

Phylogenetic diversity of archaeal 16S rRNA and ammonia monooxygenase genes from tropical estuarine sediments on the central west coast of India

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Abstract

Phylogenetic diversity analyses of archaeal 16S rRNA and ammonia monooxygenase subunit A (*AamoA*) genes were carried out from the sediment samples of Mandovi and Zuari estuaries in the central west coast of India. The 16S rRNA gene libraries revealed quite high diversity of archaea in these sediments compared to previous reports from tropical and temperate estuarine sediments. Uncultured members of Crenarchaeota accounted for ~78% of 433 archaeal 16S rRNA gene clones from both the estuaries. We detected archaeal 16S and *amoA* genes related organisms capable of ammonia oxidation. Among Crenarchaeota, Marine Group I (MGI) was the most predominant. Clones matching with the uncultured methanobacteria were predominant among the ribogroups of Euryarchaeota. Our results indicate that archaeal diversity in tropical estuarine sediments is influenced by the mangrove vegetation bordering the lower stretches of both the estuaries. Higher diversity may be related to elevated land drainages during monsoon particularly in Mandovi estuary sediments. Also, diversity of *AamoA* sequences was higher in Mandovi sediments than those from Zuari and other tropical and/or temperate estuaries studied previously.

Keywords: Tropical estuaries, Sediment, Phylogeny, Archaea, Marine Group I, DGGE, 16S rRNA, OTU, *amoA*, ammonia oxidation

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1. Introduction

Application of molecular methods has changed our perceptions on diversity, distribution and function of archaea in natural marine ecosystems. Analyses of ribosomal RNA gene sequences from environmental samples have revealed that archaea are ubiquitous (Stein and Simon, 1996) and, far more abundant (Karner et al., 2001) than previously assumed. The 16S rRNA gene-based archaeal diversity analyses reveal the existence of archaea in the open-ocean, marine sediments, soils and freshwater lake sediments (Schleper et al., 2005). Mesophilic archaea in the sub-kingdoms, Crenarchaeota and Euryarchaeota, are now recognized to be widespread in the oceans and, reported to contribute significantly to cycling of carbon and nitrogen, the two biologically most essential elements (e.g., Francis et al., 2007; Ingalls et al., 2006).

Biological fixation and microbial denitrification losses of nitrogen are ultimately connected by nitrification, which is an important process throughout the oceans and coastal systems (Ward, 2005). While decomposition of NO_3^- to N_2 gas leads to N loss, the conversion of ammonia (NH_4^+) to nitrite (NO_2^-) has been shown to be predominantly done by archaea, in particular in the soils and sediments (Ando et al., 2008; Leininger et al., 2006). Recent discoveries from metagenomic sequences and pure cultures also suggest that the archaea are capable of performing chemoautotrophic nitrification (Könneke et al., 2005; Venter et al., 2004). Many studies have shown the presence, diversity and distribution of archaeal *amoA* (*AamoA*) gene in different ecosystems and geographical locations (Beman and Francis 2006; Beman et al., 2008; Bernhard et al., 2005; Bothe et al., 2000; Dang et al., 2008). The *amoA*-encoding archaea are increasingly recognized as important for their potential of ammonia-oxidization in terrestrial and marine ecosystems (Dang et al., 2008; Leininger et al., 2006). However, the extent of occurrence and composition of ammonia oxidizing archaea (AOA) in the tropical marine systems, and their relative importance in N cycle, need more investigations.

Phylogenetic studies using 16S rRNA- gene, the traditional marker, suggest a certain level of differentiation of bacterial and archaeal OTUs between different ecosystems (Nicol and Schleper, 2006). Since two monophyletic lineages are reported for 16S rRNA gene of ammonia oxidizing bacteria (AOB), it is used for describing the AOB phylogeny as well (Bothe et al., 2000). Recently, a substantial congruence of crenarchaeal 16S rRNA and *AamoA* genes has also been shown (Prosser and Nicol,

2008). However, 16S rRNA gene diversity within Crenarchaeota is far greater than that of AOA (Schleper et al., 2005).

Coming under the influence of both the ocean and rivers, the estuaries are reported to harbor high microbial diversity. Recent phylogenetic analyses on archaea from temperate (Abreu et al., 2001) and tropical (Vieira et al., 2007; Zeng et al., 2007) estuaries harbor remarkable diversity perhaps as a consequence of the presence of estuarine, riverine and coastal-ocean assemblages. Though some tropical regions are believed to be hot-spots of biodiversity, our knowledge of archaeal diversity from these ecosystems is limited owing mainly to the lack of use of molecular phylogenetic investigations (Vieira et al., 2007; Zeng et al., 2007). In view of lack of such information, we aimed to examine the archaeal assemblages and compare their phylogenetic relationships in two tropical and ecologically similar estuaries through culture-independent 16S rRNA- and *AamoA*- gene analyses. These moderately to highly productive estuaries are fringed by mangroves in their lower stretches and, receive substantial land runoff during the monsoon months of June to September (Shetye et al., 2007). We aimed to elucidate the differences in archaeal community structure in the sediments of these estuaries and to detect the archaeal *amoA* (*AamoA*) gene which is functionally important in ammonia oxidation, one of the very crucial steps in marine nitrogen cycle.

2. Methods

2.1. Description of sampling sites

Mandovi and Zuari are the two major rivers of Goa in the central west coast of India. Before draining into the Arabian Sea, they form a confluent estuarine system between 15°09' and 15°33' N and between 73°45' and 74°14' E (Fig 1). River Mandovi has many tributaries and, receives a greater runoff than Zuari (Shetye et al., 2007). Both experience semi-diurnal tides and higher freshwater influxes during the monsoon months. A hilly terrain with dense vegetation girdles the lower stretches of both estuaries, due to which there is rich input of humus. Sediments in the lower stretches of these estuaries are reported to be mostly sandy (Mandovi: sand: 65-70 > clay 15-20 > silt:10-15%; Zuari: sand 60-65 > clay 25-30 > silt 5-10%). The annual variations in salinity range from close to freshwater ones during monsoon months (June-September) to 34 during pre-monsoon months (March-May). Annual fluctuation of water temperature is between 20 and 29 °C (Shetye et al., 2007). The high nutrient content, along with low wave action and mild currents near the banks lead also to a favorable environment for the

growth of mangroves. During the post-monsoon (October-February) months, total organic carbon is reported to be in the range of 0.8-1.2% in Zuari (Dessai et al., 2009) and, 1.6-3% in Mandovi (Nasolkar et al., 1996) sediments. In these estuaries, the average total bacterial counts are $\sim 6.31 \times 10^6 \text{ ml}^{-1}$ water and $3.02 \times 10^9 \text{ g}^{-1}$ dried sediment (Ramaiah et al., 2002).

2.2. Sampling

Sediment samples were collected during January 2005 from the locations shown in Fig 1. They were collected using van-Veen grab from three sites each in Mandovi ($15^\circ 29' \text{ N}$; $73^\circ 48' \text{ E}$; water depth 5 m; salinity in overlying water ~ 32 psu and temperature 24° C) and Zuari ($15^\circ 25'$; $73^\circ 51' \text{ E}$; depth, 5m; salinity of overlying water ~ 34 psu and temperature 25° C) estuarine mouths. The top 2.5 cm sediment layer were aseptically removed and stored separately in sterile Falcon tubes. These sediment samples were transported to the laboratory on ice for DNA extraction and stored at -70° C until taken up for further processing.

2.3. DNA extraction

Two replicates of ~ 0.5 to 1 g sub-samples of all six samples were subjected to DNA extraction. The MOBIO soil DNA extraction kit (MO BIO, USA) was used for extraction as per the protocol provided by the manufacturer. DNA extracts from each sub-sample were pooled and stored at -20° C .

2.4. DGGE Analysis

DGGE-PCR was carried out using primers, PARCH 340f: 5'-CCCTACGGGG(C/T)GCA(G/C)CAG with GC clamp : 5'-TCGCCCCGCCGCGCGGGCGGGGCGGGGGCACGGG and PARCH 934R: 5'-GTGCTCCCCCGCCAATTCCT as described by Ovreas et al. (1997). The sediment-DNA extracts were amplified in triplicate to maximize the diversity. Five hundred nanogram pooled and purified PCR products were loaded on 6% (w/v) polyacrylamide gels containing a 40 to 60% denaturing gradient of urea and formamide. The gel was run in D-Code universal mutation detection system (Bio-Rad, USA) for 16 h at 60° C at 60 V. The gel was stained in SYBR Green. Intra- and inter- estuarine variations were examined by running all 6 DNA samples on a single gel. Bands were defined manually as well as by Quantity-one software (Version 4.66, Bio-Rad, USA).

2.5. PCR Amplification of 16S rRNA gene and *AamoA* gene

The archaeal 16S rRNA gene was amplified using primers 21F: 5'-TTCCGGTTGATCCYGCCGGA and 958R: 5'-YCCGGCGTTGAMTCCAATT (DeLong, 1992). This primer set has been widely used for archaeal diversity analyses from different geographic locations. Although Teske and Sørensen, (2008), reported primer mismatch with deep sea archaeal 16S rRNA gene, we resorted to using primers of DeLong, (1992) to be able compare our results with those in literature. The amplification was carried out by following DeLong, (1992) for 25 cycles to minimize the bias. DNA from each sub-sample was amplified separately for maximizing the amplification of archaeal 16S rRNA genes from rare/minor components. The PCR products from all three samples from each estuary were then pooled and purified with QIAquick PCR purification kit (Qiagen, USA) as per manufacturer's instructions.

PCR primers Arch-amoAF: 5'-STAATGGTCTGGCTTAGACG and Arch-amoAR: 5'-GCCGCCATCCATCTGTATGT were used for amplifying *AamoA*-gene fragments following Francis et al. (2005). The PCR products were pooled and purified as done for 16S rRNA gene.

2.6. Construction of libraries and sequencing

From each estuary, both 16S rRNA-and *AamoA*- gene libraries were constructed. Pooled and purified PCR products were cloned into pGEM-T easy cloning vector (Promega, U.S.A.) according to manufacturer's instructions. Transformation was done in chemically competent *Escherichia coli* JM109 cells. Positive colonies from the libraries were randomly picked out and screened by colony PCR using M13 forward and reverse primers. The amplification reaction was carried out as follows: incubation for 7 min at 94°C (for rupturing cells), 35 cycles each of 1 min at 94°C, at 55°C, at 72°C and, a final extension step for 10 min at 72°C. The PCR products were electrophoresed in 1% agarose gel for checking and selecting the positive clones. These were purified and sequenced in both directions using M13 forward and M13 reverse sequencing primers. In all, 196 clones from Mandovi and 257 from Zuari sediments were sequenced from the 16S rRNA gene libraries. Sequences from Mandovi and Zuari were named respectively, MES-1 to MES-196 and ZES-1 to ZES-257. Similarly, 83 clones of *AamoA* (39 from Zuari and 44 from Mandovi) were sequenced. After sequencing in both the directions, good quality sequences of ~635 bp were obtained.

2.7. Phylogenetic analyses

PINTAIL program (Ashelford et al., 2005) was used to check chimera formations due to PCR artifacts. Sequences were also examined manually for chimerae, which were excluded from further analyses. Also, two short, non-chimeric sequences ZES-6 and ZES-57 were not included for further analysis.

The DOTUR program (Schloss and Handelsman, 2005) was used for grouping the sequences as operational taxonomic units (OTU). The 16S rRNA gene sequences having similarity $\geq 97\%$ were considered as the same OTU (or phylotype or ribogroup). From the combined grouping, only one representative clone from each OTU was taken for phylogenetic analyses. These representative ribogroups were checked for their similarity with sequences in GenBank using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences from this study were aligned with their matching ones in the GenBank database using ClustalX 1.83 (Thompson et al., 1994). Aligned sequences were edited manually to remove the gaps as well as those sequences with ambiguous alignment. The Neighbor-Joining method was followed to construct the tree using the software, MEGA4 and, maximum likelihood method was adopted for calculating the evolutionary distance (Tamura et al., 2007).

All the 83 *AamoA* sequences were aligned and grouped using DOTUR program at 95% cut-off level. Only group representative-sequences were used for phylogenetic tree. We used nucleic acid sequences (rather than predicted amino acid sequences) for this analysis to highlight the genetic level heterogeneity among archaeal communities. Bootstrap analysis (1000 replicates) was done to confirm the reliability of the phylogenetic tree which was constructed by following the methods described above.

2.8. Statistical analyses

From all libraries, the rarefaction analysis, richness, diversity index and non-parametric diversity index (Chao I) were carried out using DOTUR program (Schloss and Handelsman, 2005). Percentage of Good's coverage (C) was calculated using equation $C=1-n/N \times 100$ (where n is the number of phlotypes occurring only once and N, the total no. of populations). For statistical analyses, the OTUs were defined at 97% and 95% nucleotide similarity level for 16S rRNA- and *AamoA*- gene, respectively.

2.9. Nucleotide sequence accession numbers

Accession numbers DQ641722 to DQ641900 correspond to our 16S rRNA gene clones from Mandovi and, EF367439 to EF367694 to clones from Zuari estuarine sediments. Accession numbers FJ601548 to FJ601630 (in all 83) correspond to *AamoA* gene sequences of clones from Mandovi (MS-x-x) and Zuari (ZS-x-x).

3. Results

The DGGE band-pattern from all six samples (Supplementary Figure, S1), is useful to infer that there is hardly any difference in the archaeal community structure of both tropical estuarine sediments. In all, there were 12 or 13 bands in each sample.

3.1. Diversity of 16S rRNA gene

Of the 433 sequences available for phylogenetic analysis, 382 were found to belong to Crenarcheota (Fig 2a and 2b) and only 51 to Euryarcheota (Fig 2b). In general, the 16S rRNA gene sequences from the Mandovi and Zuari estuarine sediments showed a divergence of 46 % to each other. Estuary-wise numbers of archaeal phylotypes/ operational taxonomic units (OTUs) is listed in Table 1. In all, there were 91 OTUs from both estuaries. Among these, 70 (88% of total OTUs) belonged to Crenarcheota and, 21 (12 %) to Euryarcheota.

Among Crenarchaeota, six lineages were evident. These were: Marine Group I (MG I); Miscellaneous Crenarchaeota Group (MCG); Marine Benthic Group A (MBG A); Marine Benthic Group B (MBG B); Marine Benthic Group C (MBG C) and, C3 group (Fig 2a). There were four lineages of Euryarchaeota represented by 21 ribogroups: Marine Group II (MG II); Halobacteriales; Methanobacteriales; and, Uncultured Euryarchaeota Group (UEG; Fig 2b).

3.1.1. Crenarchaeal phylogeny

Members of Crenarcheota were by far the most dominant in the sediment samples of both estuaries and, their 16S rRNA gene sequences matched closely with environmental sequences reported from various geographic locations (Fig2a). In addition, a high percentage of archaeal sequences from these estuaries were phylogenetically related to uncultured archaea reported from mangrove soil (Yan et al., 2006).

Among crenarchaeotes, the MGI accounted for 60% of total clones sequenced. As many as 13 phylotypes clustered within it into four sub-clusters with strong bootstrap value of 99%. Ribogroups ZES-43, ZES-176, ZES-11, ZES-59, ZES-28 and MES-124 were represented respectively by 22, 17, 10, 6, 3 and 1 clone(s) which clustered into MG I. Ribogroup MES-151, represented by a single clone, also clustered within MG I. Further, with a total of 124 sequences, MES-65 was the most abundant ribogroup within MG I followed by MES-22, MES-137 and MES-9.

Three ribogroups, MES-95 (two clones), MES-97 (single clone) and ZES-8 (single clone) clustered within MBG A. Clones MES-192 and ZES-241 aligning into MBG B clustered with the previously reported AB177262.1 from subsurface sediment off Peru and, DQ363757 from mangrove soil clones. In a total of 14 OTUs comprising 34 clones, ZES-39 (11 clones) and ZES-252 (five clones) were the two major ribogroups aligning into MBG C.

Three ribogroups represented the C3 group. They were all from Mandovi samples (two clones of MES-185 and, one each of MES-31 and MES-152).

Clones akin to MCG from Mandovi and Zuari libraries formed 36 OTUs. They clustered with previously defined MCG clones AB177020 and AB177268 from subsurface sediment off Peru. Most of them also showed very close similarity with archaeal clones isolated previously from mangrove soil.

3.1.2. *Euryarchaeal phylogeny*

The uncultured members of Methanobactriales were the dominating clades among Euryarchaeota. Ribogroups MES 122, 24, 138, 87, 141, 72, 158, 174, 13 and ZES 127 were monophyletic with *Methanobacterium* sp with strong bootstrap values. Halobactriales was represented by only one sequence, MES 23. Six ribogroups namely MES-139 (three clones), ZES-126 (two clones), MES-81, ZES-79, ZES-71 and ZES-102 constituted the MG II. These ribogroups were monophyletic with Thermoplasmatales (*Picrophilus oshimae*). Ribogroups ZES-23, 41, 121 and MES-14 of UEG group were monophyletic with clones from hydrothermal vent (Fig 2b)

3.2. *Diversity and phylogeny of AamoA gene*

At 95% similarity, there were 27 unique phylotypes from 83 individual *AamoA* sequences from both the estuaries. All these 27 phylotypes were included in the phylogenetic analyses after matching their

571 bp region to the *AamoA* gene sequence in GenBank. These phlotypes formed two distinct clusters (Fig 3) with 95 % of the clones aligning within cluster I. Cluster II was represented by 4 phlotypes. They all are from Mandovi sediment DNA-library and, monophyletic with the sequences obtained from soil.

Diversity of *AamoA* gene sequences was higher in Mandovi samples. There were 22 *AamoA* phlotypes from 44 clones sequenced than only nine from 39 Zuari clones. The *AamoA* sequences in cluster I matched closely with environmental sequences obtained from estuarine sediments, deep marine sediment and freshwater regions. Phlotype MS_22F3 was monophyletic with clones from an estuary in Mexico (Francis et al., 2005) and from Changjiang estuary (Dang et al., 2008). Phlotype MS_11C2 with four clones is highly similar to the OTU from Qinghai Lake sediment. MS_23G3 (12 clones) is monophyletic with clones from an eelgrass-zone sand sample (Ando et al., 2008). The ZS11_C2 (with 8 homologous clones) bears high similarity with clones from North Pacific subtropical Gyre ammonia oxidizers (Mincer et al., 2007).

3.3. Statistical analyses

The Chao I richness estimator suggested high richness in both the estuaries. Shannon-Weaver index correlated positively with the results obtained from rarefaction analysis and Chao I estimator (Table 1). The difference in the diversity of *AamoA* sequences between these estuaries was far more than that between 16S rRNA gene sequences. This analysis estimated the maximum number of 134 *AamoA* gene sequences for Mandovi. Interestingly, from all 39 *AamoA* clones from Zuari sediment samples the estimated maximum number is 34, with 92% coverage (Table 1).

Rarefaction analysis, done for comparing sampling effort and phlotypes obtained, also implies that more ribotypes were found in Mandovi than in Zuari at equal sampling effort (Fig 4a). Both observed and extrapolated *AamoA* richness varied widely between the sediment libraries. In that, the rarefaction curve turned asymptotic with only 39 clones from Zuari unlike the one generated by 44 from Mandovi library (Fig 4b).

4. Discussion

4.1. Archaeal community structure

Most ribogroups of Crenarchaeota from Mandovi and Zuari estuarine sediments bear high similarity with the environmental sequences. Results of this study are similar to the ones from surface sediments from the temperate estuaries where crenarchaeotal ribogroups are reported to be predominant (Abreu et al., 2001).

Presence of MCG, MBG A-C and C3 groups in these estuarine sediments and their very high similarity ($\geq 99\%$) with those reported from different geographical locations ascertains ubiquitous distribution of these lineages (Inagaki et al., 2006). Most MCG-related phlotypes, $\sim 17\%$ of total clones, obtained in this study are closely related and/or identical to phlotypes reported from mangrove soil (Yan et al., 2007). Such a large fraction of MCG in our study region might be taken to indicate high similarity of this group in mangroves soils and estuaries. It is likely that the MCG is a predominant group in the mangrove ecosystems and allochthonous to estuarine/coastal habitats. This proposition is also supported by the fact that members of MCG have been found quite frequently in 16S rRNA gene surveys from both marine and terrestrial environments (Teske and Sørensen, 2008).

There were fewer clones relating to MG II in our library, although Euryarchaeota has been reported to be abundant in the estuarine waters (Zeng et al., 2007) and, MG II in particular to be preponderant in surface waters (DeLong, 1992; Karner et al., 2001). Many clones clustering within Euryarchaeota were monophyletic with Methanobacteriales indicating the presence of methanotrophic archaea in the sediments of these tropical estuaries. Decadal/long term monitoring of abundance and establishing the functional roles of MCG, MG II and such predominant phlotypes is essential in a concerted manner from estuaries, and from mangroves that are on rapid decline globally. This would be of significance from the climate change researches perspective. Such efforts would essentially bring forth the role of microbial communities in the feedback and/or sinking fluxes of CO_2 , CH_4 , N_2O , for instance.

From the DGGE banding pattern, major differences between the sediment-DNA samples of Mandovi and Zuari estuaries were not evident. It is thus inferrable that almost the same members of archaea are dominating in both the estuaries. One most likely reason for a fewer DGGE bands (maximum of 13 bands) in the sediments is the predominance of MG I. While useful to recognize the

dominant phylotypes, the DGGE can lead to underestimation of diversity in particular in the highly dynamic environments with very few copy numbers of ‘sparse’ archaeal species.

4.2. Diversity of *AamoA*

Recent studies suggest greater abundance of archaea than bacteria possessing *amoA* in both marine (Beman et al., 2008; Francis et al., 2007) and terrestrial (Leininger et al., 2006; Prosser and Nicol, 2008; Schleper et al., 2005) ecosystems. Further, the archaeal clades with *amoA* from estuaries are shown to be both from soil and marine sediments (Francis et al., 2005). Transport through tidal action and riverine discharges would lead to the prevalence of diverse archaea in estuaries which are related to marine sediment and soil clones.

Mandovi and Zuari estuarine sediment *AamoA* matched well with those reported from Monterey Bay, San Francisco Bay, Elkhorn Slough (USA) and Bahía del Tóbari, a Mexican estuary in the Gulf of California (Beman and Francis, 2006; Francis et al., 2005). Our *AamoA* sequences share 95–100% identity at the DNA level and, may represent the ubiquity of *AamoA* group in coastal and estuarine sediments of most geographic regions. Such close similarity of *AamoA* sequences of this study with sequences from soil, sediment, coastal sand from very distant geographical region might indicate minimal genetic diversity among dominant estuarine ammonia oxidizers.

Diversity of *AamoA* is higher in Mandovi than in Zuari sediments. The Chao I values of 30-134 from the *AamoA* clones in the Mandovi estuary are higher than the maximum of 71 from Bahía del Tóbari (Beman and Francis, 2006) and 4-46 from San Francisco Bay (Mosier and Francis, 2008). Among the phylotypes, MS_22F3 (represented by 8 clones from Mandovi and six from Zuari; Supplementary Table 1) was the most abundant. It is monophyletic in origin with the clones from Gulf of California (Francis et al., 2005) and Changjiang estuary (Dang et al., 2008). While the detection of *AamoA* is used to ascribe the role of archaea, quantitative measurements to establish ammonia oxidation rates are necessary.

Salinity is emphasized to be the important factor governing in the spatial variation of diversity of ammonia oxidizers in the estuarine environments (Dang et al., 2008; Mosier and Francis, 2008; Santoro et al., 2008). Studies in the Chesapeake Bay (Francis et al., 2003), Plum Island Sound (Bernhard et al., 2005) and Ythan (Scotland) estuaries (Freitag et al., 2006) have shown shifts in bacterial communities

possessing *amoA* along the salinity gradients. While there were no remarkable differences in salinity and/or temperature between our sampling locations during the sampling month, the difference in diversity indices between the estuaries was quite large. Higher input of monsoonal discharges laden with suspended particles into Mandovi (Shetye et al., 2007) might be the cause of higher diversity. From the data of Nasnolkar et al. (1996) it is also clear that organic carbon in Mandovi estuary is higher which could be sustaining higher diversity of archaeal (and *amoA* bearing) phylotypes. The relative abundance of both archaea and bacteria possessing *amoA* across a physiochemical gradient has been also reported in a subterranean estuary (Santoro et al., 2008).

Our results from this first time analysis of phylogenetic diversity from tropical Mandovi and Zuari estuarine sediments serve useful for recognizing high diversity of uncultured archaea with the dominance of MG I. The cosmopolitan MCG, the predominant MG I, the allochthonous archaea (e.g., MES-31 clustering in C3, ZES-200 in MBG C, ZES-252 in MCG II) from the surrounding environment and, the endemic ZES-176, MES-151 (both clustering in MG I) were the major archaeal ribotypes recovered. As most clones in the 16S rRNA gene libraries from Mandovi and Zuari estuarine sediments were related to mangrove soil, we believe that the mangroves flanking both the estuaries add the ammonia oxidizers in to these estuaries.

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References:

1. Abreu, C., Jurgens, G., De Marco, P., Saano, A., Bordalo, A.A., 2001. Crenarchaeota and Euryarchaeota in temperate estuarine sediments. *J. Appl. Microbiol.* 90, 713-718.
2. Ando, Y., Nakagawa, T., Takahashi, R., Yoshihara, K., Tokuyama, T., 2008. Seasonal changes in abundance of ammonia-oxidizing archaea and ammonia-oxidizing bacteria and their nitrification in sand of an eelgrass zone. *Microbes. Environ.* doi:10.1264/jsme2.ME08536.
3. Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., Weightman, A.J., 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Appl. Environ. Microbiol.* 71, 7724-7736.
4. Beman, J.M., Francis, C.A., 2006. Diversity of Ammonia-Oxidizing Archaea and Bacteria in the Sediments of a Hypernutrified Subtropical Estuary: Bahía del Tóbari, Mexico. *Appl. Environ. Microbiol.* 72, 7767-7777.
5. Beman, J.M., Popp, B.N., Francis, C.A., 2008. Molecular and biogeochemical evidence for ammonia oxidation by marine Crenarchaeota in the Gulf of California. *ISME J.* 2, 429-441.
6. Bernhard, A. E., Donn, T., Giblin, A. E., Stahl, D. A., 2005. Loss of diversity of ammonia-oxidizing bacteria correlates with increasing salinity in an estuary system. *Environ. Microbiol.* 7, 1289-1297.
7. Bothe, H., Jost, G., Schloter, M., Ward, B.B., Witzel, K.P., 2000. Molecular analysis of ammonia oxidation and denitrification in natural environments. *FEMS Microbiol. Rev.* 24, 673-690.
8. Dang, H., Zhang, X., Sun, J., Li, T., Zhang, Z., Yang, G., 2008. Diversity and spatial distribution of sediment ammonia-oxidizing crenarchaeota in response to estuarine and environmental gradients in the Changjiang Estuary and East China Sea. *Microbiol.* 154, 2084-2095.
9. DeLong, E.F., 1992. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. USA.* 89, 5685-5689.
10. Dessai, D.V.G., Nayak, G.N., Basavaiah, N., 2009. Grain size, geochemistry, magnetic susceptibility: Proxies in identifying sources and factors controlling distribution of metals in a tropical estuary, India. *Estuar. Coast. Shelf. S.* 85, 307-381.
11. Francis, C.A., Beman, J.M., Kuypers M.M.M., 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal oxidation. *ISME J.* 1, 19-27.
12. Francis, C.A., O'Mullan, G.D., Ward, B.B., 2003. Diversity of ammonia monooxygenase (amoA) genes across environmental gradients in Chesapeake Bay sediments. *Geobiology* 1, 129-140.
13. Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., Oakley, B.B., 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc. Natl. Acad. Sci. USA* 104, 14683-14688.

14. Freitag, T.E., Chang, L., Prosser, J.I., 2006. Changes in the community structure and activity of betaproteobacterial ammonia-oxidizing sediment bacteria along a freshwater-marine gradient. *Environ. Microbiol.* 8, 684–696.
15. Inagaki, F., Nunoura, T., Nakagawa, S., Teske, A., Lever, M., Lauer, A., Suzuki, M., Takai, K., Delwiche, M., Colwell, F.S., Nealson, K.H., Horikoshi K., D’Hondt, K.S., Jorgensen, B.B., 2006. Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. *Proc. Natl. Acad. Sci. USA* 103, 2815–2820.
16. Ingalls, A.E., Shah, S.R., Hansman, R.L., Aluwihare, L.I., Santos, G.M., Druffel, E.R.M., Pearson, A., 2006. Quantifying archaeal community autotrophy in the mesopelagic ocean using natural radiocarbon. *Proc. Natl. Acad. Sci. USA*. 103, 6442-6447.
17. Karner, M.B., DeLong, E.F., Karl, D.M., 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature*. 409, 507-510.
18. Könneke, M., Bernhard, A., Torre, J.R.D., Walker, C.B., Waterbury, J.B., Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature*. 427, 543-546.
19. Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature*. 442, 806–809.
20. Mincer, T.J., Church, M.J., Taylor, L.T., Preston, C., Karl, D.M., DeLong, E.F., 2007. Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. *Environ. Microbiol.* 9, 1162–1175.
21. Mosier, A.C., Francis, C.A., 2008. Relative abundance and diversity of ammonia-oxidizing archaea and bacteria in the San Francisco Bay estuary. *Environ. Microbiol.* 10, 3002-3016.
22. Nasolkar, C.M., Shirodkar, P.V., Singbal, S.Y.S., 1996. Studies on organic carbon, nitrogen and phosphorous in the sediments of Mandovi Estuary, Goa. *Indian J. Mar. Sci.* 25, 120-124.
23. Nicol, G.W., Schleper, C., 2006. Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? *Trends Microbiol.* 14, 207–212.
24. Ovreas, L., Forney, L., Daae, F. L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* 63, 3367–3373.
25. Prosser, J.I., Nicol, G.W., 2008. Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. *Environ. Microbiol.* 10, 2931–2941.
26. Ramaiah, N., Kenkre, V.D., Verlecar, X.N., 2002. Marine environmental pollution stress detection through direct viable counts of bacteria. *Water Res.* 36, 2383-2393.
27. Santoro, A.E., Francis, C.A., de Sieyes, N.R., Boehm, A.B., 2008. Shifts in the relative abundance of ammonia-oxidizing bacteria and archaea across physicochemical gradients in a subterranean estuary. *Environ. Microbiol.* 10, 1068-1079.

28. Schleper, C., Jurgens, G., Jonuscheit, M., 2005. Genomic studies of uncultivated archaea. *Nat. Rev. Microbiol.* 3, 479-488.
29. Schloss, P.D., Handelsman, J., 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl. Environ. Microbiol.* 71, 1501–1506.
30. Shetye, S.R., Kumar, M.D., Shankar, D., 2007. The Mandovi and Zuari Estuaries. National Institute of Oceanography, Dona Paula, India.
31. Stein, J.L., Simon, M.I., 1996. Archaeal ubiquity. *Proc. Natl. Acad. Sci. USA.* 93, 6228-6230.
32. Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596-1599.
33. Teske, A., Sørensen, K.B., 2008. Uncultured archaea in deep marine subsurface sediments: have we caught them all? *ISME J.* 2, 3-18.
34. Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W:improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673-4680.
35. Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., Wu, D.Y., Paulsen, I., Nelson, K.E., Nelson, W., Fouts, D.E., Levy, S., Knap, A.H., Lomas, M.W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y.H., Smith, H.O., 2004. Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304, 66-74.
36. Vieira, R.P., Clementino, M.M., Cardoso, A.M., Oliveira, D.N., Albano, R.M., Gonzalez, A.M., Paranhos, R., Martins, O.B., 2007. Archaeal Communities in a Tropical Estuarine Ecosystem: Guanabara Bay, Brazil. *Microb. Ecol.* 54, 460-468.
37. Ward, B. B., 2005. Molecular approaches to marine microbial ecology and the marine nitrogen cycle. *Annu. Rev. Earth Planet. Sci.* 2005. 33:301–33
38. Wuchter, C., Abbas, B., Coolen, M.J., Herfort, L., van Bleijswijk, J., Timmers, P., 2006. Archaeal nitrification in the ocean. *Proc. Natl. Acad. Sci. USA.* 103, 12317–12322.
39. Yan, B., Hong, K., Yu, Z., 2006. Archaeal Communities in Mangrove Soil Characterized by 16S rRNA Gene Clones. *J. Microbiol.* 44, 566-571.
40. Zeng, Y., Li, H., Jiao, N., 2007. Phylogenetic diversity of planktonic archaea in the estuarine region of East China Sea. *Microbiol. Res.* 162, 26-36.

Legends to Figures:

Fig. 1. Sampling sites in Mandovi and Zuari estuaries

Fig. 2. Neighbor-joining phylogenetic tree of Crenarcheota (2a) and Euryarcheota (2b) inferred from 16S rRNA gene sequences obtained from composite sediments of Mandovi (MES) and Zuari (ZES) estuaries. The tree is drawn using 91 representative ribogroups of 433 clones from these sediments. Values at nodes are the percent occurrence of the sequences in the same cluster (Bootstrap values <50% are not shown). Figures in parentheses are the number of clones related to that particular ribogroup. Scale bar represent the 5% nucleotide substitution. *Aquifex aeolicus* was used as the out group.

Fig. 3. Neighbor-hood joining phylogenetic tree of archaea ammonia monooxygenase subunit A gene sequences (*AamoA*) from the sediment samples of Mandovi (MS) and Zuari (ZS) estuaries. Only representative phylotypes were taken for tree construction after grouping with DOTUR. Figures in parentheses are the numbers of clones related to that particular phylotype. Values at nodes are the percent occurrence of the sequences in the same cluster. Only bootstrap values >50% are shown. Scale bar represents 5% substitution. *Nitrosomonos europea* and *Nitrospira briensis* were used as the out-groups.

Fig. 4. Rarefaction curves of operation of taxonomical units (OTU) of archaea derived from (a) 16S rRNA gene sequences and (b) Archaeal ammonia monooxygenase subunitA (*AamoA*) gene sequences from the sediment samples of Mandovi and Zuari estuaries.

Table 1: Distribution of ribogroups, diversity indices and coverage of operation of taxonomical units (OTUs) from sediment samples from Mandovi and Zuari estuaries

Diagnostic	16S rDNA clones		<i>amoA</i> clones	
	Mandovi	Zuari	Mandovi	Zuari
No. of clones	178	254	44	39
OTUs (no)	58	48	22	9
Shannon-Weaver Index	3.18±0.21	2.52±0.20	2.82±0.27	1.91±0.23
Simpson's Index	0.08	0.17	0.06	0.15
Chao I	110- 386	67-171	30-134	9- 34
Good's Coverage (C)	78%	88%	68%	92%

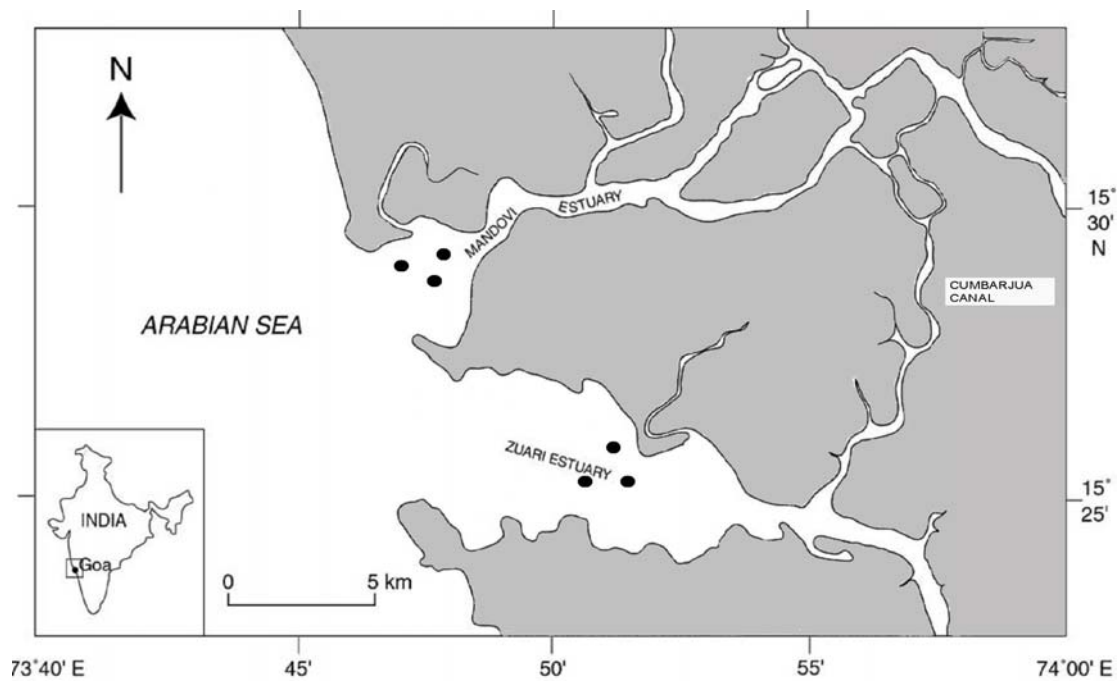


Fig. 1.

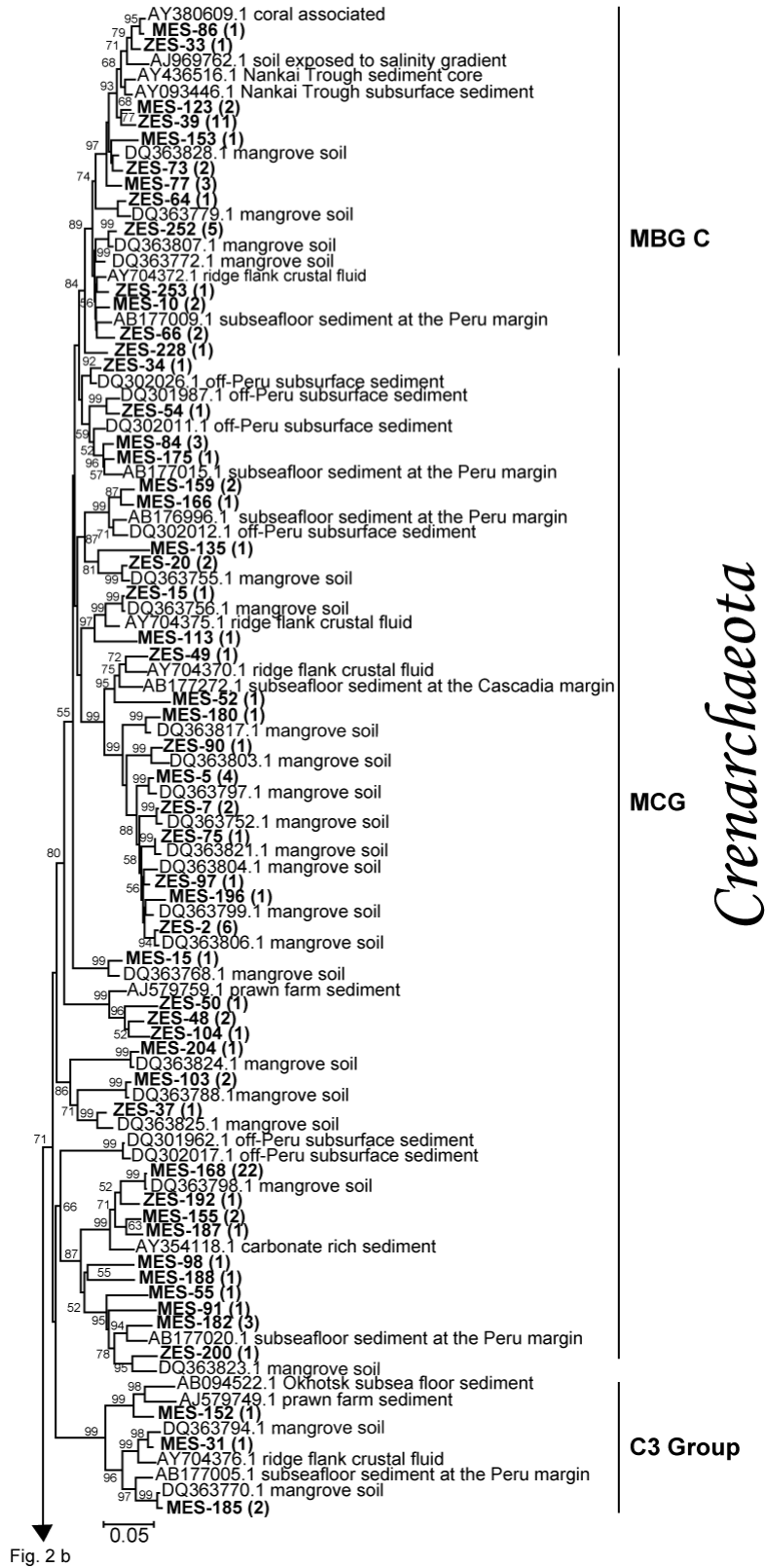


Fig. 2 b

Fig. 2a.

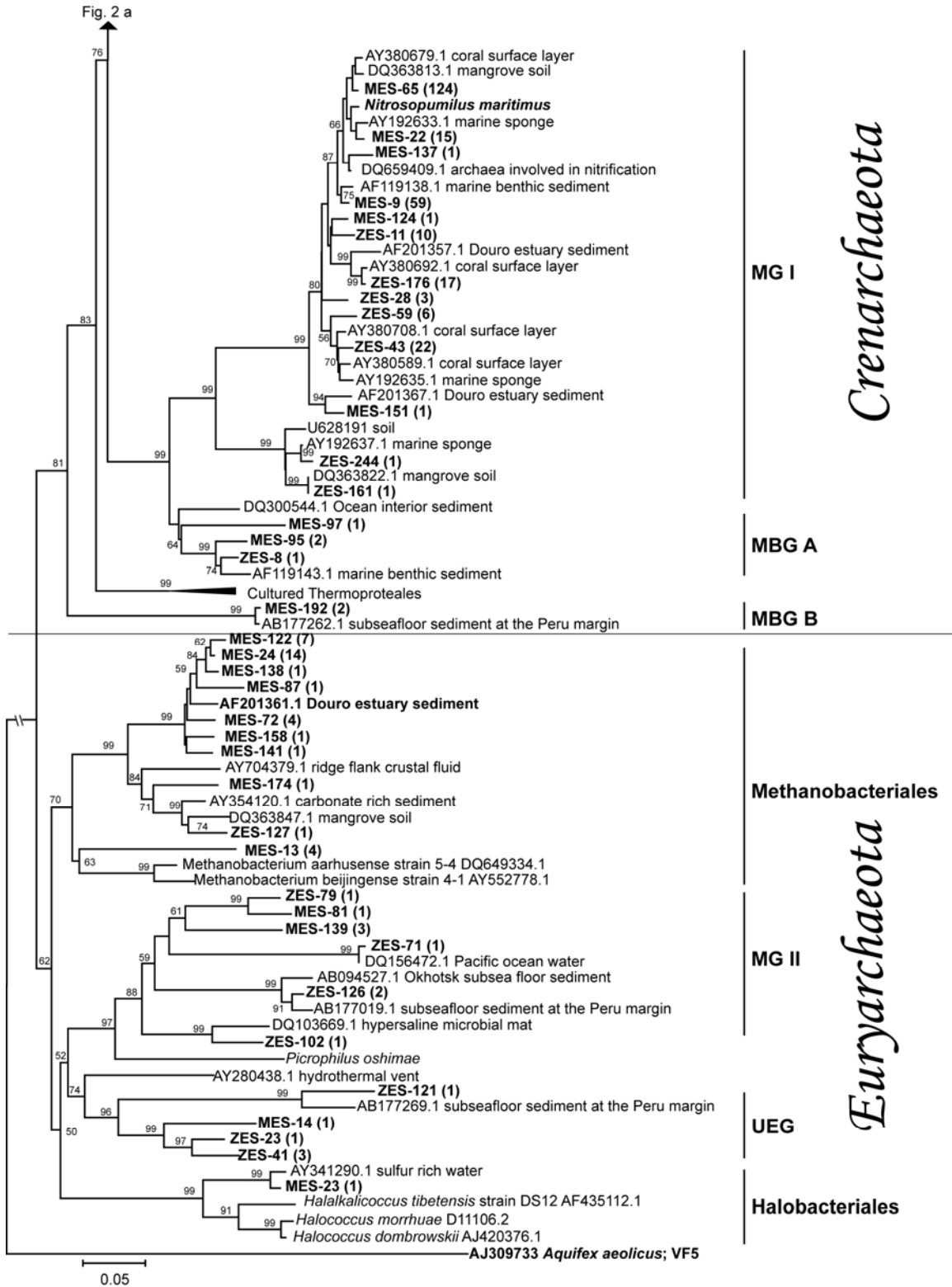


Fig. 2b.

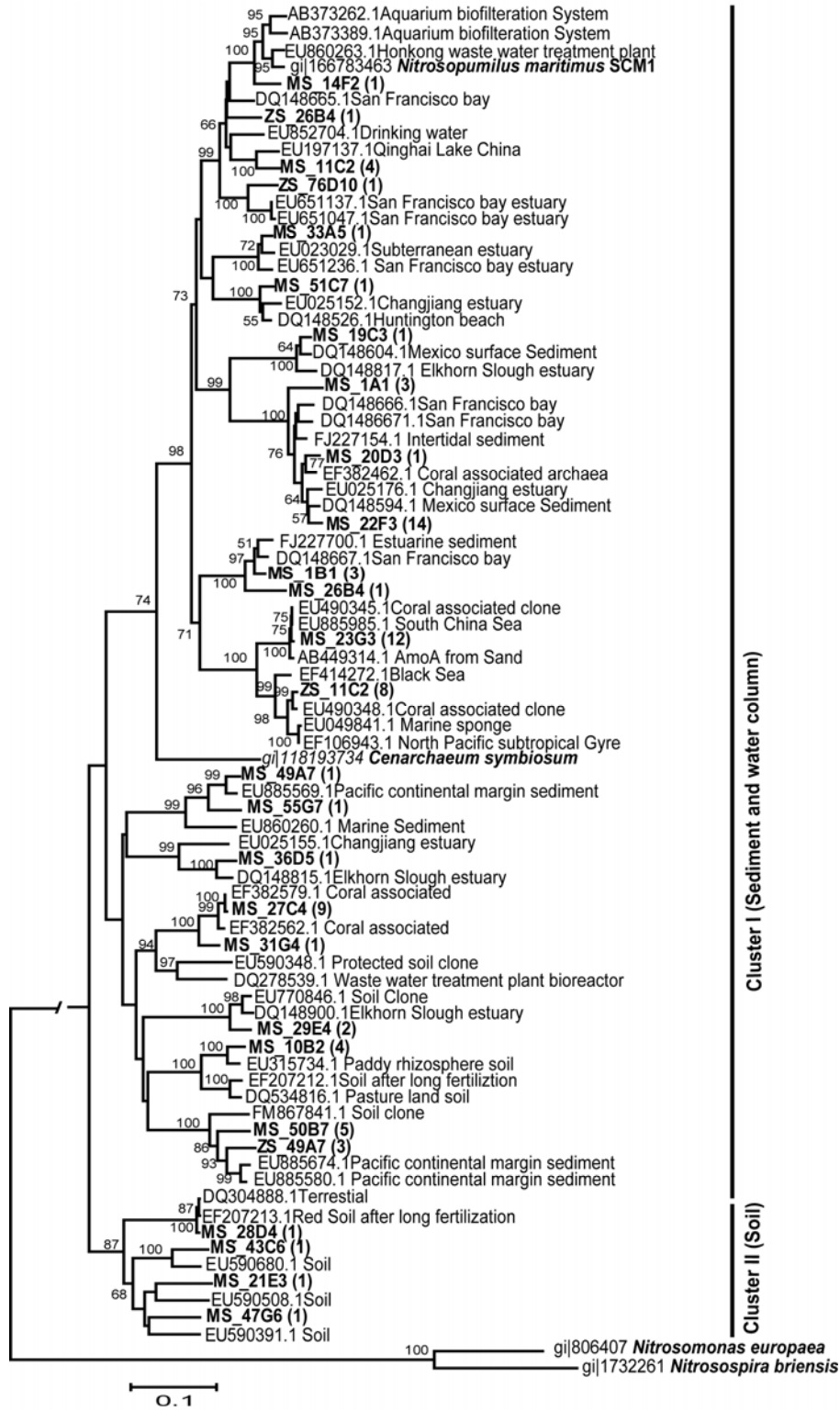


Fig. 3

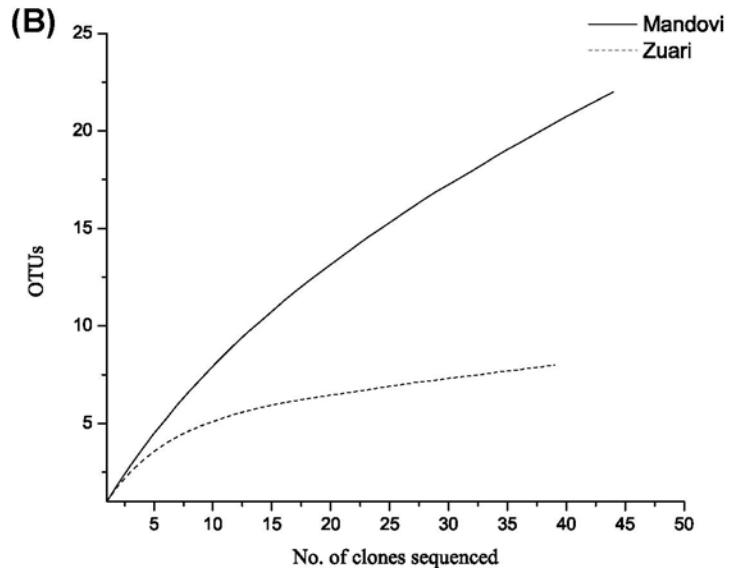
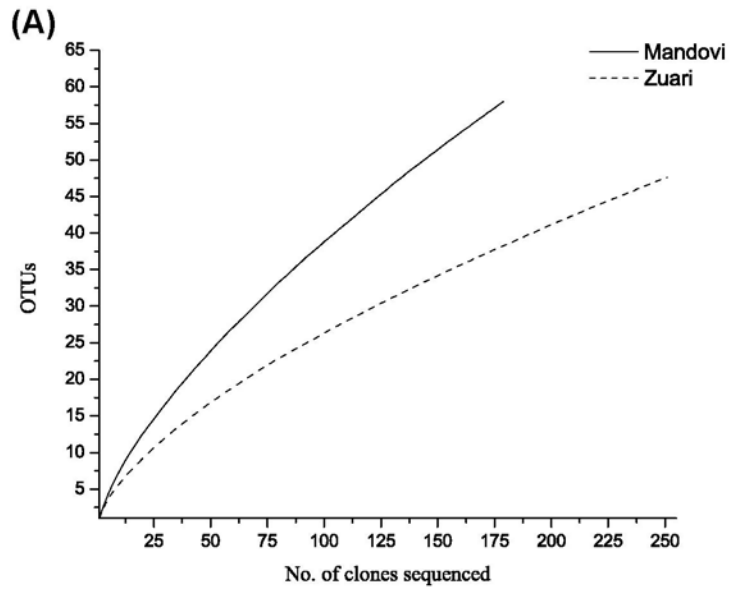
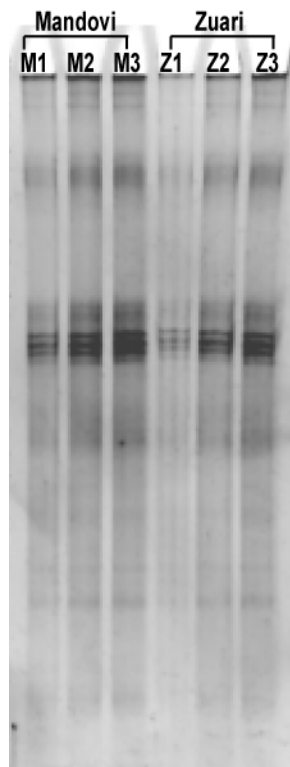


Fig. 4.

Supplementary Material

Supplementary Fig1: DGGE profiles of sediment archaeal community from Mandovi (M) and Zuari (Z) sediment DNA.



Supplementary Table 1. Details of *AamoA* gene clones from Mandovi and Zuari estuarine sediments forming OTUs with $\geq 95\%$ sequences similarity

Sl. No.	<i>AamoA</i> gene sequences with $\geq 95\%$ similarity level	No. of sequences in the group
1	MS_1_A1, MS_18B3, MS_25A4	3
2	MS_1_B1, MS_46F6, MS_94F12	3
3	MS_10B2, MS_13E2, MS_3_C1, ZS_51C7	4
4	MS_11C2, MS_39G5, MS_58B8, MS_57A8	4
5	MS_14F2	1
6	MS_19C3	1
7	MS_20D3	1
8	MS_21E3	1
9	MS_22F3, MS_54F7, MS_35C5, MS_52D7, MS_44D6, MS_45E6, ZS_43C6, ZS_57A8, ZS_29E4, ZS_68D9, ZS_89A12, ZS_9A2, ZS_93E12, ZS_74B10	14
10	MS_23G3, MS_9A2, ZS_66B9, ZS_50B7, ZS_75C10, ZS_10B2, ZS_42B6, ZS_5E1, ZS_18B3, ZS_25A4, ZS_28D4, ZS_52D7	12
11	MS_26B4	1
12	MS_27C4, MS_30F4, MS_34B5, MS_53E7, MS_95G12, MS_42B6, ZS_27C4, ZS_59C8, ZS_73A10	9
13	MS_28D4	1
14	MS_29E4, MS_4_D1	2
15	MS_31G4	1
16	MS_33A5	1
17	MS_36D5	1
18	MS_43C6	1
19	MS_47G6	1
20	MS_49A7	1
21	MS_50B7, ZS_19C3, ZS_21E3, ZS_2B1, ZS_77E10	5
22	MS_51C7	1
23	MS_55G7	1
24	ZS_11C2, ZS_53E7, ZS_82B11, ZS_58B8, ZS_61E8, ZS_69E9, ZS_81A11, ZS_90B12	8
25	ZS_26B4	1
26	ZS_49A7, ZS_65A9, ZS_92D12	3
27	ZS_76D10	1