RESEARCH ARTICLE

A novel haloarchaeal lineage widely distributed in the hypersaline marshy environment of Little and Great Rann of Kutch in India

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Archaea (Archaebacteria) are a phenotypically diverse group of microorganisms distributed mainly in extreme environments throughout the world. To study the community of archaea present in the extreme salty marshes of the Rann of Kutch, Gujarat, India culture-dependent approach was employed for isolating archaea by mimicking extreme hypersaline environment and 25 extreme halophilic archaea were obtained. Phylogenetic analysis revealed the presence of a number of uncultivated and unidentified members of archaea among the isolates present. Phylogenetic analysis involving the sequence data of 16S rRNA of 25 haloarchaeal isolates, keeping *Methanospirillium hungatei* DSM864 (M60880) as the out-group, also identified a novel lineage of three isolates. The 16S rRNA sequences of the strains 3A1-DGR, H9-DGR and 2ANA-DGR showed less than 93% similarity with the available known type strains and these three strains belong to a distinct novel lineage within the family Halobacteriaceae, near an uncultivated environmental cluster.

Keywords: Diversity, haloarchaea, marshy environment, novel lineage, phylogeny.

ARCHAEA have been the subject of intensive study owing to their prevalence in extreme environments. These organisms not only contribute to planetary biomass, but their metabolism might be essential for bio-geochemical cycles[1]. Being recognized as 'the third domain of life'[2], archaea are believed to have evolved on this planet nearly 4 billion years ago. Evolutionarily, the domain archaea has learnt to thrive in extreme conditions including extremities of pH, temperature, pressure, salt, etc.

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 overall diversity and population of physiological groups of microorganisms, therefore, decrease to few taxonomical groups when the concentration of salt gradually reaches the saturation value (5.5–6.0 M).

The salt pans are 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[3,4].

The present study is focused on the archaebacteria-dominated extreme environments of the Little and Great Rann of Kutch, which have not yet been fully explored. In this context, morphological, physiological, biochemical and molecular analysis, especially 16S rRNA gene sequences, were studied to evaluate the diversity of archaea in these hypersaline conditions and to identify the existence of novel haloarchaeal lineage, if any.

Materials and methods

Collection and characterization of samples

Soil and water samples, 59 in number, were collected from different accessible regions of the Little and Great Rann of Kutch during summer months (April–June) of 2007–2010. Each location was marked by a GPS device; and the region covered lies between 23°10.727′ N, 070°43.333′E of the Little Rann and 23°57.7215′N, 069°43.7611′E of the Great Rann of Kutch. Soil samples were collected from the top 15 cm, including the salt crust, and stored in sterile polythene bags. Water samples were also collected following standard protocol. Samples were transported to the laboratory immediately and processed or kept at 4°C until further processing. The variation in water samples was analysed by physiological and biochemical parameters, especially temperature, ratio of ions, pH, salinity, etc. following standard protocols throughout the study period.

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Enumeration of microbial population

The total population of culturable archaea was determined by directly spread-plating the samples onto different known standard halophilic media[5] like complex medium, minimum growth medium (MGM), modified growth medium (MGM) for haloarchaea, halophilic medium (HM), standard growth medium, DSMZ medium 1184, DSMZ medium 97, etc. These media contained NaCl ranging from 18% to 23%, thus mimicking the salinity levels of Kutch[6,7]. The total microbial community count was taken from brine samples by direct counting of cells in a Neubauer-improved counting chamber (Paul Marienfed, Lauda-Königshofen, Germany) using Phase Contrast Microscope (Olympus BX41, Olympus Corporation, Japan). Each sample was analysed for > 50 microscopic fields. For the isolation of halophilic archaea, water samples were serially diluted and spread-plated on different halophilic media as mentioned above. Replicates of the plates were incubated in different BOD incubators at different temperatures (28°C, 37°C and 42°C). Individual colonies were picked and streaked until a pure colony was obtained in the respective medium. The cultures were maintained at 4°C as slants and glycerol stock was maintained at –20°C for further use.

Isolation of genomic DNA and amplification of 16S rRNA

The genomic DNA of purified archaea was isolated using the genomic DNA isolation kit obtained from Promega (Promega, Madison, WI, USA) according to the manufacturer's instructions with minor modification, in which washing and lysis buffer was not used. The purified genomic DNA was used as template for amplification of 16S rRNA using known primers, 21F (5′-TTCCGGTTGATCCYGCCGGA-3′) and 958R (5′-YCCGGCGTTGAMTCCAATT-3′), as previously described[8], for amplifying haloarchaeal 16S rRNA by polymerase chain reaction (Mastercycler Gradient, Eppendorf, Germany). The PCR amplification conditions were as follows: 50 µl of total volume, 20 pmol of each primer, 2 U of *GoTaq* polymerase (Promega, Madison), 2/25 volume of dNTPs (2 mM), and a 1/5 volume of 5× *GoTaq* buffer provided with the enzyme. After a denaturation step of 5 min at 94°C, amplification reactions were performed with 30 cycles of denaturation (1 min, 94°C), primer annealing (1 min, 55°C) and primer extension (1 min, 72°C) with a final 7 min extension step at 72°C. The PCR products were resolved in agarose gel (1%). For isolation of near full-length 16S rRNA, specific primer combinations like 21F and 1492R as well as arc8f (5′-TCCGGTTGATCCTGCC-3′) and arc1492r (5′-GGCTACCTTGTTACGACTT-3′) were used[9–11]. All the PCR-amplified products were gel-purified after excising the expected amplicons from 1% agarose gel using the QIAquick® Gel extraction kit (Qiagen, Chatsworth, California, USA), according to the manufacturer's instructions.

Sequencing of 16S rRNA and phylogenetic analysis

The sequencing reaction of gel-purified PCR amplicons was carried out with a set of primers using BDT v3.1 Cycle sequencing kit, and sequencing was done in ABI 3730xl Genetic Analyser at Xcelris Labs Ltd, Ahmedabad. All the sequences were aligned using the multiple alignment program[12] Clustal W; the consensus sequence was generated and checked for chimeric artefacts with the Check Chimera program as available in the Ribosomal Database Project (RDP)[13]. The sequences were compared with the GenBank non-redundant database, using the BLASTn program available in the National Centre for Biotechnology Information (NCBI), USA (http://www.ncbi.nlm.nih.gov/BLAST/). Similarity matrices were constructed by pairwise analysis, and evolutionary distance matrices were generated according to a method described elsewhere[14]. Phylogenetic trees were then constructed by neighbour joining (NJ) method[15,16]. A bootstrap analysis was performed, in 1000 trial replications, in order to provide confidence estimates for the topology of the phylogenetic tree[17]. Evolutionary analyses were conducted using MEGA5 program[18]. The sequences obtained in this study were submitted to the GenBank database at NCBI, and were designated accession numbers JF802138, JF802141, JF802142, JF802144–JF802165.

Results

Physico-chemical properties of the samples

The colour of water of the salt crystallizers was light red to deep red indicating the dominance of archaeal population. The concentration of NaCl in majority of the water (brine) samples collected from hypersaline environments of the Rann of Kutch was near saturation, indicating the extremity of the environment. The pH of the samples varied from location to location and ranged from 6.34 to 8.70. The total dissolved solids (TDS) of brine samples varied from 110 to 470 g 1000 ml^{-1} of water, electrical conductivity (EC) of brine samples varied from 8.25 to 118.7 mS cm^{-1}, and that of soil or mud samples from 9.62 to 115.9 mS cm^{-1}. The temperature of samples ranged from 8°C in winter to 45°C in summer. The amount of anions and cations varied from time to time and location to location. Table 1 shows the overall mean values of the samples collected from the Great and the Little Rann of Kutch. The trend in the concentration of cations throughout the Rann of Kutch was $Na^+ > Mg^{2+} > K^+$, and at a few locations in the Great Rann of Kutch it was $Mg^{2+} > Na^+$. In the case of anions, however, the trend

Table 1. Comparison of physico-chemical properties of different hypersaline and marine ecosystems worldwide, including the Little and Great Rann of Kutch

Ecosystem	pH	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	SO_4^{2-}
Solar Saltern (Puerto Rico)	Nd	65.4	5.2	20.1	0.2	144.0	1.9
Great Salt Lake (USA)	7.7	105.0	6.7	11.1	0.3	181.0	27.0
Lake Assal (Djibouti)	Nd	77.8	5.4	8.0	14.6	164.0	2.3
Dead Sea	7.8	40.1	7.6	44.0	78.2	225.0	0.44
Wadi Natrum (Egypt)	11.0	142.0	2.3	nd	nd	155.0	22.6
El Golea Salt Lake (Algeria)	9.0	107.0	nd	0.3	0.4	198.0	Nd
Lake Magadi	11.0	161.0	0	2.3	0	111.0	23.4
Great Rann of Kutch	6.9	46.0	2.0	16.1	Trace	157.0	47.0
Little Rann of Kutch	8.1	65.0	2.8	15.8	Trace	119.0	32.0
Sea water	8.2	10.8	0.4	1.3	0.4	19.4	2.7

Concentration of ions in thalassohaline and athalassohaline brines: concentration of different salts (g l^{-1}). nd, Not done; data obtained from the literature[30,31]. Data for Little and Great Rann of Kutch are from the present study.

$Cl^- > SO_4^{2-} > Br^- > PO_4^{3-} > F^-$ was maintained throughout the samples and locations. In comparison to the other known hypersaline environments, the samples collected from the Little and the Great Rann of Kutch contained more SO_4^{2-} and Mg^{2+} ions. Deposition of Ca^{2+} could not be detected. NaCl content in the samples collected from the Little Rann of Kutch was very high and the total salt concentration in majority of the samples was near the saturation level. There was wide variation in the composition among the samples, indicating dynamism in the areas in terms of salt deposition.

Population of haloarchaea

The population of extreme halophilic archaea was very low in dry and wet sediments compared to water samples collected from both the locations. During the plate count, only coloured colonies were counted and the mean of three replications was depicted as CFU (cfu g^{-1} or ml^{-1}). The population of archaea in the hypersaline water and soil samples ranged from 6.26×10^3 to 3.42×10^4 CFU across locations. Direct microscopic count of total population (CFU ml^{-1}) in brine, however, ranged from 2.01×10^7 to 2.90×10^7 across samples collected from all the locations. This indicated that 0.1% of the total population can be cultured. In few brine samples, where total salt concentration was less, some eubacterial isolates were also obtained. In most of the brine samples, *Dunaliella*, a red-coloured alga, was observed. All the isolates were able to grow well in halophilic archaeal medium and majority of the isolates showed maximum growth when the medium was supplemented with 18–23% NaCl. The prevalence of hard, sticky colonies was also observed from almost all the samples, the colour of which varied from light pink to cream.

Identification of isolates on the basis of 16S rRNA sequence data

Out of 73 archaeal isolates, 25 were finally selected on the basis of morphological characterization, including differences in colony characteristics, pigmentation, growth characters, level of tolerance of NaCl, etc. and then characterized physiologically and biochemically (data not shown). Identification of the isolates on the basis of near-complete 16S rRNA sequences revealed the prevalence of common haloarchaeal genera like *Halobacterium*, *Natrinema*, *Haloferax*, *Haloarcula*, *Halorubrum*, *Halogeometricum*, *Halomicrobium*, and unclassified and unidentified phylotype within the haloarchaeal cluster, most similar to the sequence of uncultured haloarchaeal clones.

Phylogenetic analysis and identification of novel lineage

For identification of a novel lineage within the present pool of isolates, based on 16S rRNA gene sequences, all the 16S rRNA sequences of all the type strains of 33 genera were retrieved from NCBI and used as reference. The analysis, using the NJ method, consistently placed the isolates within the phylum Euryarchaeota and family Halobacteriaceae, keeping *Methanospirillum* as the outgroup. Comparison of the sequences of 16S rRNA of 25 isolates obtained from the Rann of Kutch with those retrieved from the NCBI database showed less than 93% similarity of three isolates (2ANA-DGR, 3A1-DGR and H9-DGR) to the nearest neighbour *Halorubrum* (Figure 1), indicating a novel lineage.

Morphological and physiological properties of isolates of novel lineage

The representative isolates of this new lineage were present in almost all the hypersaline water samples collected from the Little and the Great Rann of Kutch, indicating their prevalence, abundance and distribution in these salty marshes. The cell shape varied from irregular to coccoid (Table 2), compared to the rod shape of *Halorubrum*, with an outer diameter (OD) of 2.1–2.4 μm. The organisms required a minimum of 12% salt concentration,

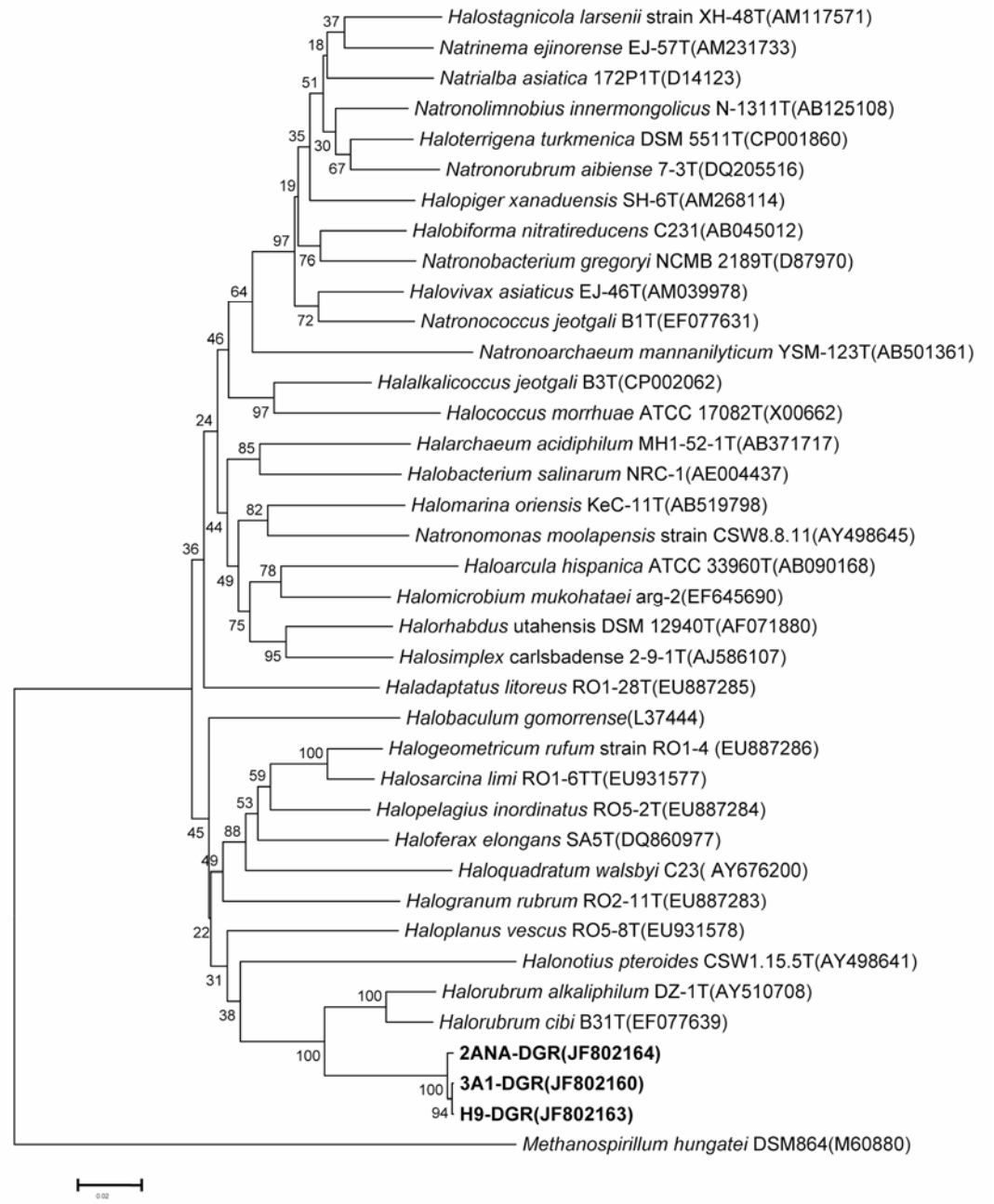

Figure 1. Construction of phylogenetic tree of the family Halobacteriaceae by neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches; bootstrap performed with 1,000 replications to provide confidence. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. *Methanospirillum hungatei* (DSM864) was kept as out-group.

optimum temperature of 37–42°C and pH range 7–8 to reach log phase of growth in 5 days.

Confirmation of novel lineage

To confirm the phylogenetic position of the new clade, this monophyletic clade was further analysed by including distantly related archaeal sequences of both cultured and uncultured groups. The sequences of the representative isolates of the new clade were identical to few reference sequences of unclassified taxa (Figure 2). Few of them were found to be closely related to sequences obtained from uncultured halophiles which were previously isolated from various marine sediments and hypersaline environments elsewhere.

Analysis of the sequences of the new clade with the retrieved sequences from cultured organisms showed the phylogenetic affiliation of these individuals close to

Table 2. Comparison of the new lineage of haloarchaeon with the nearest genera *Halorubrum*

Trait	Organism/isolate			
	Halorubrum	2ANA-DGR	3A1-DGR	H9-DGR
Shape of cells	Rod	Irregular cocci	Irregular cocci	Irregular cocci
Pigmentation	Deep red	Creamy in dark and light red in illuminated condition	Creamy in dark and light red in illuminated condition	Creamy in dark and light red in illuminated condition
Toughness of the colony	Smooth without toughness	Hard and tough	Hard and tough	Hard and tough
Motility	Motile	Non-motile	Non-motile	Non-motile
Minimum and maximum range of NaCl (M) which supports growth	2–5	2–6	2–6	2–6
Optimum temperature for growth (°C)	37–50	37–42	37–40	40–42

Halorubrum that belonged to the Euryarchaeotal clade. This analysis confirmed the existence of a new lineage, sequences of which are clearly distinct from known classified haloarchaeal sequences available in the NCBI database, consisting of a few new, expected genera in salt-rich, marshy environment in the Little and the Great Rann of Kutch.

The new lineage consisted of unique, hard, sticky, brown-coloured haloarchaeon isolates (3A1-DGR, 2ANA-DGR and H9-DGR), phylogenetically belonging to Halobacteriacea in the phyla Euryarchaeota, having least similarity to the cultivated existing lineages. The archaeal isolates of this new lineage were unique in the sense that they could adapt to wide variations of fluctuating salty settings of the Little and the Great Rann of Kutch, and showed discrepancy in growth from low to high concentrations of NaCl, high concentrations of other divalent cations (especially Mg^{2+}) and high concentrations of fluoride and bromide. They were also able to sustain wide variations in the concentration of salts formed from water-logged condition during monsoon to formation of white salt desert during summer months.

Discussion

Considering the relatively unexplored, conserved and unique nature of the hypersaline environments and their extremities in the Little and the Great Rann of Kutch, attempts were made to evaluate the group of organisms that would thrive in the extreme conditions with special emphasis on archaebacteria. It was evident that with gradual increase in the concentration of salts in the Rann environments, both the total microbial count and the representative groups of organisms changed dramatically. At near the saturation level of salts only few groups of organisms could survive (unpublished data from our laboratory) which included extreme halophilic archaea.

The population of extreme halophilic archaebacteria was very low in the soil (nearly 10 times less) or sediment samples compared to the water samples. Though direct count was several thousand times more than the *in vitro* culturable population, it did not reflect the total variability present in the population, relative abundance of individual organisms and also the total population of archaea because all of them could not be cultured.

Therefore, within the limited pool of culturable archaea obtained from the Little and the Great Rann of Kutch, the present analysis was undertaken to find out the presence of novel lineage, if any. So far, a large number of novel archaeal phylotypes have been isolated and studied in a variety of microbial habitats, including those in open ocean waters[19], salt crystallizers[20], coastal waters[8,21], polar seas[10,22], salt marshes[23], freshwater lakes[24], agricultural and forest soils, including the rhizosphere[25], paddy-field soil[26], hot springs[27], deep-sea hydrothermal vents[28], mine water and deep subsurface geothermal pools[29].

In this study, detailed analyses of the 16S rRNA sequence data of the present isolates and sequence data of all the genera of archaea retrieved from the NCBI gene bank accessions led to the identification of a novel lineage which was distinct from any previously described archaeal group. The novel lineage of three archaeal isolates, viz. 2ANA-DGR, 3A1-DGR and H9-DGR represented a potentially important and unidentified group of haloarchaeons. With the identification of the new lineage, the phylogenetic diversity of archaea has thus been extended substantially. The new lineage was found to be abundant and widely distributed in the extreme salty environments as prevalent in the Little and the Great Rann of Kutch. The undisturbed ecosystems of the Great Rann and continuous exploration of salts from the salterns of the Little Rann did not affect their abundance and distribution, indicating that evolutionarily the new lineage was conserved. Thus, the new lineage might be playing significant roles, not yet known, in the biogeochemical cycles in the extreme hypersaline situations of this region.

In addition to identification of a new lineage of haloarchaeons in the Little and the Great Rann of Kutch, the

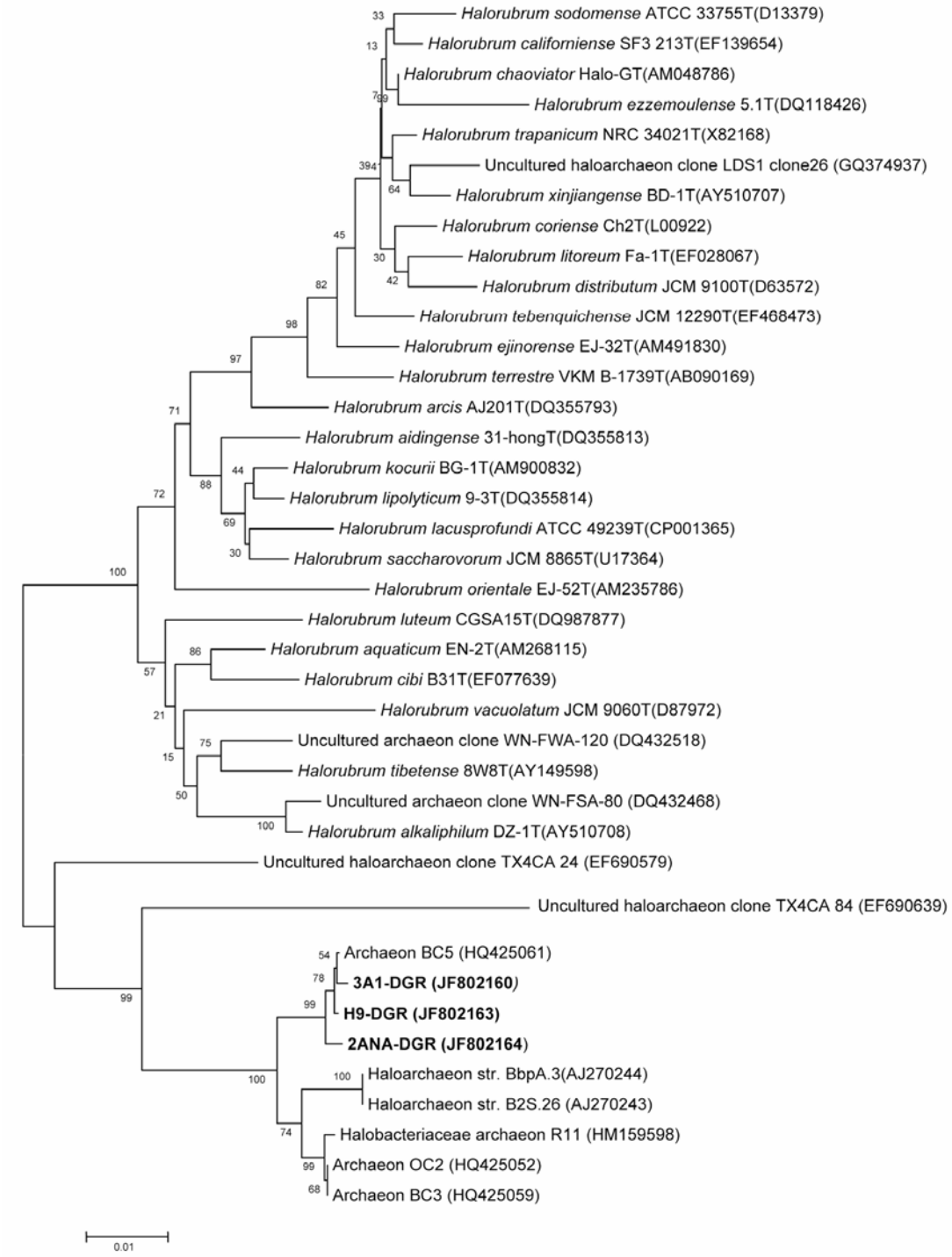

Figure 2. Reconstruction of phylogenetic tree of the unclassified 16S rRNA sequences belonging to the new lineage along with all species of genera *Halorubrum* by neighbour-joining method; bootstrap performed with 1,000 replications to provide confidence.

analysis has also provided partial insights into the unculturable groups of archaea in these conditions. However, more extensive sampling and modification of the culturing techniques will be required to unravel the entire haloarchaeal communities in Kutch ecosystems. This new lineage will provide the required impetus to our quest for unravelling the total archaeal community structure in the hypersaline settings, the role played by representative groups in the sustenance and biogeochemical cycles, the mechanisms of osmoadaptation, and mining of relevant genes of agricultural and industrial importance.

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