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RESEARCH ARTICLE

PCR-DGGE BASED DIVERSITY ANALYSIS OF BACTERIA AND ARCHAEA FROM MUD VOLCANOES ON ANDAMAN ISLANDS, INDIA

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ABSTRACT

Mud volcanoes at Baratang and Kattan started ejecting mud after the 2004 Tsunami incidence. In this study, prokaryotic diversity in the flowing mud was investigated by using PCR-DGGE approach. Presence of 88 organisms was detected of which, 36% population detected was affiliated to archaea. All the archaea detected were representatives of Euryarchaeota. 64% population was affiliated to bacteria, mainly dominated by Proteobacteria. In Proteobacteria, alpha proteobacteria contributed 26.785%, beta proteobacteria was found to be 28.57%, gamma proteobacteria contributed 16.071% and delta proteobacteria was 5.357%. Actinobacteria contributed 7.14% followed by firmicutes being 12.5%. Sphingobacteriales contributed 1.78% and ungrouped organisms contributed 1.78%. Statistical analyses of all DGGE gels indicate that the diversity of Kattan mud volcano was comparatively greater than Baratang mud volcano. The results indicate that a large number of unexplored microbial groups may exist in this unique ecosystem.

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INTRODUCTION

Mud volcanoes are the channels for releasing the pressurised mud, gases and water from beneath the surface of earth. Mud volcanoes are both submarine and subaerial in nature and are found all around the globe. They are often created at points of weakness in the Earth's crust, along fault lines. A close relationship between earthquakes and mud volcanic activity is also predicted and often both occur in close succession to each other. Volcanoes sometimes contain traces of oil, together with associated mud from great depths within the earth. About 86 percent of the gases that are released are methane along with carbon dioxide and nitrogen in traces. Chemically, volcanic mud is composed primarily of silica (55-70 %), which can support the growth of microbes and no potential inhibitors of microbial growth are reported to be present. Interest in mud volcanoes is tied to the following four reasons: potential risk for drilling operations, the tendency to occur in areas of high petroleum or gas hydrate potential, the relationship with tectonics and their rich biologic community. The application of molecular-phylogenetic methods to study natural microbial ecosystems without the traditional requirement for cultivation has resulted in the discovery of many unexpected evolutionary lineages. Members of some of these lineages are only distantly related to known organisms. They are so abundant that they are likely to have impact on the chemistry of the biosphere. So, considering the traditional limitations of yet to be cultured bacteria, molecular biology tools are helpful in portraying better pictures of the biogeospheres. Molecular fingerprints of diversity can be achieved by coupling PCR amplification of taxonomic targets with sequence dissimilarities analysed by DGGE. DGGE enables separation of DNA fragments of identical length but of different sequence. Various diversity indices can be used for statistical analysis of DGGE gels. Shannon Weiner Index for abundance, Simpsons index for dominance and range weighted richness analysis for range of denaturants used for sequence specific separation was studied. Aim of the paper is to document bacterial and archaeal diversity of mud volcanoes of Andaman using PCR based DGGE approach, after elimination of various biases like DNA extraction bias, primer bias and annealing temperature bias etc.

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MATERIALS AND METHODS

Sample collection, processing and transportation to laboratory

Flowing mud samples were collected in sterile sample containers from the mud volcanoes located at Baratang and

Kattan (12.18°N, 92.80°E). The samples were degassed at regular intervals so as to avoid any unnecessary conditions favouring growth of particular group of organisms.

Fig. 1. Flowing mud from volcano at Baratang

Microscopic analysis

Neubaeur's chamber was used for enumeration of soil bacteria. Very low cell counts were seen on microscopic analysis of the volcanic slurry. Therefore an efficient method that could lyse all the bacteria and archaea present in the sample was necessary. Genomic DNA isolation was performed by various methods. These methods rely on various combinations of chemical (detergent or guanidium isothiocyanate based), physical (temperature shock based), mechanical (bead beating based) processes.

Total genomic DNA isolation

Genomic DNA isolation in prokaryotes greatly relies on the susceptibility of cell wall to lysis. Seven different methods were performed on the same amount of sample to check for the best DNA isolation method. CTAB method- Anionic detergent (Zhou *et al.,* 1996)

1) Chromous kit-Detergent mediated lysis of cells at high temperature (protocol performed according to manufacturer's instructions)
2) MoBio Microbial DNA kit- Detergent based lysis followed by mechanical bead beating (protocol performed according to manufacturer's instructions).
3) Mobio Soil DNA kit – Guanidium isothiocyanate based lysis followed by mechanical bead beating (protocol performed according to manufacturer's instructions).
4) Sigma bacterial DNA isolation kit – Lysozyme based chemical lysis (protocol performed according to manufacturer's instructions).
5) Zymo Research kit- Detergent based chemical lysis (protocol performed according to manufacturer's instructions).
6) Direct lysis-Freeze-Thaw cycle based lysis.

On loading the DNA sample on 0.7% agarose gel, no DNA could be detected, indicating insufficient amount of detectable DNA or complete absence of it. To confirm the presence of DNA, PCR using various primers specific for different hypervariable regions were used. The risk of primer bias towards specific DNA templates was also taken care of in this experiment.

PCR amplification of the 16S rRNA genes

Genomic DNA purified from soil was used as template for PCR. Amplification of 16S rRNA genes was done by PCR using universal primer set. 27 FDD2 and 1492 RPP2 primers yielded PCR products of about 1.5 kb. 600 bp universal amplicons were obtained using primers GC clamp and com 2. Primers used for amplification of 400 bp were GC com1 and com 2. GC clamp and SRV3-2 were used to amplify 200 bp universal amplicons. Archaea specific primers used were DRF1-DRR1, which amplified 1100 bp region. 600 bp archaea specific region was amplified using primers Arch344F and Arch915 R. 200 bp Archaea-specific amplicons were obtained using primer set Arch 344F and SRV3-2. Touchdown PCR technique was employed to find out the optimum annealing temperature. Oligonucleotide primers were synthesized by M/S Sigma Aldrich India. Amplification was carried out in a 20 µl reaction mixture containing 1.5 mM MgCl2, 0.2 mM each dNTPS, 10 pM each primer, 10 ng DNA template and 1 U Taq polymerase (Sigma Aldrich) with reaction buffer supplied by the manufacturer. The PCR cycle used was as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C (1 min) - 57°C (1 min) - 72°C (1 min). Final extension was at 72°C for 20 min. Negative control reaction contained no exogenous template and the positive control was the tube containing DNA from pure culture. PCR products were examined by gel electrophoresis on 1.5% agarose gel, prepared in 1X TAE buffer. Nested PCR was employed to increase sensitivity.

Table 1. Primer details used in the study

No	Primer	Specificity	Sequence (5'→3')
1	27 FDD2	Bacteria	CCGGATCCGTCGACAGAGTT TGATCITGGC TCAG
2	1492 RPP2	Bacteria	CCAAGCTTCTAGACGGITAC CTTGTTACGA CTT
3	GC Clamp	Bacteria	CGCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGCGG GGGG CCTACGGGAGGCAGC
4	Com 2	Bacteria + Archaea	CCGTCAATTCCTTTGAGTTT
5	SRV3-2	Bacteria	TTACCGCGGCTGCTGGCAC
6	GC com 1	Bacteria	CGCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGGGG GCAGCAGCCGCGGTAATAC
7	DRF2	Archaea	CCGGATCCGTCGACTCCAGG CCCTACGGG
8	Arch344 FGC	Archaea	CGCCCGCCGCGCCCCG CGCCCGTCCCGCCGCCCCCG CCCGACGGGGYGCAGCAGG CGCGA
9	Arch 915R	Archaea	GTGCTCCCCCGCCAATTCCT

Denaturing Gradient Gel Electrophoresis:

PCR amplicons (2–20 µL equivalent to 100–500 ng DNA for metagenomic samples) were separated by DGGE on 16.5 X 16.5 cm, 1-mm-thick polyacrylamide [37.5:1 (w/v) acrylamide/ bisacrylamide, Fluka, Ronkonkema, NY, USA] gels, using Biorad Universal mutation detection system. 100% Urea-Formamide denaturant had 7 M urea and 40% (v/v) formamide. Electrophoresis was carried out at optimum voltage for optimum time at appropriate temperature in 1X Tris–acetate–EDTA (TAE) buffer. Gels were stained (0.5

mg/mL ethidium bromide in 1X TAE for 10 min), destained (sterile distilled water for 5 min) and visualized with a 312-nm wavelength transilluminator.

Elution, Reamplification and sequencing of DGGE Bands:

Bands of interest were excised from the gel using a sterile blade and incubated overnight at 4ºC in sterile distilled water to allow DNA diffusion out of the polyacrylamide matrix. The solution was then used for further amplifications using the same set of PCR primers without GC clamp. The PCR products to be used for sequencing were purified using PEG:NaCl. Cycle sequencing reaction was setup using one of the primers and cleanup was performed according to manufacturer's instruction. The samples were analyzed on an automated DNA sequencer ABI 3100 AVANT genetic analyser (Applied Biosystems, Foster City, CA, USA). Searches were performed in GenBank using BLAST to determine the closest known relatives of the partial ribosomal DNA sequences obtained.

Statistical analysis of DGGE gels

Various statistical tests were performed like Shannon Weiner diversity index, Simpson's index to give an idea of the richness and dominance of microbial communities in the soil samples. Range weighed richness was also considered, which accounts for the range of denaturant gradient over which the DNA has spread out in a single lane. Values of 0-10 indicate lower diversity, 10-30 indicate moderate diversity habitat and values of 30 and above indicate a high diversity habitat.

RESULTS

Microscopic analysis

Very low cell counts were seen on microscopic analysis of the volcanic slurry.

Total genomic DNA isolation

Genomic DNA isolation in prokaryotes greatly relies on the susceptibility of cell wall to lysis. Seven different methods were performed on the same amount of sample to check out for the best DNA isolation method. CTAB method- Anionic detergent; Chromous kit-Detergent mediated lysis of cells at high temperature ; MoBio Microbial DNA kit- Detergent based lysis followed by mechanical bead beating; Mobio Soil DNA kit – Guanidium isothiocyanate based lysis followed by mechanical bead beating; Sigma bacterial DNA isolation kit – lysozyme based chemical lysis; Zymo Research kit- Detergent based chemical lysis ; Direct lysis-Freeze-Thaw cycle based lysis. On loading the DNA sample on 0.7% agarose gel, no DNA could be detected, indicating insufficient amount of DNA or complete absence of it. DNA isolated by 5 methods was used further for optimisation experiments.

PCR amplification of the 16S rRNA genes

Various primer sets were used for the amplification of hypervariable regions of 16S rRNA genes. In case of CTAB method, no detectable amplification seen in any of the sample.

Table 2. Optimisation of DNA isolation method and choice of hypervariable region for PCR

No	Details	Dilution	A	B	C	D	E	F	G	H
	Primer pair		FDD2-RPP2	GC clamp-com2	com1-com2	SRV3-1-SRV3-2	EC 1055-EC1392	DRF1-DRR1	A344F-A915R	A344F-SRV3-2
	Specificity		Universal	Bacterial	Bacterial	Bacterial	Bacterial	Archaeal	Archaeal	Archaeal
	Hypervariable region		V1-V9	V3-V5	V4-V5	V3	V7-V8	V1-V6	V3-V5	V3
1	Chromous kit	Undiluted	-	-	-	++	-	-	-	-
2	MoBio Microbial DNA kit	Undiluted	-	-	-	+++	+	-	-	-
3	Sigma Bacterial genomic DNA isolation kit	Undiluted	++	+	-	++	+	-	-	-
4	Zymo Research kit	Undiluted	+	+	-	+++	+	-	-	-
5	Direct lysis	Undiluted	-	-	-	-	-	-	-	-
6	Chromous kit	1:10	-	-	-	++	-	-	-	-
7	MoBio Microbial DNA kit	1:10	-	-	-	+	-	-	-	-
8	Sigma Bacterial genomic DNA isolation kit	1:10	-	++	-	+++	-	-	-	-
9	Zymo Research kit	1:10	-	-	-	-	-	-	-	-
10	Direct lysis	1:10	-	-	-	-	-	-	-	-
11	Chromous kit	1:50	-	-	-	+	-	-	-	-
12	MoBio Microbial DNA kit	1:50	-	-	-	-	-	-	-	-
13	Sigma Bacterial genomic DNA isolation kit	1:50	-	++	++	+	-	-	-	-
14	Zymo Research kit	1:50	-	-	-	-	-	-	-	-
15	Direct lysis	1:50	-	-	-	-	-	-	-	-
16	Chromous kit	1:100	-	-	+	-	-	-	-	-
17	MoBio Microbial DNA kit	1:100	-	-	+	-	-	-	-	-
18	Sigma Bacterial genomic DNA isolation kit	1:100	-	+	-	-	-	-	-	-
19	Zymo Research kit	1:100	-	-	-	-	-	-	-	-
20	Direct lysis	1:100	-	-	-	-	-	-	-	-

DGGE analysis

Table 3. Optimized conditions for DGGE

No.	Details	Archaea		Bacteria	
1	Hypervariable region	V1-V6	V3	V3-V5	V4-V5
2	Size of amplicon	1100 bp	200 bp	600 bp	400 bp
3	Denaturing gradient	30-60%	40-70%	30-65%	30-70%
4	Polyacrylamide gel percentage	6%	8%	6%	6%
5	Runtime	12 hr	4 hr 30 min	6 hr	6 hr
6	Voltage	130 V	100 V	130 V	130 V
7	Temperature	60ºC	60ºC	60ºC	60ºC

With the MoBio kit for soil DNA isolation, though direct PCR did not work efficiently (smear seen in many cases), nested PCR worked very well and gave clear bands of appropriate size. These nested PCR products were used for loading on DGGE gels.

DGGE analysis of PCR amplified V3-V5 (600 bp) SSU gene fragment of 16S rRNA gene representing microbial community associated with mud volcano

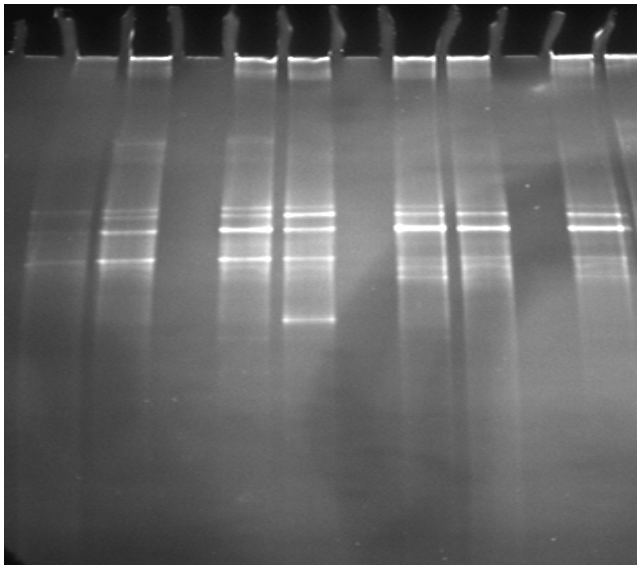


Figure 2. DGGE profile of amplified V3-V5 region of 16S rRNA gene of bacteria

Samples loaded were Lane 1: Mud from Baratang mud volcano; Lane 2: Baratang mud volcano site 1; Lane 3: Baratang mud volcano site 2; Lane 4: Volcanic mud from Kattan mud volcano site 1; Lane 5: Volcanic mud from Kattan site 2. Denaturing gradient gel electrophoresis gel of V3-V5 hypervariable region of bacterial 16S rRNA gene polymerase chain reaction products (600 bp, bases 341 to 906 relative to *E.coli* rRNA gene sequence) amplified from mud volcanic DNA extracts. Primer pair used for amplification GC-SRV3-1 – com2. Gel gradient on 6 % gel ranging from 30 % to 65 % denaturant, run for 6 hours at 130 volts.

the diversity (Hurlbert, 1971). Intensity of each band on the DGGE gel was considered to be proportional to the abundance. Following indices were used in the study:

Shannon Weiner Index (H'): Measures the number of species and how they are distributed i.e. proportional abundances of species,

- Species evenness (J): It is a measure of the relative abundance of the different species making up the richness of an area,
- Simpson Index (D): Measures dominance of each species in the population.
- Range weighted richness: Measures the range of denaturing gradients that was used for separation of individual bands. More is the range of denaturing gradient between the first and last band in a well, more is the diversity. Here, diversity implies differences in sequences, which were separated differently.

Calculations for statistical analysis of DGGE gels

The following calculations were performed to arrive at numerical values. Comparison of various samples becomes easier by the use of these statistical methods. Intensity of bands on the DGGE gels was considered to be proportional to abundance of particular species.

Table 5. Statistical analysis of the DGGE gel

No.	Shannon Weiner index	Maximum diversity	Evenness	Simpsons index of Dominance $D=\Sigma\ pi^2$	1/D	Range weighed richness
1	2.2476	2.4849	0.9045	0.1213	8.2379	7.2
2	2.1121	2.1972	0.9612	0.1309	7.6394	7.29
3	0.2105	2.9444	0.0715	0.1042	9.5920	54.15
4	0.1916	3.18	0.0602	0.0851	11.75	51.84
5	0.2535	2.6390	0.0960	0.1572	6.3613	11.76
6	2.0597	2.6390	0.7804	0.1865	5.3592	11.76
7	2.4399	2.9957	0.8144	0.1360	7.3491	24

As indicated in the range weighed richness, Baratang mud volcano and Kattan mud volcano are the most diverse habitats with good microbial load.

Table 4. BLAST analysis of well resolved individual DGGE bands (600 bp fragments – V3-V5 region bacterial amplicons using primer pair GC-SRV3-1 com2) (Batch 2)

No	Band designation	Closest phylogenetic affiliation in NCBI database	Homology (%)	Group
1	MV1-3	Methylobacterium species	97	Alpha Proteobacteria
2	MV1-4	Caulobacter endosymbiont	93	Alpha Proteobacteria
3	MV2-1	Thiobacillus sayanicus strain	98	Beta Proteobacteria
4	MV3-3	Caulobacter endosymbiont	94	Alpha Proteobacteria
5	MV4-1	Acidothiobacillus ferrooxidans	88	Gamma Proteobacteria
6	MV4-2	Acidothiobacillus ferrooxidans	90	Gamma Proteobacteria
7	MV4-3	Uncultured Nitrospira species	85	Actinobacteria
8	MV4-4	Uncultured Nitrospira species	85	Actinobacteria
9	MV5-2	Thiobacillus denitrificans	91	Beta Proteobacteria
10	MV5-3	Alkalispirillum mobile	94	Gamma Proteobacteria
11	MV5-4	Arthrobacter arilaitensis	94	Actinobacteriae

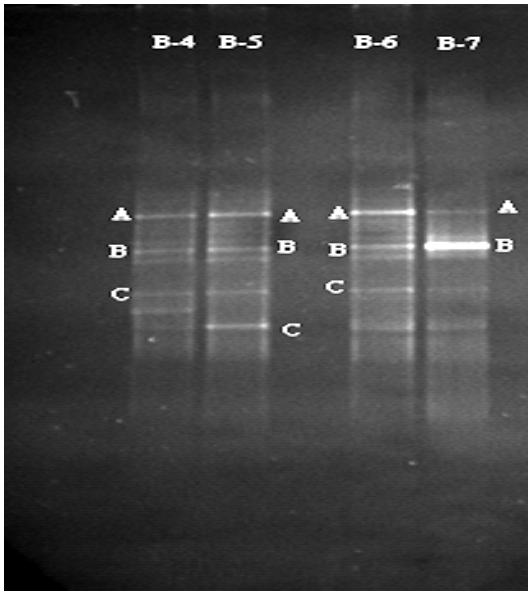
Statistical analysis of DGGE gels

DGGE gels can be analyzed statistically by using various indices. These indices are the estimates of distribution and abundance of species, within that community. No single index is available that can adequately summarize and exactly depict

DGGE analysis of PCR amplified V4-V5 (400 bp) SSU gene fragment of 16S rRNA gene representing microbial community associated with mud volcano

Denaturing gradient gel electrophoresis gel of V4-V5 hypervariable region of bacterial 16S rRNA gene polymerase

chain reaction products (400 bp, bases 519 to 906 relative to *E.coli* rRNA gene sequence) amplified from mud volcanic DNA extracts. Primer pair used for amplification was com1-com2. Gel gradient on 6% gel ranging from 30% to 65 % denaturant, run for six hours at 130 volts.



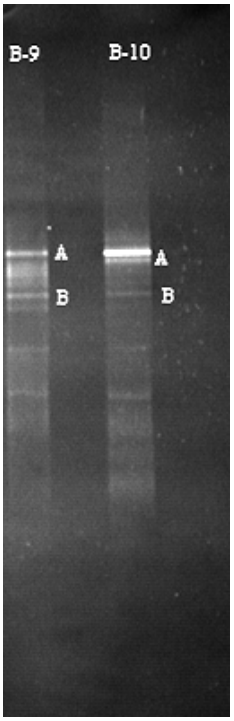


Figure 2. DGGE profile of amplified V4-V5 region of 16S rRNA gene of bacteria

Table 6. BLAST analysis of well resolved individual DGGE bands (600 bp fragments – V3-V5 region bacterial amplicons using primer pair GC-SRV3-1 com2)

No.	Band designation	Closest phylogenetic affiliation in NCBI database	Homology	Group
1	1b (B)	Iodide Oxidizing bacterium	99%	Alpha proteobacteria
2	2a (B)	Sphingobacteriales bacterium	91%	Sphingobacteriales
3	2c (B)	Thioalkalispira microaerophila	89%	Gamma Proteobacteria
4	2e(B)	Iodide oxidizing bacterium	100%	Alpha proteobacteria
5	3a (B)	Iodide oxidizing bacterium	100%	Alpha proteobacteria
6	3c (B)	Hydrogenophaga spp	97%	Beta Proteobacteria
7	3d (B)	Iodide oxidizing bacteria	100%	Alpha proteobacteria
8	4a (K)	Rhodocyclaceae bacterium	87%	Beta Proteobacteria
9	4b (K)	Thioalkalispira microaerophila	94%	Gamma Proteobacteria
10	4d (K)	Iodide oxidizing bacteria	89%	Alpha proteobacteria
11	4e (K)	Rhodocyclaceae bacterium	97%	Beta Proteobacteria
12	4f (K)	Rhodocyclaceaea bacterium	97%	Beta Proteobacteria
13	5b (K)	Thioalkalispira microaerophila	88%	Gamma Proteobacteria
14	6a (K)	Rhodocyclaceae bacterium	92%	Beta Proteobacteria
15	6b (K)	Thioalkalispira microaerophila	93%	Gamma Proteobacteria
16	6c (K)	Thiobacillus denitrificans	99%	Beta Proteobacteria
17	6f (K)	Thiobacillus denitrificans	88%	Beta Proteobacteria
18	7a (K)	Thiobacillus denitrificans	93%	Beta Proteobacteria
19	7b (K)	Thioalkalispira microaerophila	92%	Gamma Proteobacteria
20	7c (K)	Thiobacillus denitrificans	98%	Beta Proteobacteria

Table 7. BLAST analysis of well resolved individual DGGE bands (400 bp fragments – V4-V5 region bacterial amplicons using primer pair com 1- com2)

No.	Band designation	Closest phylogenetic affiliation in NCBI database	% Homology	Group
1	B4 a	Thiobacillus denitrificans	98 %	Beta proteobacteria
2	B4 b	Iodide-oxidizing bacterium	82%	Alpha proteobacteria
3	B4 c	Geothermobacter sp.	91%	Delta proteobacteria
4	B5 a	Thiobacillus denitrificans	97%	Beta proteobacteria
5	B5 b	Hyphomonas jannaschiana	74%	Alpha proteobacteria
6	B6 a	Thiobacillus denitrificans	98 %	Beta proteobacteria
7	B6 b	Iodide-oxidizing bacterium	91%	Alpha proteobacteria
8	B7 a	Iodide-oxidizing bacterium	98%	Alpha proteobacteria
9	B7 b	Candidatus Monilibacter batavus	74%	Alpha proteobacteria
10	B9 a	Hydrogenophaga bisanensis	81%	Beta proteobacteria
11	B9 b	Iodide-oxidizing bacterium	94%	Alpha proteobacteria
12	B10 a	Thiobacillus denitrificans ATCC 25259	99%	Beta proteobacteria

V3 region of archaea

When the V3 region of archaea was amplified and the amplicons were used for DGGE analysis, all organisms detected were representatives of Euryarchaeota.

With very good evenness in the diversity distribution, the range weighed richness indicates that the moderately diverse habitats are Baratang mud volcano, and a specific distance sample from Kattan sample. Here Kattan sample is indicated as a low diversity habitat.

Table 8. BLAST analysis of well resolved individual DGGE bands (200 bp- V3 region fragments)

No.	Band designation	Closest phylogenetic affiliation in EZTAXON database	% Homology	Group
1	1a (B)	*Haloterrigena limicola* AX-7;	84.44%	Euryarchaeota
2	1b (B)	*Haladaptatus paucihalophilus* DX253	83.33%	Euryarchaeota
3	1c (B)	*Halobacterium noricense* A1	83.26%	Euryarchaeota
4	1d (B)	*Halobacterium noricense* A1	84.05%	Euryarchaeota
5	2a (B)	*Halobacterium jilantaiense* NG4	83.90%	Euryarchaeota
6	2b (B)	*Methanoculleus palmolei* DSM 4273	85.40%	Euryarchaeota
7	2c (B)	*Methanofollis liminatans* GKZPZ	84.87%	Euryarchaeota
8	2d (B)	*Haladaptatus paucihalophilus* DX253	84.24%	Euryarchaeota
9	2e (B)	*Methanofollis liminatans* GKZPZ	86.13%	Euryarchaeota
10	3a (B)	*Natronolimnobius innermongolicus* N-1311	87.75%	Euryarchaota
11	3b (B)	*Methanococcoides methylutens* DSM 2657	83.24%	Euryarchaeota
12	3c (B)	*Haladaptatus paucihalophilus* DX253	84.88%	Euryarchaeota
13	3d (B)	*Halalkalicoccus jeotgali* B3	82.81%	Euryarchaeota
14	4a (K)	*Haloterrigena limicola*AX-7	82.48%	Euryarchaeota
15	4b (K)	*Haladaptatus paucihalophilus* DX253	84.03%	Euryarchaeota
16	4c (K)	*Methanofollis aquaemaris* N2F9704	82.05%	Euryarchaeota
17	4d (K)	*Methanofollis liminatans* GKZPZ	85.93%	Euryarchaeota
18	5a (K)	*Methanothrix thermophila* PT	86.89%	Euryarchaeota
19	5b (K)	*Methanolobus oregonensis* WAL1	86.66%	Euryarchaeota
20	6b (K)	*Methanoculleus submarinus* OCM 780	85.15%	Euryarchaeota
21	6c (K)	*Methanogenium cariaci* DSM 1497		Euryarchaeota
22	7a (K)	*Methanocalculus chunghsingensis* K1F9705b	84.21%	Euryarchaeota
23	7b (K)	*Methanogenium organophilum* DSM 3596	85.40%	Euryarchaeota

Key: (B): Baratang Mud volcano, (K): Kattan Mud volcano.

Table 9. Statistical analysis for DGGE profile of V3 region of archaea

No.	No of bands detected	Shannon Weiner index	Maximum diversity	Evenness	Simpsons index of Dominance D=Σ pi2	1/D	Range weighed richness
1	11	1.9984	2.3978	0.8334	0.18173	5.5028	12.1
2	11	2.0835	2.3979	1	0.1765	5.6657	12.1
3	13	2.2038	2.5649	0.8592	0.1531	6.5306	16.9
4	15	2.4938	2.7080	0.9208	0.1047	9.5428	2.25
5	14	2.5	2.6391	0.9536	0.0902	11.0814	19.6
6	18	2.7206	2.8903	0.9412	0.0763	13.0922	35.64
7	12	2.4132	2.4849	0.9711	0.0957	10.4415	12.96
8	3	0.9251	1.0986	0.8421	0.4516	2.2143	0.99

Table 10. BLAST analysis of well resolved individual DGGE bands (1100 bp fragments - V1-V6 region amplicons using primer pair DRF1-DRR1)

No.	Band designation	Closest phylogenetic affiliation in EZTAXON database	% Homology EZTAXON database	Group
1	1a (B)	*Methanoculleus palmolei* DSM 4273	82.72%	Euryarchaeota
2	2a (B)	*Methanosaeta harundinacea* 8Ac	81.53%	Euryarchaeota
3	2b (B)	*Methanosarcina vacuolata* Z-761	81.50%	Euryarchaeota
4	4a (K)	*Methanosarcina lacustris* ZS	82.14%	Euryarchaeota
5	4b (K)	*Methanococcoides alaskense* AK-5	82.33%	Euryarchaeota
6	4c (K)	*Archaeoglobus fulgidus* VC-16	80.30%	Euryarchaeota
7	6a (K)	*Methanomethylovorans thermophila* L2FAW	80.88%	Euryarchaeota
8	7a (K)	*Methanohalobium evestigatum* Z-7303	80.25%	Euryarchaeota

Key: (B): Baratang Mud volcano, (K): Kattan Mud volcano.

Table 11. Statistical analysis for V1-V6 region of archaeal DGGE profile

No.	No of bands seen	Shannon Weiner index	Maximum diversity	Evenness	Simpsons index of Dominance D=Σ pi2	1/D	Range weighed richness
1	17	2.6897	2.8332	0.9493	0.0767	13.0247	5.78
2	18	2.4633	2.8904	0.8522	0.1102	9.0663	6.48
3	21	2.6159	3.0445	0.8592	0.1032	9.6899	8.82
4	20	2.7065	2.9957	0.9034	0.0817	12.2339	16
5	18	2.4909	2.8903	0.8617	0.1220	8.1948	6.48
6	19	2.637	2.94	0.8955	0.1014	9.8539	7.22
7	14	2.3431	2.6390	0.8878	0.1339	7.4631	3.92
8	19	2.4364	2.9444	0.8274	0.1438	6.9525	7.22

V1-V6 region of Archaea

Even though the evenness obtained in the DGGE profile is more, the range weighed richness indicates that the Kattan mud volcano is a habitat of moderate diversity and all others are with low microbial diversity.

DISCUSSION

Various euryarchaeotes were found to be present in the Mud volcanoes at Baratang and Kattan, on the basis of sequencing of the 16S rRNA gene after proper resolution of individual bands on DGGE gels. Majority of the organisms showed close homology to methanogens like *Methanoculleus palmolei, Methanosaeta harundinacea, Methanosarcina vacuolata, Methanosarcina lacustris, Methanococcoides alaskense, Archaeoglobus fulgidus, Methanomethylovorans thermophila, Methanohalobium evestigatum,Methanoculleus submarines, Haloterrigena limicola, Haladaptatus paucihalophilus, Halobacterium noricense, Halobacterium jilantaiense etc.* These organisms mainly are associated with methanogenesis and many sulfate reducing bacteria (like *Methanococcoides alaskense, Archaeoglobus fulgidis*) were also found to be present. Acetate scavenging methanogens like *Methanosaeta harundinaceae,* and microbes used in dehalogenation like *Methanosarcina vacuolata* were detected. Great phylogenetic diversity of halophiles was also detected in these mud volcanoes. Moreover, it is unlikely that the inspected sequences exhaust the diversity of halophilic community. Although phylogenetic placement of some of the sequences is inexact in detail (low pair wise similarity) representatives of most of the major groups of cultivated halophilic prokaryotes are evidently present in this environment. Very low percent homologies and high BLAST scores indicate that these organisms may be novel ones. Moreover, zero E value in most of the cases indicates that the obtained homologies are not merely by chance. Further investigation on these lines is essential for reporting the novel species. As the pristine habitats of Andaman and Nicobar Islands are yet unexplored, the presence of these organisms can be reported for the first time.

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