*Available online at* www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.5352

**ISSN: 2320 – 7051**

*Int. J. Pure App. Biosci.* **5 (5):** 348-352 (2017)

*Research Article*

International Journal of Pure & Applied Bioscience

OPEN ACCESS

# Phylogenetic Studies of Unculturable Halophilic Bacteria in the Fossil Sediments of Ariyalur Basin

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Received: 30.07.2017 | Revised: 27.08.2017 | Accepted: 4.09.2017

**ABSTRACT**

*Metagenomics is a habitat based method to study the unculturable mixed microbial populations which are difficult for cultivation based approaches. In the present investigation, an attempt was done to study the uncultivable microbial diversity from the fossil sediments of Ariyalur through 16S rRNA gene sequencing. Two uncultured bacterial 16S rRNA gene sequences were cloned in the vector respectively and subjected to 16S rRNA sequencing. The revealed two 16S rRNA gene sequences showed the high similarity to Bacillus oceanisediminis and Acinetobacter indicus. The microbial data suggested the sea invasion theory of Ariyalur.*

***Keywords:*** *Metagenomics, Ariyalur, Acinetobacter indicus, Bacillus oceanisediminis, microbial diversity.*

## INTRODUCTION

Majority of bacterial cells in the biosphere are difficult to isolate and culture due to diverse factors such as the lack of nutrients, toxic metabolites accumulation and excess inhibitory compounds secretion, faulty combinations of environmental conditions and slow growth rate compared to other microbes[1]. Due to these limitations, traditional methods are not ideal for the isolation and culture of microbes. Hence an alternative method called metagenomics could be utilized to study the bacteria that are recalcitrant to isolate and culture.The Cretaceous Formation of Ariyalur is one of the best developed sedimentary sequences in South India and has been attracting the scientific community from early 1920s for its fossil repositories. The Sea invasion theory of Ariyalur suggests the ingression of sea occurred about 120 million years ago and later receded towards the east, thus emerged the present Ariyalur land. Excavations of Ariyalur basin has resulted in the identification of fossils related to the remains of teeth, vertebrae, limb bones of Hyena, Bos, Sus, Rhinoceros[2], dinosaurs[3] and fossils of marine organisms such as *Nautilus*, *Rostellum gregareum*, *Rostellum carinatum*, *Ammonite*, *Astarte legans*, *Pseudopectin equivlvis*, *Astarte gueuxi* and *Gryphaea*[4], thus substantiate the sea invasion theory. The present study was carried out to characterize the microbial phylogeny in the fossil sediments of the Ariyalur basin and correlating the microbiological data to substantiate the sea invasion theory.

**Cite this article:** Edward, A., Phylogenetic Studies of Unculturable Halophilic Bacteria in the Fossil Sediments of Ariyalur Basin, *Int. J. Pure App. Biosci.* **5(5):** 348-352 (2017). doi: http://dx.doi.org/10.18782/2320-7051.5352

## RELATED WORK

Many research related to Metagenomics of uncultured microbes in salt rich environment has been reported in Lonar lake sediments[5], saline desert of Kutch[6], hexachlorocyclohexane contaminated soils[7] and the diversity of *Bacillus thuringenesis*[8] in the western ghats. There were also reports about the culturable diversity of bacteria from the fossilized remains of Ariyalur basin. Prabhu *et al*.,[9,10] has reported the isolation and characterization of two bacterial isolates, *Bacillus halodurans* and *Stenotrophomonas maltophilia* from the soils of Ariyalur basin that has shown resistance/tolerance against antimicrobials. Recently the diversity of Arbuscular Mycorrhizal Fungi was reported in the cement contaminated sites[11] and the calcerous soils of Ariyalur[12]. The present work involves studying the unculturable microbial diversity of halophilic bacterial communities in Ariyalur basin to dissipate the sea invasion theory which is currently lacking in literature.

## MATERIALS & METHODS

### 1. Collection and storage of samples

Samples were collected from the upper layer of soils, from different geographic locations. Soil that consists of mixture of sand and fossil aggregates were collected, composited, homogenized by sieving and stored at 4º C till the extraction of environmental DNA.

### 2. Extraction of Soil genomic DNA

The environmental genomic DNA was isolated using HiPurA™ Soil DNA Purification Kit according to the manufacturer's instructions. The isolated genomic DNA was dissolved in molecular biology grade water and stored at -20°C for further use.

### 3. PCR analysis

Amplification of 16S ribosomal sequence from environmental genomic DNA was carried out by using the primers 27F (5′AGAG TTTGATCMTGGCTCAG3′) and 1492R (5′ TACGGYTACCTTGTTACGA CTT 3′) (13) in a thermal cycler (Gradient PCR, Eppendorf). The cyclic conditions were as follows: initial denaturation at 94 °C for 3 min, denaturation with 35cycles at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 2 min and finally held at 4 °C. The PCR products (1500 bp) of the sample were identified by 1% agarose gel electrophoresis[13,14, 15].

### 4. Clone library and sequencing

PCR products were purified using the FavorPrep GEL/ PCR Purification Kit (Favorgen Biotech Corporation, Taiwan) and cloned into pMD20-T vector using the Mighty TA-cloning Kit (TAKARA BIO INC, Japan) as per manufacturer's instructions and transformed in to *E.coli* CJ236 (TAKARA BIO INC, Japan) competent cells using standard protocol. Two colonies were picked randomly and the plasmid was isolated using FavorPrep Plasmid Extraction Mini Kit (Favorgen Biotech Corporation, Taiwan) and processed for sequencing. Automated sequencing was carried out according to the applied Biosystems automated sequencer by Dideoxy chain termination method using Synergy Scientific Services. 2.11. The representative sequences obtained were searched using NCBI BLAST to find the nearest matched species. The uncultured bacterial 16S rRNA sequences were deposited in the GenBank nucleotide sequence databases. A phylogenetic tree was constructed by the neighbor-joining distance matrix method in the Clustal X program with 1000 bootstrap replicates and displayed using the Molecular Evolutionary Genetics Analysis package (MEGA6)[19].

## RESULTS AND DISCUSSION

Comparing the uncultured 16S rDNA gene sequences with the sequences submitted in Genbank by Nucleotide BLAST revealed that our uncultured bacterium clone MN02 16S rDNA sequence (KX059329.1) has shown 99% similarity to *Acinetobacter indicus* (KX950813.1) and uncultured bacterium clone MN04 16S rDNA sequence (KX059328.1) has shown 94% similarity to *Bacillus oceanisediminis* (JQ799086.1) respectively. A neighbor-joining tree of our two sequences with other Genbank 16S rRNA sequences of

bacterial isolates from Ariyalur basin showed that our sequence occupies specific clade with *Bacillus* and *Acinetobacter*. Kimura-2 parameter was used as the nucleotide substitution model. The bootstrap values (%) presented at the branches was calculated from 1000 replications. *Citrococcus alkalitolerans* strain AFB10 (HQ848268.1) was used as an out-group. Scale bar indicates 0.1 substitutions per nucleotide position.

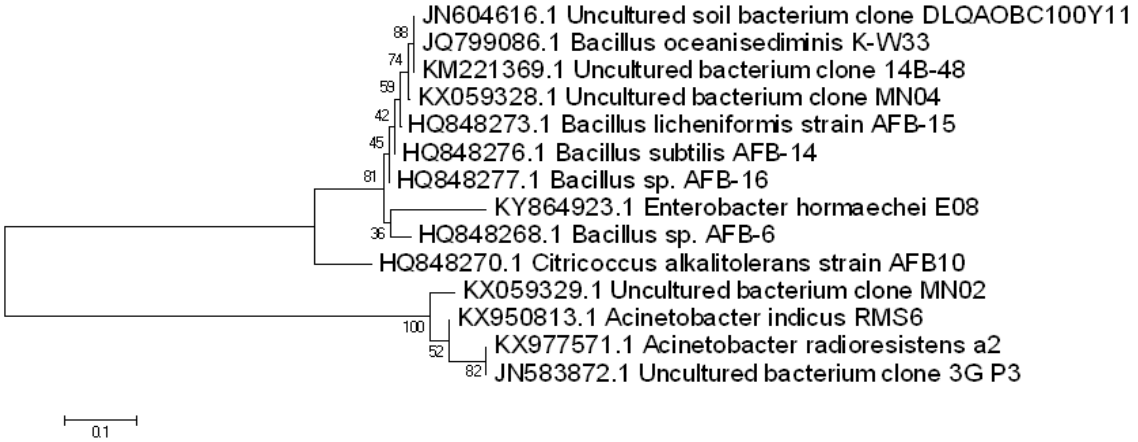


**Fig. 1: 16S rDNA phylogenetic tree, showing the relationship between uncultured MN02 & MN04 clones and other bacterial species isolated from Ariyalur basin, forming distinct clusters**

The evolutionary history was inferred using the Neighbor-Joining method[16]. The optimal tree with the sum of branch length = 1.50051028 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches[17]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [18] and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 220 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[19].

*B. oceanisediminis* is a halophilic, gram positive, spore forming, rod-shaped aerobic, surviving at a temperature range of 4°C-45°C and pH fluctuation of 6-10. Since it is a decomposer bacterium in marine environment, it plays an important role in the cleaning of sediments in the ocean ecosystem. Also it has been reported to contain copper, cobalt-zinc-cadmium and tellurium resistance proteins connected to biomineralization and isolated from a sample of sediment from the South Sea in China at a depth of 823 metres[20, 21]. *Acinetobacter indicus* has been reported to be isolated from Hexachlorocyclohexane contaminated sites which is a salty environment[22]. The presence of *B. oceanisediminis* could helps in the formation of Ariyalur marine fossils by biomineralization. Also the presence of these two halophilic bacteria suggests that the Ariyalur basin was once inundated with sea water as B. *oceanisediminis* reported in the abyss of sea.

## CONCLUSION

The present study concludes the sea ingression and regression theory of Ariyalur through the microbiological data. Further sequencing of other metagenomic 16S rRNA genes could reveal more unknown uncultured bacterial communities in Ariyalur basin, thus strengthening the sea invasion theory.

**Acknowledgement**

We acknowledge the University Grants Commission (UGC, Government of India) for the financial assistance sanctioned through Minor research project [5825/15 (SERO/UGC) January 2015].

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