Saudi Journal of Biological Sciences 28 (2021) 3783–3788

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Characterization of archaeal symbionts of sponges from the coral reef ecosystems of the Gulf of Mannar, Southeast coast of India

Jasmin Chekidhenkuzhiyil [a], Abdulaziz Anas [a,*], P.A. Thomas [b], Balu Tharakan [a], Shanta Nair [a]

[a] CSIR-National Institute of Oceanography, Regional Centre, Cochin, Kerala 682 018, India
[b] Panachammoottil, Gandhipuram, Thiruvananthapuram, Kerala 695017, India

ARTICLE INFO

Article history:
Received 24 December 2020
Revised 10 March 2021
Accepted 21 March 2021
Available online 27 March 2021

Keywords:
Archaea
Sponges
Gulf of Mannar
Coral reef
Nitrosopumilus

ABSTRACT

Sponges accommodate a diverse group of microorganisms with varied metabolic capabilities. The bacterial associates of sponges are widely studied while our understanding of archaeal counterparts is scanty. In the present study, we report the archaeal associates of two sponges, *Pseudoceratina purpurea* (NCBI barcode: KX454492) and *Cinachyra* sp. (NCBI barcode: KX454495), found in the coral reef ecosystems of Gulf of Mannar, India. Archaea in the water column was predominated by members of class Halobacteria of Phylum *Euryarchaeota* (97%) followed by a minor fraction (3%) of *Nitrosopumilus* sp. of phylum *Thaumarchaeota*. Interestingly, *Thaumarchaeota* was identified as the sole archaeal population associated with the two sponges studied, among which *Nitrosopumilus* sp. occuppied 80 and 100% of the sequences in the clone library of *P. purpurea* and *Cinachyra* sp. respectively. Other archaea found in the *P. purpurea* were *Nitrososphaera* (10%) and unclassified ones (10%). The study identified *Nitrosopumilus* sp. as a unique symbiotic archaeon of sponges, *P. purpurea* and *Cinachyra* sp. The existence of host driven factors in selecting specific associates from a diverse group of archaea in the environment may need further investigations.

1. Introduction

Microorganism being the coevolved partner of higher organisms (host) in the marine environment contribute significantly to the existence of their hosts through providing secondary metabolites, enzyme etc (O'Brien et al., 2019; Vijayan et al., 2017; Vinothkumar and Parameswaran, 2013). Each host can be considered as a micro-niche where a consortium of microorganisms live and interact. Any alterations in the composition of this consortium can impart significant changes to the health of the host (Clemente et al., 2012; Cryan and Dinan, 2012). However, the discussions on such interactions were restricted to the discipline of pathogenesis until the report of Woese and George Fox in1970s (McFall-Ngai et al., 2013). Host-microbe interactions are much relevant in the marine environment, especially when the resilience of many organisms such as sponges towards environmental stressors are attributed to the microbial flora associated with them.

Sponges are the ideal organisms to study the host-microbe interactions as a significant volume of their biomass are contributed by associated microorganisms (Hentschel et al., 2012). Many of the ecological functions such as remineralization of organic matter and participation in nitrification, which were originally thought to be carried out by sponge are actually performed by the microorganisms inhabiting in their body (Bayer et al., 2008; Erwin et al., 2011). Sponges accommodate a diverse community of microorganisms in their body representing all three domain of life viz Archaea, Bacteria and Eukarya and are recently documented to contain viruses as well (Chaib De Mares et al., 2017). A maximum of 52 phyla of bacteria, including candidate phyla such as *Poribacteria* and *Tectomicroba* and two archaeal phyla are reported from different sponges (Schmitt et al., 2012; Thomas et al., 2016; Webster and Taylor, 2012). The interactions of these microorganisms with respective sponges are quite diverse that ranging from mutualistic to commensalistic or exploitative (parasitism/pathogenesis) in nature. Apart from participating in nutrient cycling and translocation, certain secondary metabolites of associated microorganisms enhance the chemical defence of sponges to predators and epibionts, which is thought to be the underlying

* Corresponding author.
E-mail address: anas@nio.org (A. Anas).

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.03.054

factor to the ecological and evolutionary success of sponges (Webster and Thomas, 2016).

Although less diverse compared to bacteria, symbiotic archaea are known for performing specific functions in sponges, especially participation in nitrogen metabolism (Auguet et al., 2010; Radax et al., 2012; Turque et al., 2010; Zhang et al., 2014). Interestingly, the abundance of single archaeal lineage have been found exceeding the bacterial abundance in several sponges (Jackson et al., 2014; Preston et al., 1996). However, we know little about the diversity of archaea associated with marine sponges globally. Majority of the studies are restricted to sponges from Mediterranean, Caribbean and Pacific region with less reports from Indian subcontinent (Auguet et al., 2010; Chaib De Mares et al., 2017; Feng et al., 2016; Jackson et al., 2013, 2014; Radax et al., 2012; Turque et al., 2010; Zhang et al., 2014). Most of these studies reported the dominance of *Thaumarchaeota* and or *Crenarchaeota* in sponges while more diverse group of archaea were present in the environment in which they were grown. Investigations on archaeal symbionts of sponges from different ecosystems across the globe will support in defining the role of host driven factors in sponge-archaea interactions. In the current study, we report the archaeal diversity associated with two sponges *Pseudoceratina purpurea* and *Cinachyra* sp in comparison with the water column in proximity in the coral reef ecosystems of Gulf of Mannar situated at the southeast coast of India.

2. Materials and methods

2.1. Sample collection and transportation

The sponge samples were collected by scuba diving from (5–10 m depth) the coral reef ecosystems of Gulf of Mannar, India. Once sampled, the sponge samples were handled with all precautions (wearing nitrile glows) to minimize contamination, sand and debris found on the surface were removed by washing with sterile calcium-magnesium-free artificial seawater (CMF-ASW, pH7.2) and transported to the laboratory in an ice chest. Reef water (1000 ml) were collected from within 5 m periphery of the sponge and transported to the laboratory in an ice chest. The reef water was filtered through a mixed cellulose ester membrane filter (0.2 μm) and the filter paper was stocked at −20 °C until use. Further details on sample preparation and transportation may be found elsewhere (Jasmin et al., 2015).

2.2. Extraction of DNA from sponge holobiont and water

Genomic DNA was extracted from reef water and sponge tissue separately following the methods of Boström et al. (2004) and Ouyang et al. (2010) respectively with slight modifications. The sponge tissue (100 mg) was homogenized with 400 μl of lysis buffer (500 mM NaCl, 100 mM EDTA and10 mM Tris pH 8.0). Similarly, one half of the filter paper (cut into small pieces) were subjected for bead beating for 1–2 min (2 mm diameter beads) in the presence of 500 μl lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM EDTA and 50 mM Tris, pH 8.0). The lysed samples were further treated with lysozyme (25 U) for one hour at at 37 °C followed by SDS (1%) and proteinase K (500 $\mu g ml^{-1}$) for 2 h (for tissue) or 5 h (for filter paper) at 55 °C. Both the reaction mixtures were further extracted twice with Chloroform:isoamyl alcohol (24:1). The aqueous phase was recovered into another microcentrifuge tube and supplemented with 0.6 times volume of isopropanol and was kept −20 °C for 60 min to precipitate the DNA. The precipitated DNA was washed thrice with ethanol (70%), dissolved in TE buffer (pH 7.4) and stored at −20 °C until used. The quality and purity of DNA was assessed using agarose gel electrophoresis (on 0.8% agarose gel) and spectrophotometry (absorbance at 260/280 nm in a ND-1000 spectrophotometer (NanoDrop, Thermo Scientific) respectively.

2.3. Identification of sponges

The sponge samples were identified following a polyphasic approach using both morphological and molecular tools. Morphological characterization includes recording the colour, morphology and spicule characterization. The molecular identification was done by amplifying the COI gene in a PCR using universal primers; LCO1490:5′ –GGTCAACAAATCATAAAGATATTGG-3′, and cox1-R1:5′- TGTTGRGGGAAAAARGTTAAATT- 3′ (Folmer et al., 1994). The reaction was conducted in a 25 μl reaction volume containing 1 μl template DNA (50–100 ng), 1 μl of each primers (10 picomoles μl^{-1}), 10x Taq DNA polymerase buffer (2.5 μl), dNTPs (200 μM each) and DNA polymerase (0.5U Taq). The PCR was carried out in a Thermal cycler (Eppendorf, Germany) with recommended conditions for the primer (Folmer et al., 1994). The amplified PCR products were purified using a PCR cleanup kit (Genetix Biotech, India) and was cloned in to pJET 1.2 blunt vector using CloneJET PCR cloning kit (Thermo Scientific), following the instruction manual. Plasmids were extracted from ten randomly selected clones and were subjected for sequencing PCR with vector specific primers, pJET 1.2F and pJET 1.2R and ABI PRISM Bigdye terminator v3.1cycle sequencing kit (Life Technologies, USA). The Products of sequencing PCR were purified and sequenced on Applied Biosystems ABI 3730xl DNA analyser.

2.4. Archaeal community structure

The community structure of archaea associated with sponges were studied using 16S rDNA clone library method. The archaeal 16S rRNA gene was amplified from metagenomic DNA isolated from the reef water and two sponges; *Cinachyra* sp. and *P. purpurea* using universal primers [ARCH 21F: TTCCGGTTGATCCYGCCRG and ARCH 958R: TCCGGCGTTGAMTCCAATT] (DeLong, 1992). The gene amplification was performed following standard PCR conditions (30 cycles) with annealing at 55 °C for 60 s and extension at 72 °C for 1.5 min. The PCR products were purified, cloned and sequenced following the same conditions and reagents mentioned in the above section. Nearly 270 to 330 clones were there in each of the three clone libraries, out of which 100 to 130 clones were randomly chosen and grown for plasmid isolation in 5 ml LB media for overnight at 37 °C degree under shaking conditions. The recombinant plasmids were purified and sequenced as mentioned in the earlier section.

2.5. Data analysis

The sequences with high quality were selected after screening using Sequencher V4.10.1 (GeneCodes Corporation, Ann Arbor, MI USA). Contaminating vector- and chimeric-sequences were removed from the dataset using VecScreen program of NCBI and DECIPHER online tool (http://decipher.cee.wisc.edu/FindChimeras.html) respectively. The sequences were further analysed in depth and classified into OTUs in the NGS analysis pipeline of SILVA (https://www.arb-silva.de/ngs/). From the three libraries with 100–120 sequences each, a total of 81 OTUs were obtained upon SILVA analyses. The OTU data available were used to depict the bar chart which describes the diversity and abundance of microbes in each of the three libraries analysed here. Also, the representative sequences (OTUs) were searched against NCBI Genbank and the sequence data of the nearest neighbours were downloaded to be used as reference sequences. Together, the OTUs and the reference sequences were aligned (using clustal W program) and a

Phylogenetic tree was constructed using neighbour-joining method in MEGA 5.2 (Tamura et al., 2011). The difference in microbial community structure in sponges *Cinachyra sp*, *Pseudoceratina purpurea*, and the reef water sample were analysed using Library compare tool in RDP (http://rdp.cme.msu.edu). The multivariate cluster analysis of the resulting data were performed in PRIMER v.6 (Plymouth Marine Laboratory, UK).

3. Results

Two sponges used in the study were identified based on morphological and molecular tools as *P. purpurea* (NCBI barcode: KX454492) and *Cinachyra* sp. (NCBI barcode: KX454495). The *P. purpurea* is described as an yellow coloured erect sponge with laminar branches and was found attached to seaweed *Caulerpa racemosa* (Fig. 1A). The surface colour changed to yellowish brown on removing from the substratum and exposed to air, while the interior remains yellow (inset in Fig. 1A). The texture was leathery and oscules and pores were untraceable. The spicules are blunt ended with terminal dome shaped structures. The COI gene sequence of the same showed 98.86% similiarity with *Pseudoceratina purpurea* (KY565313) isolated from Oahu Island, Hawaii. The *Cinachyra* sp. was found as a massive sponge with bright yellow in color and occassional patches of greyish brown and green due to the settlement of silt/sand and algae (Fig. 1B). The *Cinachyra* sp. was found attached to the substratum with a broad base and the spherical body has unevenly arranged golf ball like depressions. *Cinachyra* sp. have tough and incompressible body with small oscules scattered over (inset in Fig. 1B). The long spicules were sharply pointed with a size of approximately 2.529 mm. The COI gene sequence showed only 92.7% of similiarity with a distant relative *Biemna fistulosa,* in the same class *Demospongiae*.

The archaea was more diverse in the water column, dominated by phylum *Euryarchaeota* (97%) followed by *Thaumarchaeota* (3%) (Fig. 2). Members of class *Halobacteria* were predominant (95%) among *Euryarchaeota*, among which a significant fraction (35%) were grouped into unclassified. The remaining members of *Halobacteria* were identified as *Halorussus* and *Halomicroarcula* (9% each); *Haloplanus, Halolamina, Halobellus, Haloarcula Halomicrobium* (6% each) and Genus *Natromonas* (4%).

The *Nitrosopumilus* sp. coming under phylum *Thaumarchaeota* were poorly represented in the clone library of reef water (3%), while they dominated in the library prepared from sponges (Fig. 3). An 80 and 100% of sequences from the clone libraries of *P. purpurea* and *Cinachyra* sp respectively were identified as *Nitrosopumilus* sp coming under order *Nitrosopumilales* of Phylum Thaumarcheaota. Remaining sequences in the clone library (10% each) of *P. purpurea* were identified as *Nitrososphaera* sp (Phylum Thaumarchaota, order Nitrososphaerales) and unclassified archaea respectively. The phylogenetic relationship between the sequences obtained from the three clone libraries are depicted as rooted neighbour joining tree using MEGA software (Fig. 3). The multivariate cluster analysis using Primer software showed nearly 40% similarity of archaeal diversity between the sponges, while they were highly distant from the reef water (Fig. 4).

4. Discussion

From the first description by Woese and Fox in1978 and till recently, Archaea were known for their existence in extreme environments. Later they were reported from marine waters and sediments as planktonic or in association with other organism (Auguet et al., 2010; DeLong, 1992; Dombrowski et al., 2019; Fuhrman et al., 1992; Santoro et al., 2019). Their association with sponges was first detected in *Cenarchaeum symbiosum* from the offshore waters of Santa Barbara in 1996 (Preston et al., 1996). Archaea associated with sponges were less diverse (Zhang et al., 2014) with only two leneages reported from many sponges studied (Hentschel et al., 2012). More studies from diverse marine environment are important to understand the diversity of archaea associated with sponges and the ecological significance of their interactions. Current study provides the first insight into the archaeal diversity associated with sponges from Indian waters. The archaea were less diverse in sponge *P. purpurea* and *Cinachyra sp*, compared to the water column in which they were living. Previous reports from Mediterranean and Caribbean waters showed that the archaeal community associated with sympatric sponges are different from that of seawater and varied between different sponges (Zhang et al., 2014). Studies from Atlantic, Mediterranean and Caribbean waters showed the higher diversity of archaea with phylum *Euryarchaeota* as ubiquitous in seawater (Garcia-Bonilla et al., 2019; Jackson et al., 2013; Zhang et al., 2014). The Thaumarchaeal reads from seawater and sponge were almost represented by order *Nitrososphaerales* and *Nitrosopumilales*. Among this, the *Nitrosopumilus* sp of *Nitrosopumilales* represented almost all the clones in *P. purpureae* and *Cinachyra* sp. respectively.

Similar to the recent observations from Mediterranean sponges (Garcia-Bonilla et al., 2019), we also found higher abundance of Thaumarchaeota in *P. purpureae* and *Cinachyra* sp. Although there are diverse archaeal genera present in reef water, the unique genus

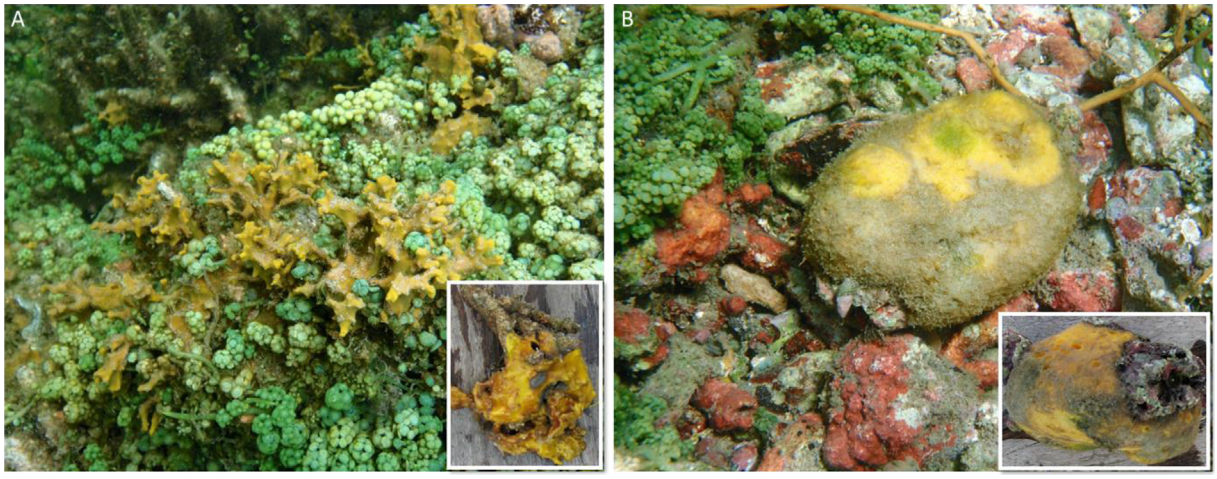

Fig. 1. Underwater images of sponges A) *P. purpurea* and B) *Cinachyra* sp. Images of the same after reaching outside water is given in the insets.

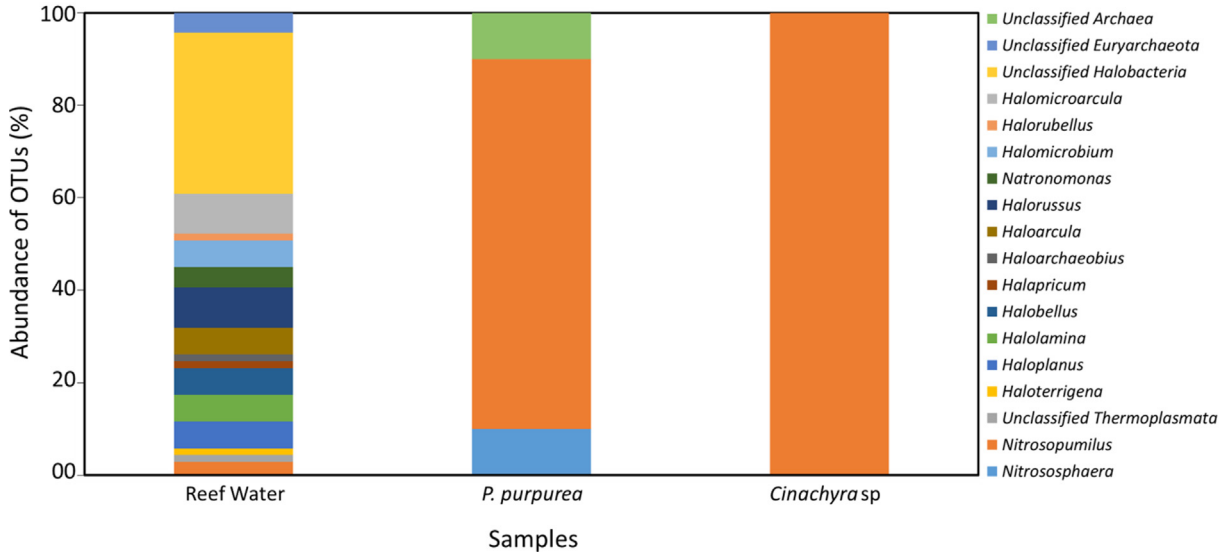

Fig. 2. Bar chart showing diversity of archaea in reef water and sponges.

Nitrosopumilus (contributed only ~3% of clone library of reef water) contributed nearly 80% of the clones in the library generated from both sponges. It is possible that the sponges catch the transient flora from water and enrich within the mesophyl tissue. A study based on the analysis of 440,000 sequence of associated microorganisms in *Xestospongia* sp from different geographical locations also reported the dominance (~60%) of core group of microorganisms in sponges (Swierts et al., 2018). Similarly, 4 and 6 OTUs were found contributing 88% of the 210 and 273 OTUs respectively of two individuals of *Inflatella pellicula* from deep sea (Jackson et al., 2013). Majority of the studies agrees to a point that archaea are less diverse in sponges compared to the surrounding seawater and certain archaeal species get enriched within sponges (Swierts et al., 2018). If the variable/core/species-specific concept of bacterial symbionts of sponges are taken into consideration, these dominant archaea can be considered as the core archaea associated with sponges.

Nitrosopumilus sp of archaea are reported from a wide range of sponges across geographical barriers and are known to participate in nitrification, specifically in the oxidation of ammonium to nitrite (Bayer et al., 2008; Moeller et al., 2019; Polónia et al., 2014; Rodríguez-Marconi et al., 2015). In a coral reef ecosystem, sponges ingest nitrogen along with their food and excrete ammonia (Brusca and Brusca, 1990). The *Nitrosopumilus* sp. colonized in sponge tissue can support the conversion of ammonium to nitrite and nitrate (Feng et al., 2016) and thus protect the host cells from ammonia toxicity. The numerical dominance of *Nitrosopumulus* sp. in sponges indicates that they could be the core archaea of sponges. However, this doesn't mean that they are species specific and their growth is obligately depended on the host. The dominance of *Nitrosopumilus* sp. was reported from the sponge *Ircinia* sp. from Mediterranean and Caribbean waters (Zhang et al., 2014). There must be a factor which promoted the selective enrichment of *Nitrosopumilus* sp. in sponge tissue from the surrounding environment, which is yet to be confirmed. There are different assumptions on microbiome and physiology of sponges, one of which says that the small sized pores, dense mesohyl and complicated canal systems inside the sponges like *Cinachyra* sp. may result in the circulation of water at high pressure through their canal system and which will promote the colonisation of species specific microorganisms (Jasmin et al., 2015). This could be a possible factor influencing the colonization of limited number of archaea in certain sponges. Based on the literature, these observations can also be connected with the functional role of sponges in reef ecosystems (Bell, 2008; James, 2007). The *Nitrosopumilus* sp. are known for their capabilities to oxidize ammonium and hence their interactions may be in support of the participation of those sponges in the biogeochemical cycling of nitrogen in the reef ecosystem. Or otherwise the enrichment of ammonia oxidizing archaea are also defined as an adaptation of sponge to increase their resistance to ammonium toxicity from eutrophication pressure (Baquiran and Conaco, 2018; Turque et al., 2010).

5. Conclusion

Present study reports the diversity of archaea associated with sponges, *P. purpurea* (NCBI barcode: KX454492) and *Cinachyra* sp. (NCBI barcode: KX454495), found in the coral reef ecosystems of Gulf of Mannar. Although the water column had a diverse group of archaea, the *Nitrosopumilus* sp. was found contributing more than 80% of the sequences in the clone library of both the sponges. This indicates the existence of a host driven factor in enriching selected group of archaea in sponges. Further studies may focus on the factors influencing this enrichment and its ecological significance.

6. Compliance with Ethical Standards

Authors declare no conflict of interest. The field collections carried out for the purpose of this manuscript did not involved endangered or protected species. Permission was received from Principal Chief Conservator of Forests and Chief Wildlife Warden for entering coral reef ecosystems of GoM. No specific permission was required to collect the analysed sponge samples.

Author contribution

The design of the work and the preparation of manuscript was done by AA, JC and SN. JC and BT contributed to the molecular biology analysis of the sample and TPA contributed to the morphological identification of sponges.

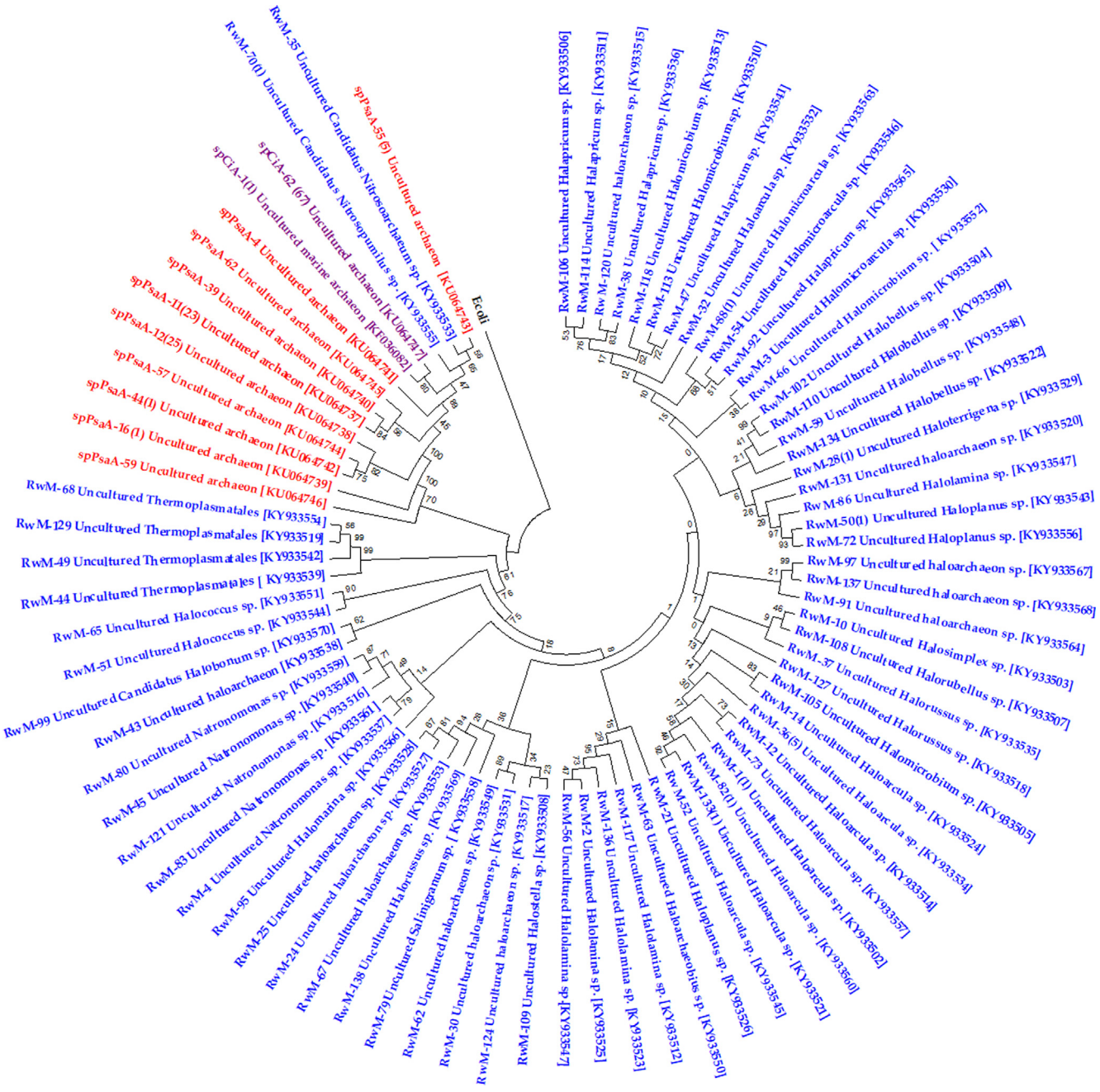

Fig. 3. Neighbour joining Phylogenetic tree representing the relationship between the sequences from the reef water (blue) and two sponges, *Cinachyra* sp (purple) and *P. purpurea* (Red).

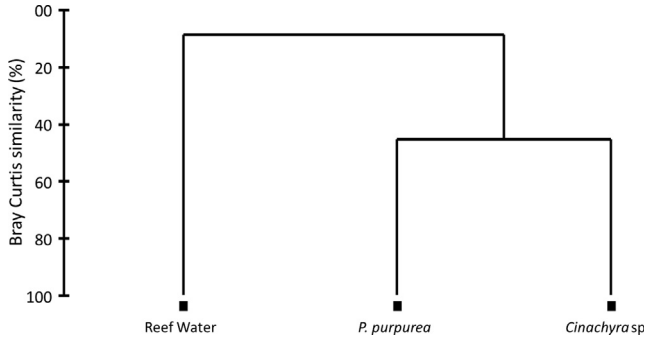

Fig. 4. Dendrogram showing the similarity of archaeal diversity in reef water and sponges, *P. purpurea* and *Cinachyra* sp.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the Director, CSIR - National Institute of Oceanography, Goa and the Scientist-in-Charge, CISR-NIO Regional Centre, Kochi, for extending all required support. The authors acknowledge Dr. Manikandan C, Scientist CSIR-NIO for the support rendered for sample collection and underwater imaging of

sponges. JC is grateful to Department of Science and Technology, Govt of India for the research grant SR/WOS-A/LS-339/2009.

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