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Genetic diversity and phylogenetic analyses of culturable extremely haloarchaea isolated from marine solar saltern pond in Mumbai, India

Dipak T. Nagrale[1, 3]*, Renu[1], Priyanka Das[2]

[1]ICAR- National Bureau of Agriculturally Important Microorganisms, Kushmaur, Maunath Bhanjan-275103, India. [2]ICAR- Central Inland Fisheries Research Institute, Barrackpore, Kolkata-700120, India. [3]ICAR- Central Institute for Cotton Research, Post Bag No.2, Shankar Nagar P.O., Nagpur, India.

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ABSTRACT

The diversity and phylogenetic analyses of culturable haloarchaea from crystallizer saltern pond was studied on the basis of 16s rDNA sequences determined with *Haloarcula* spp. Four brine samples were taken from different sites of marine solar saltern crystallizer pond in hot summer and six distinct extremely halophilic archaea were isolated on specific haloarchaea agar medium. The rDNA were amplified with specific 16S rRNA primer by PCR, identified through BLASTn and EzTaxon programme as members of family *Halobacteriaceae*. On the basis of 16S rDNA sequences results, we confirmed strains M1(1), M3(1) and M2(2) of *Haloarcula marismortui* by 98.77%, 98.98% and 98.69% respectively. Similarly, others were resembled as *Haloarcula salaria* M2(1), *Haloarcula argentinensis* M4(1) and *Haloarcula quadrata* M4(2) with 98.69%, 95.98% (BLASTn 99% similarity with *Haloarcula marismortui* ATCC 43049 chromosome II) and 98.84% respectively, under extremely haloarchaeon genera *Haloarcula*. However, present study indicated that similarity coefficient of these strains and species sequences suggesting novel strain(s) and even taxa especially for strain *Haloarcula argentinensis* M4(1). The identity of new strains of *Haloarcula* from these sites provides new horizon from marine solar saltern crystallizer ponds from coastal areas Mumbai, India.

1. INTRODUCTION

Most extremely hypersaline habitats and environments are in hot and dry areas of the world. Salt lakes can vary considerably in ionic composition; depend to a major extent on the surrounding topography, geology, and general climatic conditions. Alkaline salt lakes located in Africa, India, China and other places with pH values more than 11 and higher and salt concentrations exceeding 300g/l are teeming with life [29]. The microbial diversity in these hypersaline habitats has been studied by molecular, ecological [3, 6, 7] and culturable approaches [9, 10]. Some strains may grow at salt concentrations minimum 1.5 M at 37ºC, most of the strains grow best at concentrations of 3.5-4.5 M and grow well in saturated NaCl (5.2 M) [1]. Haloarchaea often display a reddish colouration due to presence of caretonoid pigments.

* *Corresponding Author*
E-mail: dip29unique @ gmail.com

Haloarchaea belonging to the class *Halobacteria* consisting of 14 genera in Bergey's Manual of Systematic Bacteriology [16]. The genus *Haloarcula* currently comprises eight recognized species, *H. amylolytica* [21]; *H. vallismortis* [15, 38]; *H. argentinensis* [20]; *H. hispanica* [21]; *H. japonica* [35]; *H. marismortui* [30]; *H. quadrata* [31] and *H. salaria* [27]. However, *Haloarcula mukohataei* has been renamed as *Halomicrobium mukohataei* [28]. The *Haloarcula* genus categorized under extremely haloarchaeons group which are red to orange coloured with square, triangular, rectangular, short to pleomorphic rods and irregular in shapes. The marine solar saltern pond of Mira Road is located between northern portion of Salsette Island and at the northern part of the Konkan region of Arabian Sea. It is mainly of Deccan lava terrain and consists of waterlogged and marshy areas. Mira road marine solar saltern pond located on the Basin of Arabic Sea was selected for the study with an objective to determine and analyze the genetic diversity of extremely haloarchaea from hypersaline niches of marine solar saltern pond in the closer suburban area of Mumbai based on 16S rRNA gene sequence analysis.

2. MATERIALS AND METHODS

2.1 Survey and sampling sites

Hypersaline habitats were surveyed from Mumbai suburban and Arabian Sea coastal areas. Brine samples were taken from four different sites of marine solar saltern pond located at Mira road (19°16'N 72° 51E), Mumbai. It was selected on the basis of typical pink-red colouration of hypersaline sites located in saltern crystallizer ponds.

2.2 Isolation and growth conditions

Halophilic archaea were isolated by directly spreading 0.1 ml of brine sample on haloarchaea agar plates and by using membrane filter technique [26] and [12]. Different colonies developed on haloarchaea agar and the filters were obtained and purified on haloarchaea agar plates.

2.3 Molecular characterization

2.3.1 Phylogenetic analysis

A rapid and small scale method of chromosomal DNA extraction was done [11]. The 16S rDNA of all six isolates were amplified using haloarchaea specific primers. Forward primer (Forward 27- 5'ATTCCGGTTGATCCTGCCGAAG3') [1] and reverse primer (Reverse R1521-5'AGGAGGTGATCCAGCCGCAG3') [39] were used.

The PCR volume of 20 µl was set for amplification as (sterile distilled water: 11.5 µl; taq polymerase buffer (10X): 3 µl; forward primer F 27 (20 pmoles): 1µl; reverse primer (R1521): 1µl; dNTP's (10 mM with 2.5 mM each): 1µl; target DNA template (~100 ng/µl): 1.5 µl and taq polymerase (3 units/ml): 1 µl (Merck, India). PCR programme was set with 105ºC lid temperature following step 1: initial denaturation, 94ºC for 3 minutes; step 2: denaturation, 94ºC for 30 second; step 3: primer annealing, 64ºC for 30 seconds; step 4: primer extension, 72ºC for 1 minute and 30 seconds. Step 2, 3 and 4 were repeated for 35 cycles. The final extension was given at 72ºC for 10 minutes. Amplification was performed in G-Storm thermocycler. The purity and size of each PCR product was studied by gel electrophoresis on 0.8% agarose gel made in 1X TAE (Tris Actetate EDTA) buffer containing 0.5 µg/ml ethidium bromide. 1.5 kb ladder was used as molecular weight marker for comparison of size of PCR products.

The gel was visualized in UV transilluminator. All the PCR reagents were obtained from Merck Bioscience Pvt Ltd, India. The PCR amplicon was purified by Exo-SAP solution (Affymetrix USB, US). 8 µl of Exo-SAP solution was added in each DNA amplicon for purification of each 20 µl PCR products and incubated for 10 min at 37 ºC in water bath. Then, again it was incubated at 80 ºC for 10 min in water bath for inactivating proteins and excess PCR ingredients.

Table 1: Haloarchaea isolates obtained from different sites of Mira road marine solar saltern pond.

S. No.	Site	Co-ordinates	Isolate(s)/strain ID
1	Salt pan I	19º16'40.70" N 72º 51'15.01" E	M1(1)
2	Salt pan II	19º16'41.76" N 72º 51'15.58" E	M2(1), M2(2)
3	Salt pan III	19º16'40.88" N 72º 51'15.21" E	M3(1)
4	Salt pan IV	19º16'45.96" N 72º 51'17.71" E	M4(1), M4(2)

Table 2: References and nucleotide sequence accession numbers studied.

Organism	Strain	Accession numbers	References
Haloarcula marismortui	M1(1)	KJ526219	This study
Haloarcula salaria	M2(1)	KJ526220	This study
Haloarcula argentinensis	M4(1)	KJ526221	This study
Haloarcula quadrata	M4(2)	KJ526222	This study
Haloarcula marismortui	M3(1)	KJ526223	This study
Haloarcula marismortui	M2(2)	KJ526224	This study
Haloarcula sp.	E2 strain	U68539	(4)
Haloarcula sp.	E211 strain	U68537	(4)
Haloarcula sp.	A43	DQ309094	(32)
Haloarcula argentinensis	arg-1	NR_028218	(20)
Haloarcula sp.	AB19	DQ471854	(34)
Haloarcula sp.	G10	JN112003	(23)
Haloarchaeon	EH4	FJ868736	(8)
Haloarcula californiae (rrnA gene)	JCM 8912	AB477984	(25)
Haloarcula hispanica	HLR4	DQ089683	(22)
Haloarcula sp.	A337	DQ309092	(32)
Archaeon	BC27	HQ425119	(24)
Sulfophobococcus zilligii	K1	NR_029316	(18)

2.3.2 *16S rDNA sequencing and analysis*

The amplified 16S rDNA of isolates was sequenced using an automated Applied Biosystems DNA sequencer. The PCR amplicon with 100 ng concentrations of each DNA was used for sequencing. The quality of chromatogram was checked and edited with AB Sequence scanner v 1.0 software. The contigs were made by DNA baser from forward and reverse sequences of each isolates. The reference strains of *Haloarcula* and related 16S rRNA partial gene used in the 16S rRNA analysis has shown in Table 2. Phylogenetic trees were constructed by the neighbor-joining method (NJ) in MEGA var 4.0 [36]. Bootstrap analysis was performed as described [13] on 1000 random samples taken from multiple alignments. The partial 16S rRNA gene sequences of the haloarchaea strains were compared with those available in the database like EZ taxon and NCBI BLASTn search and sequences were submitted to NCBI gene bank database. Identification to the species level was determined as 16S RNA gene sequence similarity of with that of prototype strains sequences in the gene bank. Sequence alignment and comparative analyses was performed using multiple sequence alignment program CLUSTAL W [37] considering default parameters with data conversion to PHYLIP format.

2.4 Nucleotide sequence accession numbers

The NCBI GenBank accession numbers of the isolated strains are KJ526219-KJ526224.

2.5 Biodiversity index

The biodiversity and evenness of species of microbial community was measured by the Shannon index [19].

3. RESULTS

3.1 Isolation of haloarchaea

Total 6 haloarchaea were isolated (Table 1) and screened (Fig. 1), out of which two each were from salt pans II and IV whereas, one each from salt pan I and III, respectively. The six haloarchaea isolates (accession numbers: NAIMCC-B-1790 to NAIMCC-B-1795) were deposited in recognized national repository of NAIMCC unit of ICAR-National Bureau of Agriculturally Important Microorganisms, Kushmaur, Maunath Bhanjan- 275103, India.

3.2 Amplification of 16S rRNA gene

16S rDNA from all isolates was amplified by PCR method using archaea specific primers. Amplicon size approximately 1500 bp was obtained from all the isolates.

3.3 Diversity and phylogenetic analysis

Total 16S rDNA sequences obtained from each of the six isolates examined and were compared with a database of type cultures in EzTaxon and NCBI nucleotide BLASTn search [2]. On the basis of archaeal 16S rRNA gene, 100% archaeal community was closely related to phylum Euryarcheota with different species of *Haloarcula marismortui*, *Haloarcula argetinensis*, *Haloarcula salaria* and *Haloarcula quadrata*. Comparative study of the *Haloarcula* spp. in study and strains sequences with other *Haloarcula* spp./strains in the NCBI database indicates that they are most closely related to the other species and/or strains of the genus *Haloarcula*.

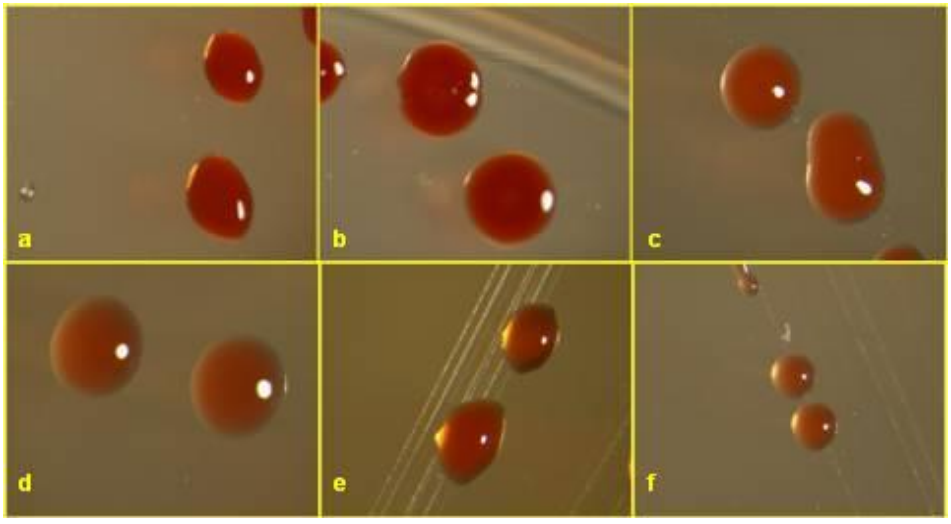

Fig. 1: Colony morphology and pigmentation of *Haloarcula* isolates on HA medium.
[a: *Haloarcula marismortui* M1(1), b: *Haloarcula salaria* M2(1), c: *Haloarcula argentinensis* M4(1), d: *Haloarcula quadrata* M4(2), e: *Haloarcula marismortui* M3(1), f: *Haloarcula marismortui* M2(2)]

3.4 Biodiversity index of extremely haloarchaea

The Shannon diversity indices measured by the diversity analysis of the isolates revealed that the cultured extremely haloarchaea from Mira road marine solar saltern pond obtained as Shannon-Wiener diversity index (H) =1.243 and evenness=0.896.

4. DISCUSSION

4.1 General features and phenotypic properties of strains

Total 6 *Haloarcula* strains were screened and selected from four different salt pan sites brine samples of marine solar saltern crystallizer pond located at Mira road suburban, Mumbai, India. All these isolates produced dark red to blood red pigmented colonies on solid HA media at optimum 37ºC after 8-10 days. However, the pigmentation slightly varied among the isolates as compared to respective type strains. On the basis of polyphasic characterization, these isolates were identified as extremely haloarchaeon members of family *Halobacteriaceae* [17].

4.2 Diversity and phylogenetic analysis

On the basis of archaeal 16S rRNA gene, 100% archaeal community was closely related to phylum Euryarcheota with different species of *Haloarcula marismortui*, *Haloarcula argetinensis*, *Haloarcula salaria* and *Haloarcula quadrata*. In earlier studies, *Haloarcula* was also designated as extreme halophilic archaeon [14, 5]. The phylogenetic tree based on 16S rRNA gene reveals a single major group of archaea, especially Euryarchaeota consists several species of *Haloarcula* with little differences of bootstrap values. In the phylogenetic tree constructed (Fig. 2), two major groups were appeared, I and II with slight difference in bootstraps. Sequence similarity matches with *Haloarcula hispanica* and Archaeon sp. lie in cluster II while *Haloarcula argentinesis*, *Haloarcula californiae* and Haloarcheon come under cluster I. M1(1) KJ526219 matches with *Haloarcula hispanica* with highest bootstrap value in cluster II. The 16S rRNA sequence analyses revealed that, M1(1) strain was analyzed to identify as *Haloarcula marismortui* by 98.77%. The identity of M3(1) and M2(2) strains confirmed to *Haloarcula marismortui* by 98.98% and 98.69% respectively. Apart from strains of *Haloarcula marismortui*, strain M2(1) resembled *Haloarcula salaria* JCM 15759^T by 98.69% [27]. Strain M4(1) resembled with *Haloarcula argentinensis* JCM 9737^T [14] by 95.98% and 99% with *Haloarcula marismortui* ATCC 43049 chromosome II by BLASTn.

Isolate M4(2) was resembled to *Haloarcula quadrata* species by 98.84% [31]. All our six isolates identified under single dominant genus *Halarcula*, most probably because of their high temperature requirement for growth, hypersaline habitat compared with other haloarchaea group. Two strains of *Haloarcula* were isolated [39] (*H. argentinensis* and *H. hispanica*) in the month of April, 2002 from Ayakekumu Lake, China. Similar results obtained in this study as brine samples collected from Mira road marine crystallizer saltern pond, Mumbai, India in the month of April, 2012 which also obtained only one dominant genus *Haloarcula* as extremely haloarchaeon group. In the present study we have isolated and characterized 6 different strains of single dominant genus *Haloarcula* in hot summer from a marine solar saltern pond located at Mira road, Mumbai, India. According to 16S rRNA gene sequence analysis and phenotypic characteristics, these six strains were clustered in four different species of *Haloarcula* as *Haloarcula marismortui* [M1(1), M3(1) and M2(2)] *Haloarcula salaria* M2(1), *Haloarcula argentinensis* M4(1) and *Haloarcula quadrata* M4(2).

The extremely halophilic archaeon genus *Haloarcula* is the dominant inhabitant (>15% NaCl w/v conc.) of hypersaline environments, is highly recombinogenic and reported rapid genomic variation [33]. The results presented in this study may contribute to genetic diversity of *Haloarcula* archaeal taxonomy from marine solar saltern pond and hypersaline habitats of India. This extreme Indian hypersaline niches elucidating and indicating the abundance of salt tolerant extremely haloalkaliphilic archaeal species and strains with several biotechnological potentials.

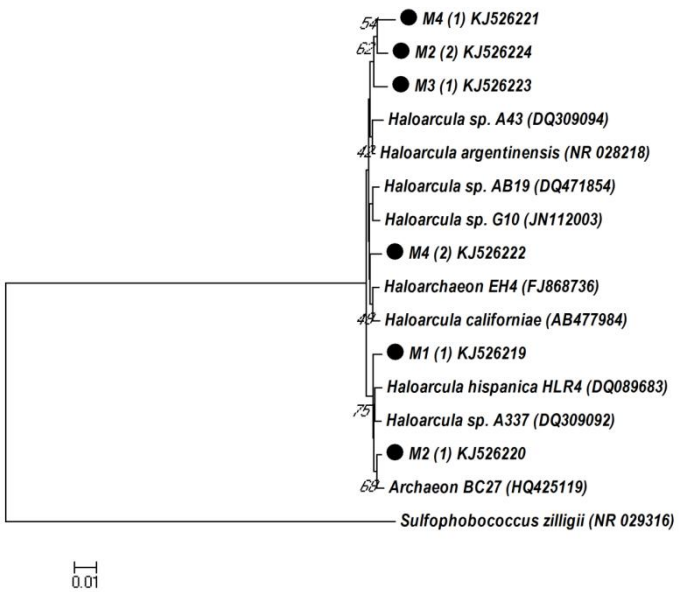

Fig. 2: Phylogenetic tree based on 16S rRNA gene sequence similarity data of six *Haloarcula* isolates in this study and other species of the family *Halobacteriaceae*. *Sulfophobococcus zilligii* was included as out-group representative of non halophilic archaea. The scale bar determined by measuring the lengths of horizontal lines connecting any two strains/species.

5. CONCLUSION

The genetic diversity of extremely halophilic archaea isolates indicated that one genus is dominant i.e. *Haloarcula* but with four different species in hot summer of Mira road marine solar saltern ponds. Recent and advanced techniques are necessary to study culturable extremely halophilic archaea and possibility of novel strains from coastal regions saltern ponds of Arabian Sea, indicating taxonomic lineage in area specific niches.

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Conflict of Interests: There are no conflicts of interest.

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