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Study of microbial diversity in Indian Ocean water samples by the use of metagenomics technique

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Abstract
The characterization of global marine microbial taxonomic and functional diversity is a primary goal of this study. The Indian Ocean water sampling expedition is a part of this study. Four water samples were collected and genomic DNA was extracted by metagenomics technique. The extracted DNA samples were amplified using 16s rRNA and then analyzed by using BLAST and phylogenetic tree was prepared to determine relationship between all types of bacterial sequence and provides unprecedented insight into bacterial diversity, which implausible to culture. The results show four strains *Escherichia coli*, *Pseudomonas meridian*, *Bacillus indicus* and *Bacillus velezensis* were obtained in the study.

Keywords: Metagenomics, Indian Ocean, 16s rRNA etc.

Introduction
The characterization of global marine microbial taxonomic and functional diversity is a primary goal of the study made after obtaining the samples from Indian Ocean. Marine bacteria play an important role in providing energy and nutrients in the water. So an understanding of their distribution and seasonal diversity is essential. Many studies have investigated microbial distribution and diversity with regard to the particular environmental and geographical conditions in various microbial habitats [1, 2].

The world's oceans are teeming with microscopic life shapes. Nominal cell counts of >105 cells per ml in surface sea water [3]. With more than 1030 microbial cells, prokaryotes are the predominant life form on Earth [4, 5]. Bacteria are ubiquitous, inhabiting typical soil and water environments as well as radical environments such as deep-sea hydrothermal vents, glacier ice, and waters with high salt content [6-8].

An explosion of knowledge in the field of microbial physiology and genetics happened during 1960s to mid-1980s wherein some scientists came to believe that cultured microorganisms did not represent the whole microbial world. The isolating and sequencing the genome of an individual organism from a consortium might not be adequate as the single isolate cannot be a representative of the full genetic makeup. Moreover, achieving culture conditions for isolating a single member from a consortium would be a daunting task [9]. New non-culture based approaches have recently been developed that can be extensively used for comprehensive analysis of different communities in a microbial consortia. The term "Metagenomics" was first coined by Handelsman and his colleagues in their study of natural products from soil microbes [10]. Recent advances in next-generation sequencing techniques have enabled large-scale exploration of the taxonomic diversity and geographic distribution of marine bacteria. High-throughput pyrosequencing techniques for phylogenetically informative marker genes, including the rRNA genes, have recently provided evidence that marine bacteria may indeed exhibit spatial patterns akin to those of larger organisms, in terms of distribution and abundance, but their temporal patterns remain relatively unexplored in the ocean water column [11, 12].

Materials and Methods
1. Date and place of collection of the water samples
Four surface water samples were collected from Indian Ocean from the coastal region of Chennai, Tamil Nadu during January 2016. The four water samples were designated as E, F, G and H.

2. Extraction of DNA by metagenomics technique from the water samples collected from Indian Ocean

The microbial DNA was extracted by metagenomics technique from the water sample obtained from Indian Ocean. The DNA was isolated directly without culturing the microbes into culture medium. Debris was removed from the collected water sample (100 ml) then it was poured through Micro cloth filtration material which is having size of 0.45µm per pore. This step was conducted to trap the microbes onto the filter membrane. 2µl of Tween 20 was added to 1 ml Distilled water. Here Tween 20 acts as filter wash buffer. Immediately, vortex the tube and transfer the cell suspension to a clean micro centrifuge tube. Resuspend the cell pellet in 300 µl of TE Buffer, than add 2µl of Ready-lyse lysozyme solution and add 1µl of RNase A to the cell suspension. Mix them by vortexing and incubate at 37 °C for 30 minutes. Now add 300µl of Meta-lysis solution (2X) and 1µl of Proteinase K to the tube. Mix them by vortexing and treat with 570µl of isopropanol. Now add 500µl of 70% ethanol to the pellet and re-suspend the pellet in 50µl of TE Buffer. Now centrifuge the sample at different time frames. Re-suspend the DNA pellet in 50 µl of TE Buffer and store for further work.

3. Agarose gel electrophoresis

Dissolve agarose powder in 1X TAE buffer. Wipe the gel casting tray & comb with ethanol. Pour the agarose solution in the gel casting tray and leave to solidify for 15-20 minutes. Mix the DNA sample (pellet) in 50µl of TE buffer. Load the samples in the wells carefully using pipettes in order that the gel should not be broken. Run the sample for 20-30 minutes. Remove the gel from the electrophoresis tank and observe the bands on UV transilluminator.

4. Polymerase chain reaction

Amplification of DNA was be performed in a total volume of 30µl along with 16s rRNA.

5. Sequencing analysis by bioinformatics tools

Sequencing analysis was performed by using NCBI BLAST tool and CLUSTAL omega tool. To construct the phylogenetic tree CLUSTAL OMEGA was used. Homepage of CLUSTAL OMEGA was opened from the URL www.ebi.ac.uk. Selected all the aligned sequences and click on the distance tree of result. A phylogenetic tree will appear along with your query sequence. Click on the name of any species, then on layout and finally on radial tree to get the structure of the phylogenetic tree.

Results and Discussions

The result shows that good amount of genomic DNA was extracted from the samples obtained from Indian Ocean and similar results were recorded by [13]. The results obtained from after visualizing the bands in the gel electrophoresis have shown genomics DNA of sample E, F, G and H (Figure 1). It also shows that genomic DNA of more than 10kb, 8kb, 6kb, 5kb, 3kb, 1kb.

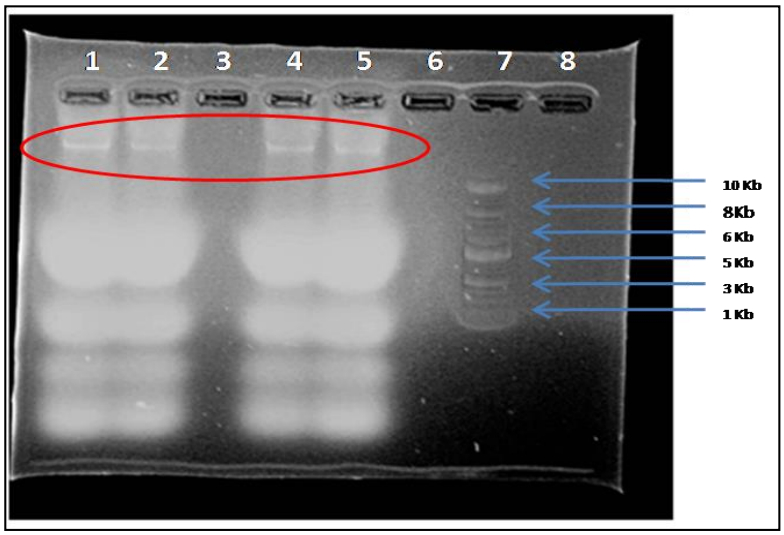

Fig 1: Results of the agarose gel electrophoresis for genomic DNA extracted from the Indian Ocean water samples designated as E, F, G and H.

Lane 7: 1kb DNA Ladder

Lane 1: Sample E (Genomic DNA) Size: More than 10 kb
Lane 2: Sample F (Genomic DNA) Size: More than 10 kb
Lane 4: Sample G (Genomic DNA) Size: More than 10 kb
Lane 5: Sample H (Genomic DNA) Size: More than 10 kb

2. Quantification of genomic DNA extracted from different water samples of Indian Ocean:

Quantification step was performed by using UV-Vis double beam spectrophotometer of wave length between 254-260nm and the significant DNA concentration in sample (Table 1).

Table 1: Result of the DNA concentration in water samples of Indian Ocean

S. No.	Sample ID	Variety of samples	Ratio	DNA concentration	Protein concentration
1	E	Indian ocean	1.34	0.81	1.05
2	F	Indian ocean	1.27	1.22	1.80
3	G	Indian ocean	1.29	1.65	2.36
4	H	Indian ocean	0.43	0.36	3.14

After the amplification, the genomic DNA obtained from the four water samples shows the bands at 10kb, 8kb, 6kb and 2kb (Figure 2) with DNA ladder of 10kb. The result showed similarity to result obtained by [14].

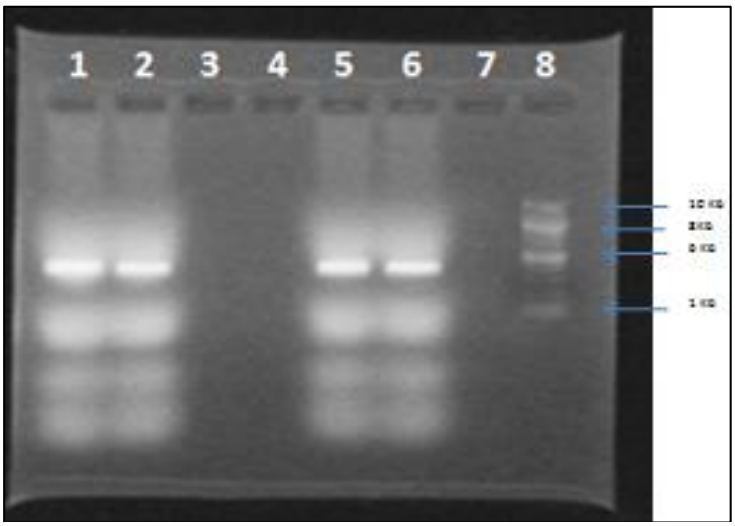

Fig 2: Agarose gel electrophoresis of amplified DNA products by PCR for the water samples namely E, F, G and H. Lane 8: 10kb DNA Ladder

Lane 1: Sample E (PCR product) Size: 1.2-1.3 kb
Lane 2: Sample F (PCR product) Size: 1.2-1.3 kb
Lane 5: Sample G (PCR product) Size: 1.3-1.4 kb
Lane 6: Sample H (PCR product) Size: 1.3-1.4 kb

3. Sequencing of the amplified DNA products of the different water samples

The sequencing was performed by Sanger's method and the sequences showed similarity to study [13]. The code sequences obtained are as follows (Table 2).

Table 2: Results of the sequences of the different water samples

S. No.	Sample ID	Samples description	Code sequence
1	E	Indian ocean	CG20160320E
2	F	Indian ocean	CG20160320F
3	G	Indian ocean	CG20160320G
4	H	Indian ocean	CG20160320H

4. Sequencing analysis by bioinformatics tools

(a) Result of the BLAST for the first sequence CG20160320E of the first water sample i.e. E

The sequence which was obtained from the sample E showed 99% similarity with *Escherichia coli* gene sequence. To understand evolutionary relationship is a fundamental aspect of modern biology, with the phylogenetic tree being a primary tool for describing these associations. The design of phylogenetic tree with *Escherichia coli* gene 16srRNA and relation with other bacteria (Figure 3).

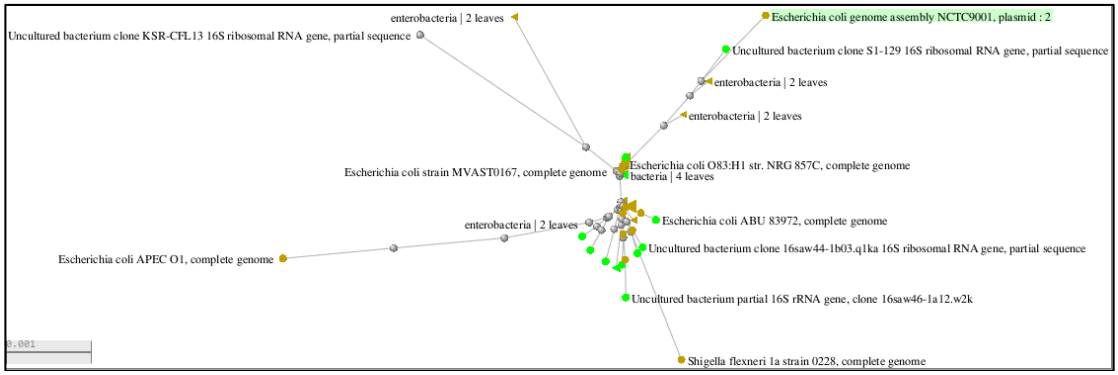

Fig 3: Phylogenetic tree of *Escherichia coli* gene 16srRNA

After the analysis with the phylogenetic tree the observed result showed similarity to *Escherichia coli* genome assembly NCTC9001, plasmid: 2 which is found closest with uncultured bacterium clone S1-129 16S rRNA gene, partial sequence. It is a gram negative, non-spore forming rod shaped bacteria. The organism is a facultative anaerobe and ferments simple sugars such as glucose to form lactic, acetic, and formic acid. *E. coli* is commonly used as an indicator in the field of water purification. The *E. coli* index can indicate about the amount of human feces in the water [15].

(b) Result of the BLAST for the second sequence CG20160320F of the second water sample i.e. F

The sequence which was obtained from the sample F showed 98% similarity with *Pseudomonas meridian* strain CMS 38. To understand evolutionary relationship is a fundamental aspect of modern biology, with the phylogenetic tree being a primary tool for describing these associations. The design of phylogenetic tree with *Pseudomonas meridian* gene 16srRNA and relation with other bacteria (Figure 4).

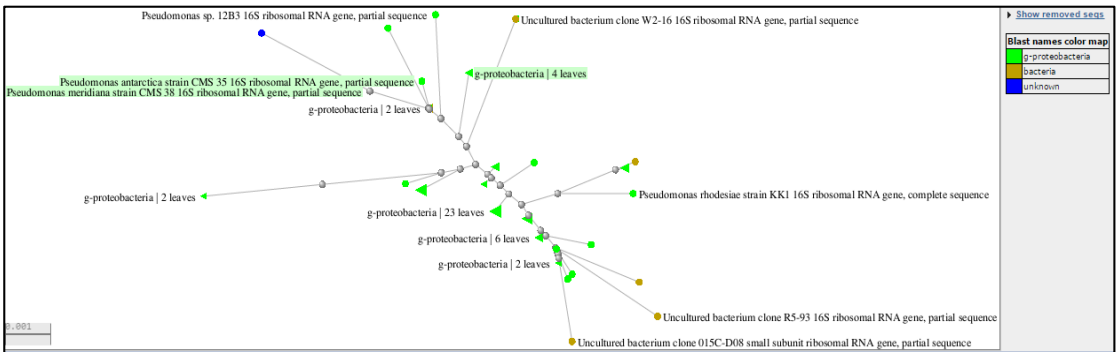

Fig 4: Phylogenetic tree of *Pseudomonas meridian* strain CMS 38.

After the analysis of the second sequence with the phylogenetic tree the observed result showed similarity to partial sequence of result with *Pseudomonas meridian* strain CMS 38. The second sequence was also similar to *Pseudomonas* strain CMS 35 16s rRNA gene. *Pseudomonas meridian* is a Gram-negative, rod-shaped bacteria. It is classified as aerobic, though it is known sometimes to thrive in conditions with little or no oxygen. This bacteria is infects through hospitals and other health care facilities, making it a nosocomial pathogen. It is also becoming increasingly resistant to treatment with antibiotics. *Pseudomonas meridian* is a frequent culprit in causing infections of the lungs, bones and joints, skin, blood and urinary tract. It is particularly dangerous in people with compromised immune systems [16].

(c) Result of the BLAST for the third sequence CG20160320G of the third water sample i.e. G

The sequence which was obtained from the sample G showed 98% similarity with *Bacillus indicus* strain sd/3rRNA partial sequence. To understand evolutionary relationship is a fundamental aspect of modern biology, with the phylogenetic tree being a primary tool for describing these associations. The design of phylogenetic tree with *Bacillus indicus* gene 16srRNA and relation with other bacteria (Figure 5).

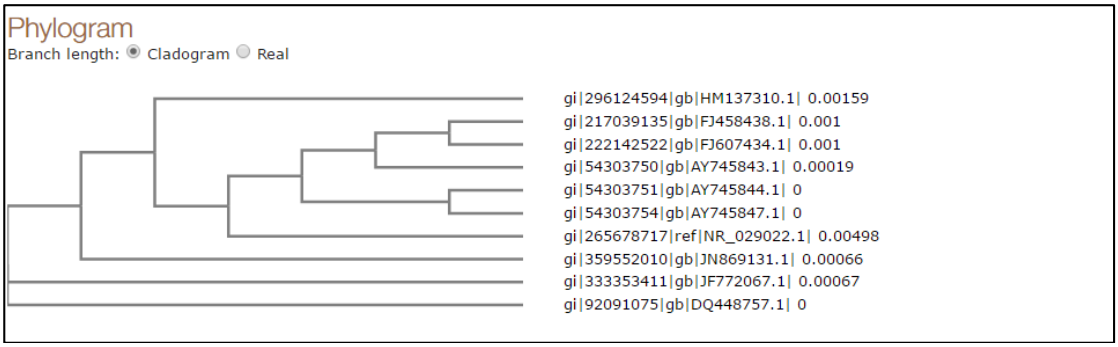

Fig 5: Phylogenetic tree of *Bacillus indicus* strain sd/3

After the analysis of the third sequence with the phylogenetic tree the observed result showed similarity to partial sequence of result with *Bacillus indicus* strain sd/3rRNA. *Bacillus indicus* is a gram-positive, rod-shaped, spore-forming bacterium. *Bacillus cibi* was once thought to be a separate species but is now classified as *Bacillus indicus*. It is an interesting bacterium in the probiotics world because it can produce carotenoids, pigments which are the sources of the yellow, orange, and red colors of many plants, algae and photosynthetic bacteria. Many of these carotenoids have known health benefits and thus the potential to include this bacterium or its products in supplements and many processed foods is exciting to the food and supplement industries [17].

(d) Result of the BLAST for the fourth sequence CG20160320H of the third water sample i.e. H:

The sequence which was obtained from the sample H showed 99% similarity with *Bacillus velezensis* strain GEN 11 16s RNA partial sequence. To understand evolutionary relationship is a fundamental aspect of modern biology, with the phylogenetic tree being a primary tool for describing these associations. The design of phylogenetic tree with *Bacillus velezensis* strain GEN 11 16srRNA and relation with other bacteria (Figure 6).

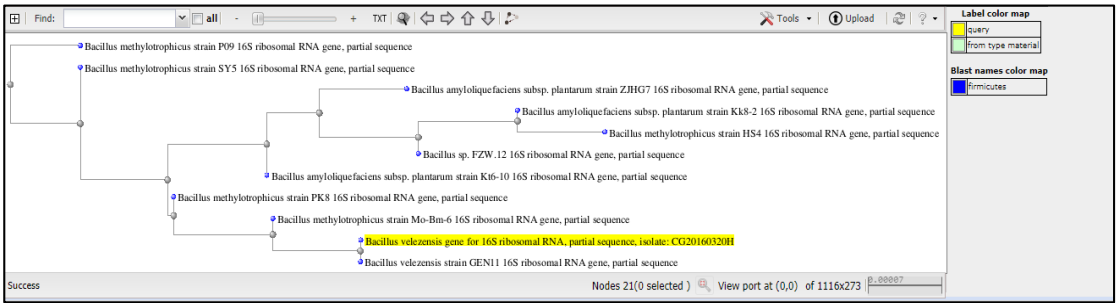

Fig 6: Phylogenetic tree of *Bacillus velezensis* strain GEN 11 16s rRNA partial sequence

After the analysis of the fourth sequence with the phylogenetic tree he observed result showed similarity to partial sequence of result with the topology of the phylogenetic tree confirmed that they were members of the *Bacillus subtilis* group. The partial sequence of result was similar to *Bacillus velezensis* strain GEN 11 16s RNA partial sequence and closest to *Bacillus methylotrophicus* strain Mo-Bm - 16r sRNA gene partial sequence and *Bacillus* methylotrophicus strain PK 8- 16r s RNA gene partial sequence represent father for at, that consider a genus of gram-positive, rod-shaped bacteria and a member of the phylum Firmicutes. Bacillus species can be (having the ability to be aerobic or anaerobic). They will test positive for the enzyme catalase when there has been oxygen used or present. They are ubiquitous in nature, both free-living (non-parasitic) and parasitic pathogenic species. Many species of Bacillus can produce copious amounts of enzymes which are used in different industries. Some species can form intracellular inclusions of polyhydroxy alkanoates under certain adverse environmental conditions. *Bacillus subtilis* has proved a valuable model for research. Other species of Bacillus are important pathogens, causing anthrax and food poisoning [18].

5. Nucleotide sequence accession numbers

All nucleotide sequence data from Indian Ocean of this study were submitted to the DNA data bank of Japan (DDBJ) for getting the accession numbers of the four water samples.

Table 3: Results of the accession numbers of various water samples

Sample No.	Sample ID	Sample description	Code Sequence	Accession Number
1	E	Indian ocean	CG20160320E	LC144549
2	F	Indian ocean	CG20160320F	LC144550
3	G	Indian ocean	CG20160320G	LC144551
4	H	Indian ocean	CG20160320H	LC144552

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