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Research Article

Phylogenetic Analysis of 16S Ribosomal DNA Sequences of Uncultured Bacterial Communities from the Creatceous Fossil Sediments of Ariyalur Basin

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ABSTRACT

Metagenomics is a process which involves the bypassing of microbial cultures in the laboratory conditions and enables to study the microbial communities that are difficult to culture. In the present investigation, uncultured microbial DNA was isolated from the fossil sediments and characterized through 16S rRNA gene sequencing. Two different 16S rRNA gene amplicons were cloned in the vector respectively and subjected to 16S rRNA sequencing. Bioinformatics analysis of two 16S rRNA gene sequences has shown the closer resemblance to Brevibacterium species and Pseudomonas species respectively. The microbial data suggest the sea invasion theory of Ariyalur.

Key words: *Fossils, Ariyalur, Pseudomonas species, Brevibacterium species, 16S ribosomal RNA.*

INTRODUCTION

Metagenomics is a study of the uncultured microbes from environmental samples in which the standard laboratory protocols will not be sufficient and efficient. Using the conventional culturing techniques, only 1% of microbes are culturable using media with 99% unable to be cultured. Hence Metagenomics are considered to be an ideal method for studying the biological diversity of unculturable microbes in an environment. Metagenomics is a multi-step process that involves: (i) Isolation of environmental DNA from microflora (ii) amplification of desired genes (iii) cloning of amplicons in the vector and library construction, and the (iv) phylogenetic analysis of the metagenomic library. Ariyalur is a treasure trove of marine fossils and is considered as Cretaceous Park of South India. Around 15 million years ago, the sea water that submerged the area receded towards the east and left behind fossilized traces of marine animal and plant origins[5]. The authors successfully unearthed the remains of dinosaur fossils from this basin. Subsequent article by Narayan in *Nature* emphasized the significance of the Ariyalur fossil basin as the remains of upper cretaceous period (i.e) approximately 65 Million years ago[16].

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Continued excavations since then identified fossil remains of teeth, vertebrae, limb bones of Hyena, Bos, Sus, Rhinoceros[21]. Molluscs dominate this group, followed by other upper cretaceous creatures including dinosaurs[3]. The taxonomy and phylogenetic relationship of all the fossils collected from Ariyalur fossil mine were discussed in relation to evolution[23]. Adding flavor to these discoveries, plant fossil has also been discovered from Ariyalur basin which supports the speculation that the region once was a marine shore environment. The present study was done to amplify the 16S rRNA sequences from the uncultured microbial DNA and proving the sea invasion theory of Ariyalur basin based on the microbiological data.

Related work

Metagenomic research works of uncultured microbes were carried out in the oldest ice of earth[4], 200 to 13,000-year-old horse bones from northern Siberia[6], *Brachylophosaurus canadensis* fossil[20] and 5,300-year-old Copper Age natural ice mummy[15]. Even though there were reports about the characterization of cultured microbes from Ariyalur fossils[1,2], still the metagenomic characterization of uncultured microbial communities was not done exponentially in the Cretaceous fossil sediments of Ariyalur basin. Two uncultured bacterial species such as *Bacillus oceanisediminis* and *Acinetobacter indicus* was reported from the fossil sediments of Ariyalur basin[7]. In the current research work, an effort was taken to prove the sea invasion theory of Ariyalur basin by charactering the uncultured microbial community data which is currently lacking in literature.

MATERIAL AND METHODS

1. Collection and storage of samples

The soil samples were collected from the Limestone Mines of Tamilnadu Cement Corporation Limited, Kallankurichi, Ariyalur district State of Tamilnadu (Latitude 11.1375959 and Longitude 79.0826431). Soil samples were collected randomly 5-10 cm beneath the surface using spatula and packed in sterile centrifuge tubes. 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. Soil samples possess many compounds that can inhibit downstream enzymatic reactions. The soil sample was lysed and homogenized in a bead-beating step. The total genomic DNA obtained was bound to silica membrane in a spin column format, washed and eluted from the membrane. The purified genomic DNA was dissolved in molecular biology grade water and stored at -20°C for further use.

3. PCR analysis

The PCR parameters were optimized for the maximum amplification of 16S rRNA genes. Amplification of 16S ribosomal sequence from genomic DNA was carried out by using the primers 27F (5′AGAGTTTGATCMTGG CTCAG3′) and 1492R (5′ TACGGYTA CCTTGTTACGACTT 3′)[10] in a thermal cycler (Gradient PCR, Eppendorf). Varying concentrations of template DNA, primers, Mg^{++} ions and different annealing temperatures were set for the PCR optimization. PCR mixtures were prepared with 1µl (100ng) of genomic DNA, 5 µl of PCR buffer 10X, 2mM of $MgCl_2$, 200µmol of each dNTPs, 25 pmol each primer, and 1U *Taq* polymerase (Himedia, Mumbai) and sterile Milli Q water to a final volume of 50 µl. PCR includes initial denaturation at 95°C for 5 min., 35 cycles of denaturation at 95°C for 1 min., annealing at 55°C for 1 min., elongation at 72°C for 1 min. and final extension at 72°C for 4 min. Approximately 1.5 kb amplicons were generated and were identified by 1% agarose gel electrophoresis[8,10,24].

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. Multiple sequence alignment of our 16S rDNA sequences with other bacterial sequences has done with ClustalW which determine the details of the phylogenetic distance between our DNA sequences and its closest relatives. The aligned data set was converted into Molecular Evolutionary Genetics Analysis (MEGA) format. 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)25.

RESULTS AND DISCUSSION

BLAST analysis of the 16S rDNA sequences of uncultured bacteria with the sequences submitted in Genbank has revealed that our uncultured *Brevibacterium* sp. clone MJC13 16S rDNA sequence (MF289398.1) has shown 91% similarity to *Brevibacterium sp.* BMT7 (JX434114.1) and uncultured *Pseudomonas* sp. clone MAV02 16S rDNA sequence (KX059336.1) has shown 86.6% similarity to *Pseudomonas sp.* strain BH-3 (JQ799086.1) respectively. A neighbor-joining tree of our two sequences with other Genbank 16S rRNA sequences showed that our sequences occupy specific clade with *Pseudomonas* and *Brevibacterium*. Kimura-2 parameter was used as the nucleotide substitution model. The bootstrap values (%) presented at the branches was calculated from 1000 replications. *Uncultured Streptomyces sp. clone MJC19* (MF289404.1) was used as an out-group. Scale bar indicates 0.1 substitutions per nucleotide position.

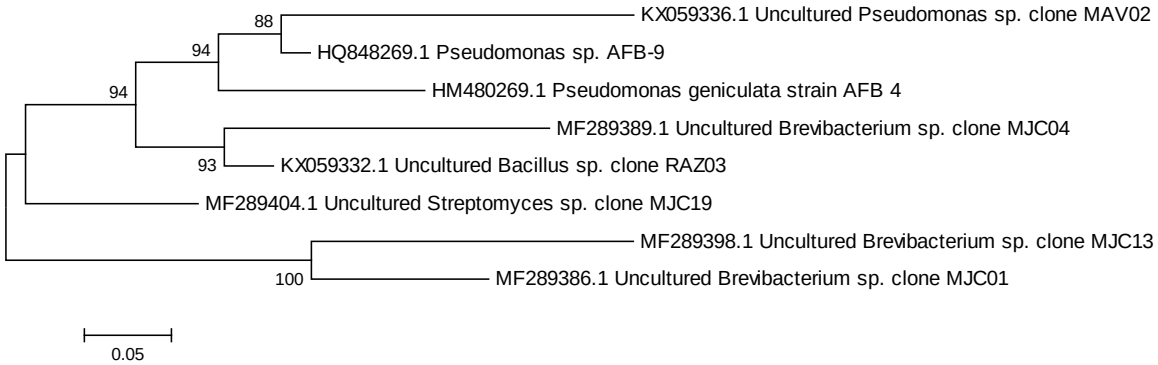

Fig. 1: 16S rDNA phylogenetic tree, showing the relationship between uncultured MN02 & MN04 clones and other bacterial species isolated from Ariyalur basin, forming distinct clusters

The evolutionary history was inferred using the Neighbor-Joining method[19]. The optimal tree with the sum of branch length = 1.31976703 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches[9]. 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[13] and

are in the units of the number of base substitutions per site. The analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 264 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[25].

Brevibacterium is a gram positive bacteria of the order Actinomycetales. It was reported that *Brevibacterium* was recovered from marine environments, such as algae and beach sediment[11,14]. A pigment forming *Brevibacterium* strains were isolated from the sea surface inter tidal zones at different sampling sites along the Visakhapatnam coastal region[22]. *Pseudomonas* is a gram-negative bacteria and is generally regarded as a freshwater or terrestrial organism. Presence of *P. aeruginosa* was first reported in marine environments i.e. Tokyo Bay[12]. Isolation of *P. aeruginosa* strains from an open-ocean site were also reported[17].

CONCLUSION

The microbiological data of the present study conclusively proved that the Ariyalur basin was once submerged under the sea. Further sequencing of other uncultured bacterial communities could strengthen the sea invasion theory.

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