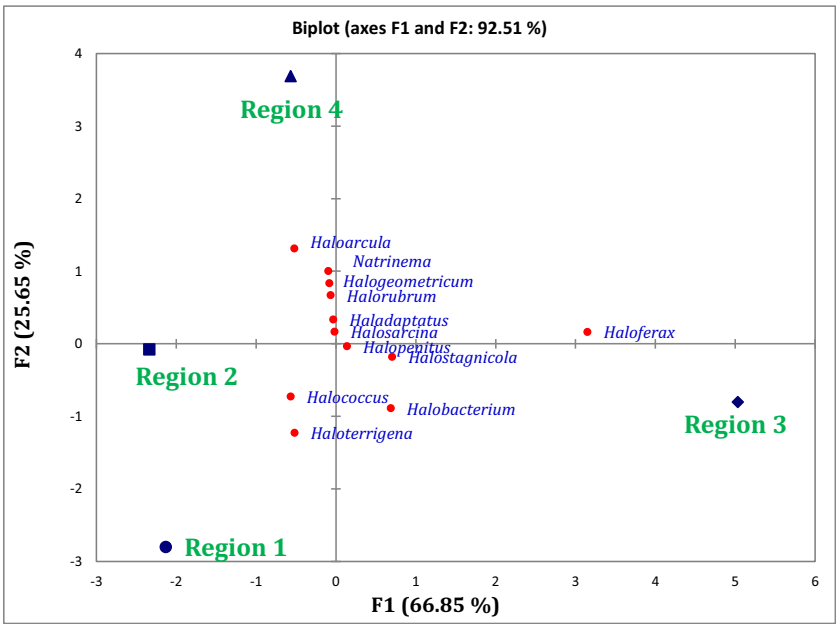

Fig. 4 Principal component analysis biplot of distribution of genera in four different regions of Rann of Kutch

land, salt pan or rhizosphere of monocots and dicots growing in extreme saline and moisture deficit regimes was taken up so as to see the fluctuations in total culturable population and genetic make up of archaeal population in different seasons. Haloarchaea were isolated characterised for population dynamics, phylogenetic diversity, community structure in rhizospheric, non-rhizospheric, hypersaline water and sediments from different region of Rann of Kutch. The data generated for plant growth promoting attributes of archaea was also correlated with the predominant archaea prevalent in all seasons (data not presented).

The hypersaline region represents hot spots of biodiversity and several novel salt tolerant archaeal and bacterial species have been isolated from different hypersaline habitats (Bodaker et al. 2010; Kanekar et al. 2015; Oren 2008; Saxena et al. 2016). In our present investigation a total of 253 putative halophilic archaea were isolated in three seasons, of which 157 was confirmed as archaea based on amplification of 16S rRNA gene using archaea specific primers. Phylogenetic analyses of 157 isolates suggest that archaea adapted to hypersaline environment forms a monophyletic group within the kingdom *Euryarchaeota*, the *Halobacteriaceae*, with 16 different genera. Culture-independent, molecular phylogenetic studies of hypersaline habitats have indicated more phylotypes and novel lineages within the *Halobacteriaceae* (Pagaling et al. 2012). In three different seasons, twenty eight distinct species of 16 genera namely *Haladaptatus, Haloarcula, Halobacterium, Halococcus, Haloferax, Halogeometricum, Halolamina, Halopenitus, Halorubrum, Halosarcina, Halostagnicola, Haloterrigena, Natrialba, Natrinema, Natronoarchaeum* and *Natronomonas* were isolated from ten different sites in four geographical regions of Rann of Kutch, India. In recent study on microbial communities from the Kutch region, using metagenomic approach, 67 known genera of archaea have been reported form different sites of Kutch (Pandit et al. 2015). The culture dependent approach followed in this study could retrieve only 24% of 67 genera reported through culture independent approach. Similar results have been reported earlier in many studies (Oren 2015; Roh et al. 2010).

Population and community structures of archaea were different in all three seasons. Highest diversity and population of archaea was recorded in winter season. Nine species of archaea namely *Haloarcula argentinensis, Halobacterium* sp., *Halococcus* sp., *Haloferax larsenii, Haloferax* sp., *Halolamina pelagica, Halolamina* sp., *Haloterrigena hispanica* and *Haloterrigena* sp. were common and routinely isolated from samples collected in all seasons (Table 3, Fig. 5a). Among these nine species, *Halococcus* sp. was found to be predominant and constituted 27% of the community deciphered through cultural approach. However following metagenomic approach, (Pandit et al. 2015) reported *Haloarcula*, *Halogeometricum*, *Natromonas* and *Halobacterium* to be the dominant genera in hypersaline habitat of Kutch. In the present study, these four genera were isolated but their percent contribution in the community structure was low- *Haloarcula* (9%), *Halogeometricum* (3%), *Natromonas* (1%) and *Halobacterium* (8%). There were certain species isolated only during two seasons like *Haloarcula tradensis, Haloferax alexandrinus, Haloferax volcanii, Halogeometricum borinquense, Halostagnicola kamekurae* and *Natrialba* sp. during summer and winter; *Natrinema* sp., *Halococcus hamelinensis* and *Halosarcina* sp. during winter

Table 3 Population dynamics of archaea: Niche specific and seasonal fluctuation

Halophilic archaea	Autumn				Winter				Summer			
	R1	R2	R3	R4	R1	R2	R3	R4	R1	R2	R3	R4
Haladaptatus paucihalophilus				●								
Haloarcula argentinensis				●		●		●		●		
Haloarcula sp.				●								●
Haloarcula tradensis						●						●
Halobacterium sp.	●		●		●		●		●		●	
Halococcus hamelinensis	●				●							
Halococcus sp.	●	●	●	●	●	●	●	●	●	●	●	●
Haloferax alexandrinus							●				●	
Haloferax larsenii			●	●			●	●			●	●
Haloferax sp.			●				●				●	
Haloferax volcanii							●				●	
Halogeometricum borinquense								●				●
Halogeometricum rufum												●
Halolamina pelagica			●		●				●			
Halolamina sp.		●	●			●				●	●	
Halopenitus persicus							●					
Halorubrum sp.								●				
Halosarcina sp.				●				●				
Halostagnicola kamekurae							●				●	
Haloterrigena hispanica	●				●							
Haloterrigena sp.	●		●		●	●			●			
Haloterrigena thermotolerans					●							
Natrialba sp.						●				●		
Natrinema altunense												●
Natrinema pallidum												●
Natrinema sp.												●
Natronoarchaeum mannanilyticum							●					
Natronomonas pharaonis							●					

Red dot-all season and all sites; black dot- any two or more season/sites, Blue dot- all seasons; Pink dot-Niche specific archaea

and autumn and *Haloarcula* sp. during autumn and summer (Table 3, Fig. 5a). There were many archaea that were recovered only in one season and were designated as season specific such as *Haladaptatus paucihalophilus* for autumn; five strains of *Natronoarchaeum mannanilyticum, Natronomonas pharaonis, Halopenitus persicus, Halorubrum* sp. and *Haloterrigena thermotolerans* for winter and three strains of *Halogeometricum rufum Natrinema altunense Natrinema pallidum* for summer (Table 3, Fig. 5a). Similar type of seasonal variations among bacteria has been reported from Bhitarkanika, a tropical mangrove ecosystem in India (Mishra et al. 2012), Lake Namco, the largest Tibetan lake (Liu et al. 2013) and Atlantic forest soils in Cardoso Island, Brazil (Pupin and Nahas 2014). However there are no reports available for seasonal variations of culturable archaea in saline habitats.

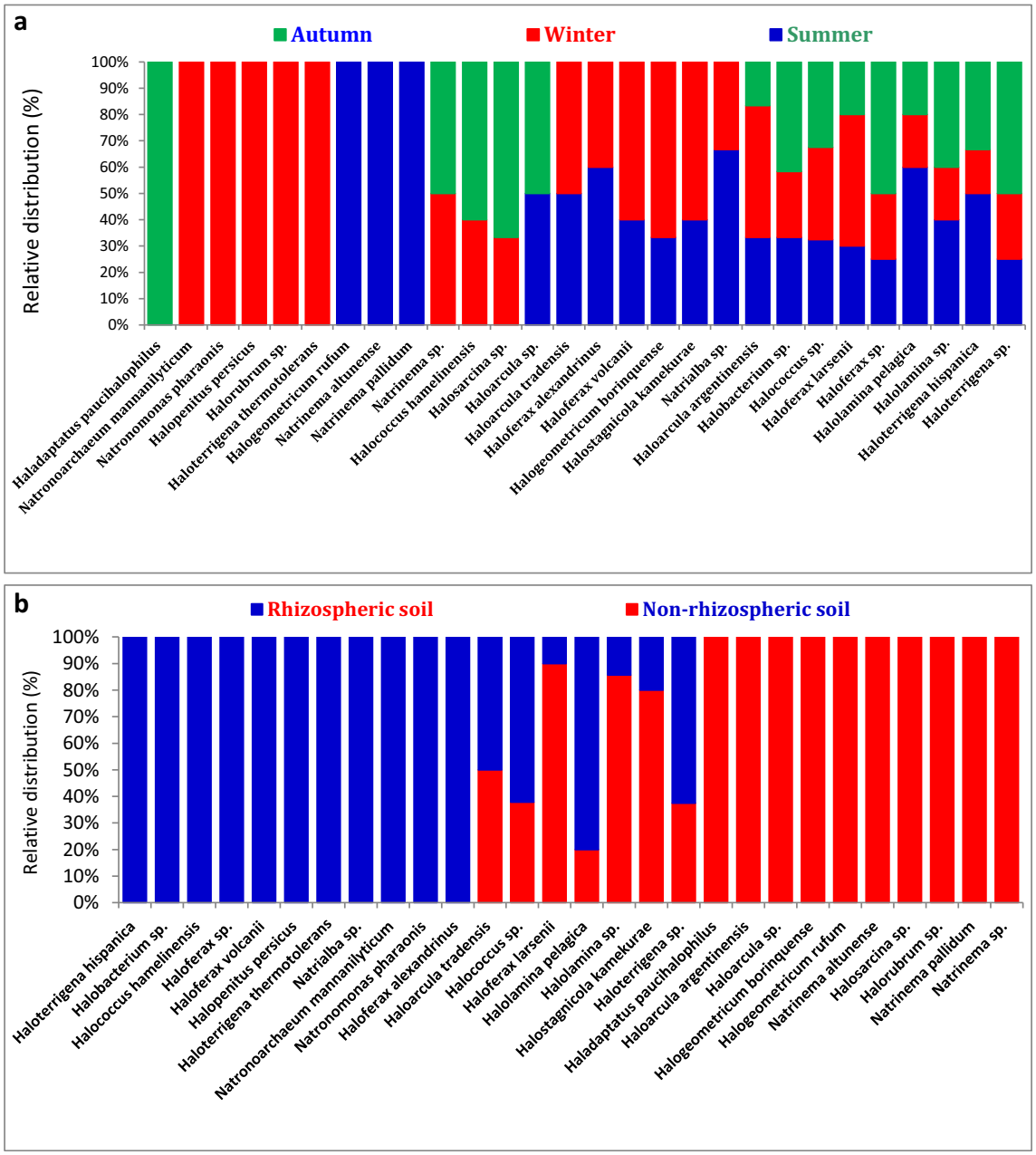

Fig. 5 Relative distribution of archaeal genera in; **a.** three seasons; **b.** rhizospheric and non-rhizospheric samples

The perusal of the data on diversity also revealed variations in the distribution of halophilic archaea among samples collected from rhizosphere and non-rhizosphere soil. Of the 28 distinct species identified in the study, only 7 were recovered from rhizospheric and non- rhizospheric samples. There were ten species specifically isolated from rhizospheric samples while 11 species were isolated from non rhizospheric samples (Table 3, Fig. 5b). The archaeal isolates obtained in the present study were characterized for seven plant growth promoting (PGP) attributes - solubilization of phosphorus and potassium, production of IAA, abscisic acids, zeatin and siderophore and ACC deaminase activity (data not presented). The most predominant genus *Halococcus* exhibited six traits- P and K solubilization and production IAA, abscisic acids, zeatin and siderophore. Among the ten isolates specifically obtained from rhizosphere, eight isolates (80%) exhibited three or more traits. However, among the 11 isolates obtained from non rhizosphere samples, only 5 showed three or more PGP attributes. The results do indicate the importance of distribution of PGP archaea in the rhizosphere. However it is difficult to correlate the prevalence of efficient PGP archaea only in the rhizosphere of plants growing in these hypersaline habitats. For example, the most efficient P solubilizer, *Natrinema* sp. strain IARI-WRAB$_2$ was isolated from non rhizospheric soil of Region 4 (Yadav et al. 2015d). It is worthwhile to mention that

the region 4 had the highest values of EC among all the sites. There are no earlier reports available with regard to distribution of PGP archaea specifically in the rhizosphere or non rhizosphere, However parallel reports are available for bacteria (Chen et al. 2015; Qi et al. 2012).

The culture dependent approach provides a baseline to study the seasonal variations in the community structure of archaea at different sites in Rann of Kutch. The correlation of the diversity data with prevalence of PGP traits among archaea gives credence to our hypothesis that archaea could play a major role in establishment of vegetation in extreme environments of Rann of Kutch.

Acknowledgments The authors are grateful to the Division of Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi and National Fund for Basic, Strategic and Frontier Application Research in Agriculture (NFBSFARA) project "Role of Archaebacteria in Alleviation of Salinity and Moisture Stress in Plants" Indian Council of Agricultural Research for providing the facilities and financial support, to undertake the investigations.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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