

Bacterial diversity in deep-sea sediments from Afanasiy–Nikitin seamount, equatorial Indian Ocean

RAKHEE KHANDEPARKER *¹, RAM MURTI MEENA¹ AND DEEPTI DEOBAGKAR²

¹National Institute of Oceanography, CSIR, Goa- 403 004, India

²Molecular Biology Research Laboratory, Centre of Advanced Studies,

Department of Zoology, University of Pune, Pune, 411007, India

* Corresponding: Tel.: +91 832 2450540; fax: +91 832 2450606; E-mail: rakhee@nio.org (Rakhee Khandeparker)

Abstract:

Deep-sea sediments can reveal much about the last 200 million years of Earth history, including the history of ocean life and climate. Microbial diversity in Afanasiy–Nikitin seamount located at Equatorial East Indian Ocean (EEIO) was investigated. Deep sea sediment collected at 150 cm depth (~67 ky BP) exhibited a dominance of clones clustering in the phylum Firmicutes (63%), followed by γ -Proteobacteria (37%). The bacterial diversity within sediment at 200 cm depth (~84 ky BP) was dominated by γ -Proteobacteria (66%) followed by β - Proteobacteria (34%), whereas no Firmicutes clones were retrieved. Notably, 29 % clones from 150 cm and 18% of clones from 200 cm sediment showed high identity to sequences previously reported from mining sites, while 21% of clones from 150 cm depth and 10% from 200 cm sediments showed identity to sequences previously reported from volcanic areas. Further, many of the sequences were from uncultured clones. Overall, these data may help elucidate the nature of microorganisms inhabiting these sites and unravel the molecular diversity of the deep sea from an evolutionary perspective.

Keywords: Deep sea, Bacteria, SSU rRNA, Afanasiy, Seamount, Phylogeny, Indian ocean

Introduction

The Afanasiy Nikitin Seamount (ANS) located at 3°S latitude and 83°E longitude in the Equatorial East Indian Ocean (EEIO) was discovered during the R. V. Vityaz cruise of 1959 (Bogorov and Bezrukov 1961). The Afanasy Nikitin Seamount is a prominent topographic high of oval shape in plan, elongated in a N-S direction for 500 km. It rises 3500 m above the abyssal plain that lies at 5000 m water depth, and is topped by a flat guyot-like summit at 1740 m minimum water depth. The seamount is built up of a low wide pedestal and of a cone-shape volcanic edifice resting on the pedestal. Slopes are irregular, steep in parts, with terraces and scarps.

The ANS has evolved as a part of an 85°E ridge (Borisova et al. 2001). Subsequent to its emplacement the ANS has undergone tectonic disturbances due to intraplate deformation episodes in the region (Srikrishna et al. 2001) as evidenced by numerous criss-cross faults of the seamount (Rudenko 1994). Geochemical data of volcanic rocks from the ANS represent a bimodal series of olivine picrite basalts and trachybasalt to trachyte (Sborshchikov 1995), indicating that the first phase of seamount volcanism occurred in the late Cretaceous adjacent to a spreading center, while the second phase of volcanic activity led to the formation of the seamount above sea level in the latest Cretaceous to Paleocene. The ANS gains significance because of its tectonic history and has presence of potentially economically important Co-enriched Fe-Mn crusts (Parthiban and Banakar 1999).

The deep sea is probably the largest habitat on earth and one of the least known biologically. Its microbial diversity is virtually unknown. Better knowledge of the types and diversity of bacteria which are dominant in ANS sediments will lead to increased understanding of the diagenesis' and evolution of organic matter in the early bio geological record. This unique habitat may also show the presence of bacteria which are unique.

Materials and Methods

Study area and collection of deep-sea sediment samples

Sediment samples were collected by dredging along short tracks of ~100m on the slopes of ANS during the cruise of the R. V. *A A Sidorenko*, CC2/GCEQ2 (AAS-65: August 2003) (Fig:1) , undertaken for exploration of Co-enriched seamount Fe- Mn crusts in the EEIO. The core used for this study was recovered from (2°29'N; 78°E) having the length of 5.62 m. The water depth in the dragging station was 3788 m. The sediment samples were transported to the lab in sterile polyethylene bags under refrigeration for DNA extraction and stored at -70°C until taken up for further processing.

DNA Extraction

Two replicates of ~ 1 g sub-samples of sediment 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.

PCR amplification and cloning procedures

The SSU rRNA gene fragments were amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-30) (Weisburg et al. 1991). The amplification was carried out in 25 µl of reaction mixture containing DNA template, PCR buffer, dNTP (10 mM), MgCl₂ (25 mM), the primers 27F (10 ppm) and 1492R (10 ppm) respectively, Taq polymerase (Sigma) (2U) and autoclaved Milli-Q water (Millipore). The amplification was carried out for 35 cycles of 94 °C, for 45 s, 60 °C for 45 s and 72 °C for 45 s, 72°C for 7 min with initial 05-min denaturation at 94 °C using a PCR-Express thermal cycler (Hybaid). The PCR amplified DNA was then ligated in pGMT vector and subjected to transformation in *E.Coli*. JM 109 cells. For screening of the recombinants, each clone was picked from LB agar plate containing 50µg/ml of ampicillin, 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG) and 20 mg/ml of 5-bromo-4-chloro-3-indoly- β -D-galactopyranoside (XGal) .

Analysis of DNA sequences and phylogenetic relationships

The DNA sequences of the PCR products were determined using a Taq Dye Deoxy terminator cycle sequence kit (Perkin–Elmer) using the protocol recommended by the supplier. Sequencing reaction products were analyzed with a model 373A automated DNA sequencer (Applied Biosystems). Databases (GenBank) were searched for sequences identity analysis of the SSU rRNA sequence obtained. A multiple sequence alignment was generated with ClustaW, and the phylogenetic analysis of the 16S DNA sequences was carried out with the MEGA 4.0 program (Tamura et al., 2007). To obtain a confidence value for the aligned sequence dataset, a bootstrap analysis of 500 replications was done. Phylogenetic trees were constructed by the neighbor-joining method based on Kimura's two-parameter distance (Kimura, 1980).

Nucleotide sequence accession numbers

Accession numbers KF280210 to KF280228 correspond to our 16S rRNA gene clones from 150 cm depth and accession numbers KF280229 to KF280236 and KF373534 correspond to 200 cm depth sediment sample.

Results

Depth profiles of the cores

A single location of ANS deep-sea sediment sample was selected for analysis at two different depth intervals from a 5 m-long core. The sediment samples from 150 cm depth of age ~ 67 ky BP and 200 cm age of ~ 84 ky BP were selected. The sediment sample from depth 150 cm represents the glacial period while the sediment sample at 200 cm represents interglacial period. 64 clones obtained from 150 cm depth while 58 clones acquired from 200 cm depth of the deep-sea were analyzed (Table 1 and 2). Molecular sequencing of SSU rRNA followed by phylogenetic analysis revealed presence of DNA sequences representing diverse microbes. A sequence similarity cutoff of 95% was selected as this value has been suggested to allow separation of sequences at the genus level (Ludwig et al., 1998). Phylogenetic trees were constructed based on all of the sequenced clones and these were classified into Firmicutes, α and γ -Proteobacteria.

Quantification of different bacterial groups

The majority of isolates from deep sea sediment of 150 cm depth were classified into the phylum Firmicutes (63%), followed by γ -Proteobacteria (37%). Of the total clones that were sequenced, γ -Proteobacteria showed sequence identity to previously cultured representatives such as *Pseudomonas* and *Shigella/ Escherichia* in a few cases, while within the Firmicutes phylum, *Bacillus* was dominant with minor presence of *Dolosigranulum-related sequences* (Fig. 2 and Fig.3). The phylogenetic analyses revealed different pattern of bacterial diversity at 200 cm depth sediment where it showed complete dominance of γ -Proteobacteria (66%) with representative of *Pseudomonas* and *Enterobacteriaceae* followed by β -Proteobacteria (34%) represented by *Limnobacter* and *Burkholderiales* (Fig.4 and Fig.5).

In this analysis no Firmicutes were retrieved from the 200 cm sample, whereas this phylum represented a major fraction of the 150 cm depth sediment. A lower proportion (65%) of clones from the 150 cm sediment sample clustered with uncultured bacterial sequences compared to 90% of clones from the 200 cm sample. In this study, 29 % clones from 150 cm and 18% from 200 cm depth samples had high identity with uncultured bacterial clones retrieved from mining sites that were primarily enriched in iron and potassium. Similarly, 21% of clones from 150 cm and 10 % from 200 cm samples had high identity with bacterial sequences from volcanic areas. Interestingly 54% of clones at cm depth sediment showed close relation to bacteria from hydrothermal vent reported from Suhiyo mount (Table 1 and Table 2).

Discussion

The tectonic history of ANS reveals that, ANS is composed of Late Cretaceous effusive rocks comprising a bimodal series of olivine picrite-basalt, and of trachybasalt to trachyte erupted near a spreading center. Conglomerates from the upper slope testify that the volcano emerged above sea level in the Paleocene, and subsided in the Eocene (Sborshchikov et al.1995). The ANS has got significance not only because of its tectonic history but also due to the presence of potentially economically important Co-enriched Fe-Mn crusts (Parthiban and Banakar 1999). Presence of bacterial phylogroups such as phylogroup 1 and 16 from 150 cm depth and Phylogroup 2 at 200 cm depth shows close relation to the microbes found in mining areas rich in iron and potassium (Table 1 and 2). The presence of bacterial fraction in this niche also shows close relation to bacteria from volcanic sites. Phylogroup 8, 12 at 150 cm depth while phylogroup 1 at 200 cm depth shows close relation to uncultured flora from volcanic areas (Table 1 and 2). Both these correlations not only strongly corroborate the presence of Co-enriched Fe-Mn crusts in ANS but also support to the fact that the ANS is a volcanic sea mount.

It is noteworthy that 52 % population at 200 cm depth of ANS showed high sequence identity to uncultured bacterial clones from Suhiyo mount. The Suiyo Seamount is one of the most active submarine volcanoes in the Izu-Bonin Arc, Western Pacific Ocean. Its caldera, which is a horseshoe-shaped structure, contains many active hydrothermal vents and is a hydrothermal volcano. This suggests that ANS might have had similar environment as that of Suhiyo seamount (Kato et al. 2009).

Our results suggest that phylotypes found from the sediment sample of Affanasy sea mount at both depths deferred in diversity. The sediment sample from 150 cm depth represents age of ~67 ky BP which is also supposed to be a glacial period had more diverse bacterial populations when compared to 200 cm depth sediment which represents age of ~84 ky BP and also the interglacial period. One noticeable variation was that complete absence of Firmicutes in 200 cm depth sediment sample while 150 cm sediment sample was dominated by Firmicutes. This suggests that Firmicutes were not predominant in such ecosystems and represent later colonisation and further analysis will help unravel the evolution and adaptation of Firmicutes. Zhang et al. (2002) observed that the isolated genera of *Bacillus* have their maximum population in the middle of the ice core, and decrease in deeper or in superficial layer when he studied ice core of Malan Glacier, China. According to their findings the discrepancy of distribution of *Bacillus* sp. may imply a change of environment that affected the growth of those bacteria.

Low bootstrap values in phylogenetic trees of both sediments reveals that there exists some microbial flora which does not show close relationship with any cultured (and hitherto identified)

organisms but show relationship with organisms reported as uncultured and thus possibly depict unusual or distant (represent new strains or species) bacteria. For example phylogroup 3 at 200 cm depth is closely associated with uncultured microflora but it also shows a distant relationship with cultured *Limnibacter* sp. which may suggest that they are distantly related to β - Proteobacteria. Overall, the data from the 150 cm and 200 cm samples suggest that not only were the microbial communities, preserved from 67 ky BP and 84 ky BP timeframe, distinct, but that specific and unique phlotypes were prevalent in each sample type.

Miteva et al, (2004) explored GISP 2 (Greenland Ice Sheet Project) ice core to study the diversity and survival of microorganisms trapped in the ice at least 120,000 years ago revealed the presence of four major phylogenetic groups: the high-G+C gram-positives, low-G+C gram-positives, *Proteobacteria*, and the *Cytophaga-Flavobacterium-Bacteroides* group. Kato et al. (2009) reported that the Suiyo Seamount was completely dominated by proteobacteria. Recently, bacterial abundance, diversity and glaciochemical composition from three depths corresponding to 1926 AD, 1989 AD and 1953 AD from an ice core of coastal Dronning Maud Land, East Antarctica has been analysed (Antony et al. 2012). Further fourteen bacterial isolates belonging to the genera *Methylobacterium*, *Brevundimonas*, *Paenibacillus*, *Bacillus* and *Micrococcus* were reported by Antony et al. (2012). The deep-sea environments of the South Atlantic Ocean was studied for culturable bacteria by Castro et al. (2013), who reported that γ -Proteobacteria and Firmicutes were the most frequently cultured with *Halomonas* the most frequent genus among the strains.

Most notably, the highest proportion of sequences identified herein clustered with other unculturable clonal sequences, particularly in the 200 cm depth sample where approximately 90% of the clones had the highest sequence identity with uncultured bacterial sequences. Studies on the genomic information of the 'unculturable' bacteria from the clones in metagenomic libraries, can help in understanding their physiology and provide insight into their role in the ecosystem. Some success has been obtained in achieving these goals in recent years. Sequence analysis of a 130 kb genomic fragment from a BAC clone that encoded the rRNA operon from an uncultivated member of marine γ -Proteobacteria (SAR 86 group) revealed the presence of rhodopsin gene. This gene encoded a protein with highest amino acid sequence identity with archaeal rhodopsins (Beja et al. 2001). Recent studies have found representatives of bacterial divisions with few or no cultivated members that are widely distributed in the environment and numerically seem to dominate many of the environments examined (Dojka et al. 2000).

Whilst little is known about the biology associated with seamounts in the Indian Ocean, studies have indicated that there exert an important influence on water circulation in the region (Rogers et al. 2012). Also the location of the sediment study core falls within well oxygenated equatorial deep water regime (>3500 m) and there are no evidences for suboxic/anoxic conditions in this region

during the past (Punyu et al., 2014). Recent studies by Punyu et al., (2014) in the same region showed comparison in marine productivity of LGM (Last Glacial Maxima) around ~ 25–17 ky BP (sediment around 40 to 60 cm depth) and Holocene period, they found the presence of two times higher organic carbon in the LGM sediment than in the Holocene sediment suggesting significantly increased productivity during LGM. Our study area which is from 150 cm and 200 cm depth from the same core represent much older sediment history that is it represents ~ 67 ky BP to ~ 84 ky BP. Punyu et al., (2014) also suggested that the increased glacial Equatorial Indian Ocean productivity appears to be the result of combination of significantly intensified Wyrski Jets enhancing the nutrient levels in the photic layer, and the increased input of soil-nutrients from Indian sub-continent by the intensified winter monsoons. The change in bacterial community observed in our results may also be due to similar reasons as the circulation pattern of ocean during glacial and interglacial period may vary which may affect the community of living organisms. Microbial community genome sequencing carried out in this analysis in an attempt to understand community structure and ecosystem function. Availability of community genome sequences will help in development of gene expression profiles and physiological studies providing a comprehensive approach to environmental biology.

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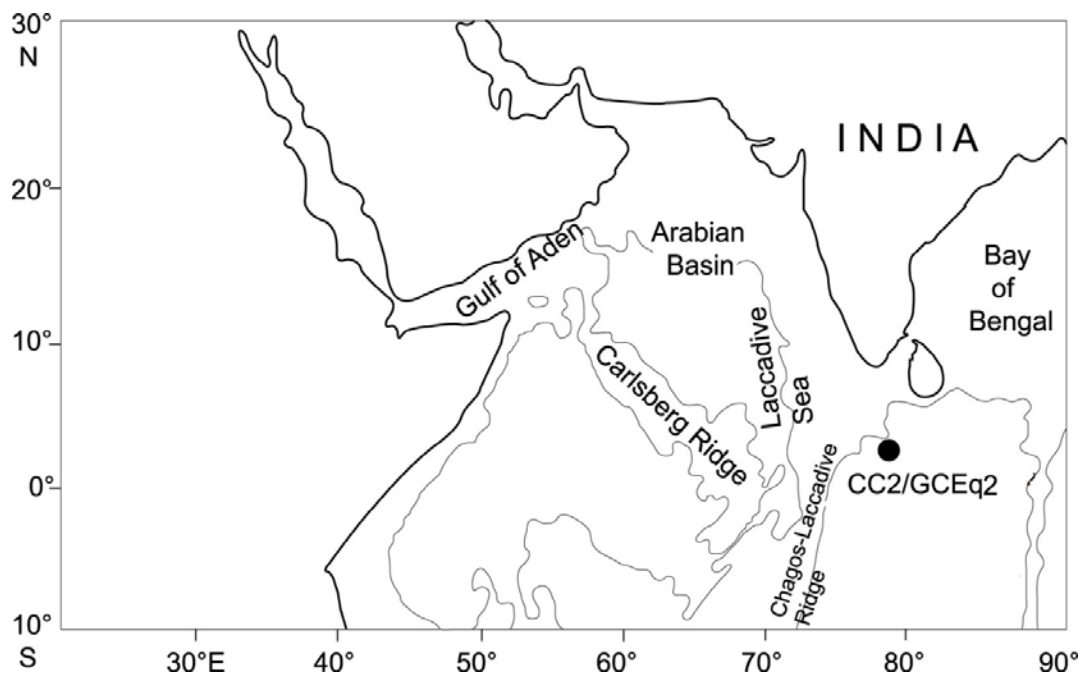


Fig:1 Sampling location at Afanasy Sea Mount

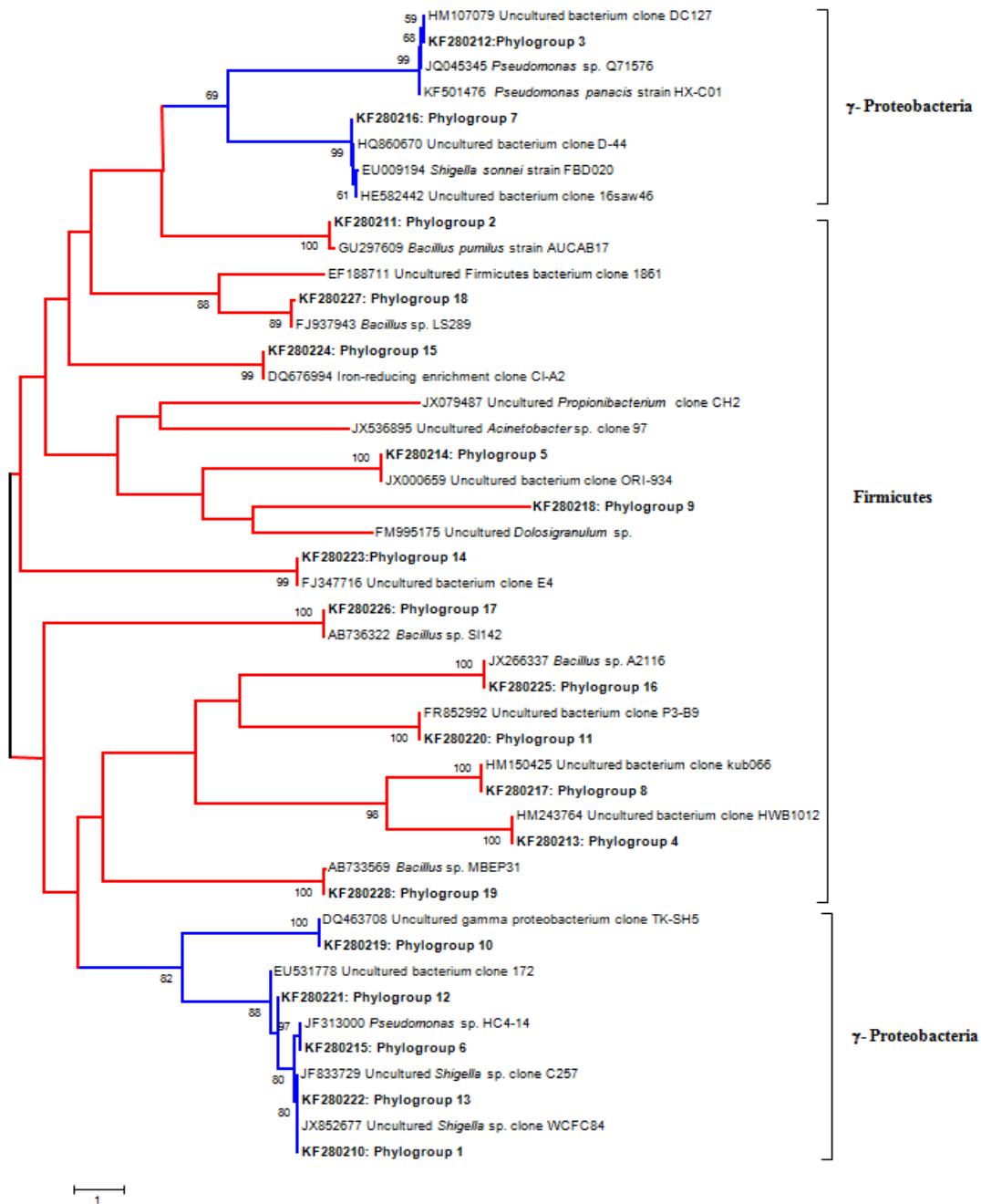


Fig: 2 Phylogenetic relationship between clonal sequences retrieved from 150 cm sediment samples collected at the Afanasie-Nikitin seamount and representative sequences from the database. This tree was generated by program MEGA 4 using neighbor joining. Values at the nodes were obtained by bootstrap analysis based on 1,000 resamplings. Bootstrap values over 50% are shown.

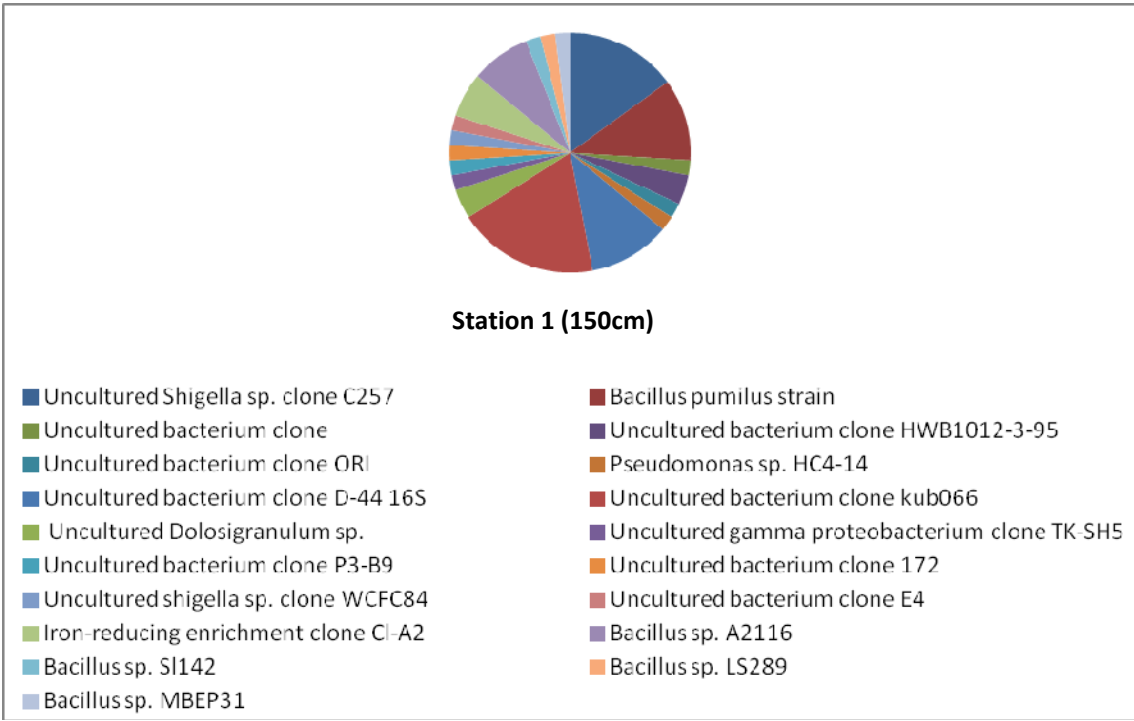


Fig. 3 Percentage of the 64 clones retrieved from deep sea sediment at 150 cm clustering in various taxonomic groups of bacteria.

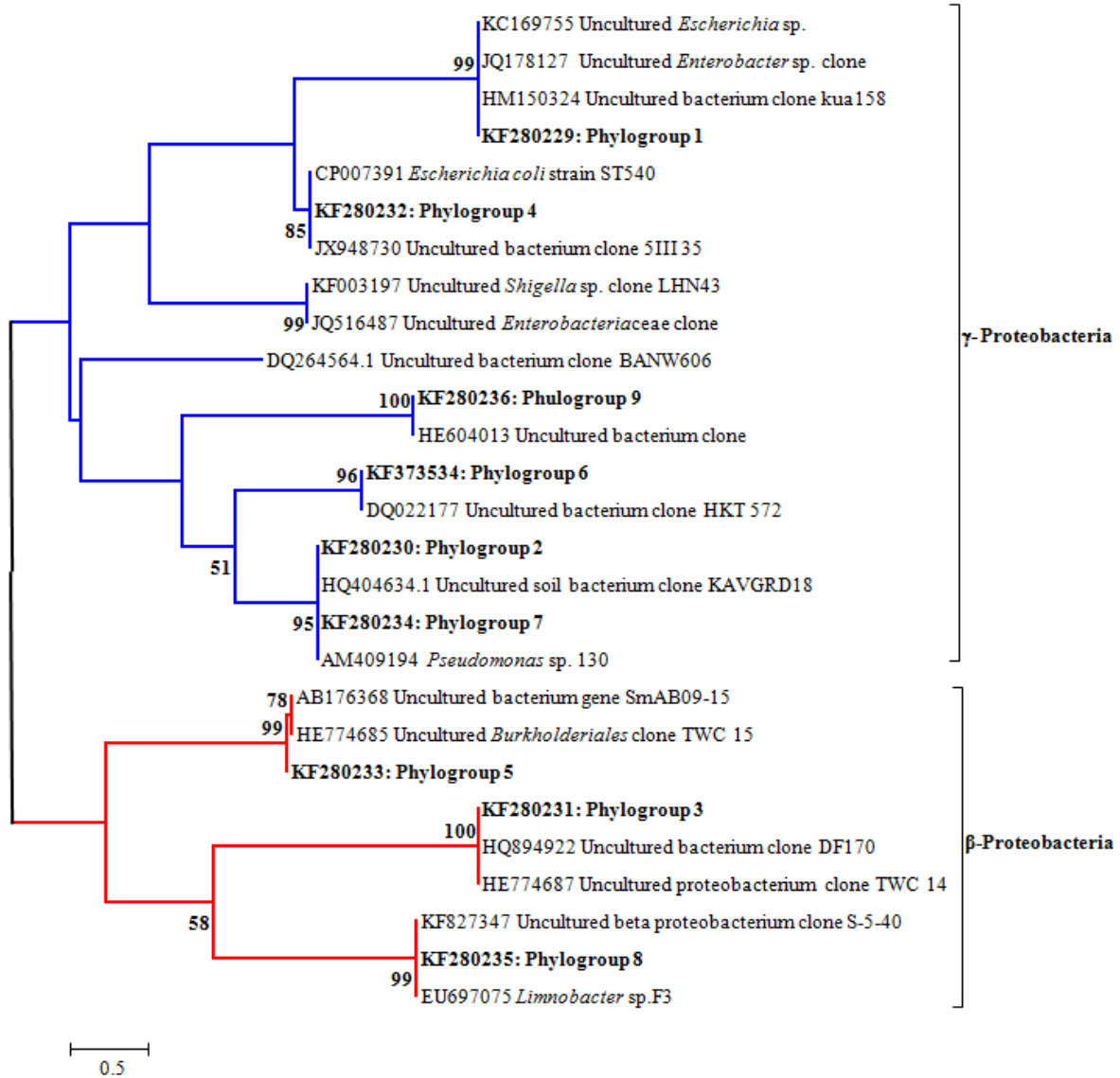


Fig: 4 Phylogenetic relationship between clonal sequences retrieved from 200 cm sediment samples collected at the Afanasie-Nikitin seamount and representative sequences from the database. This tree was generated by program MEGA 4 using neighbor joining. Values at the nodes were obtained by bootstrap analysis based on 1,000 resamplings. Bootstrap values over 50% are shown.

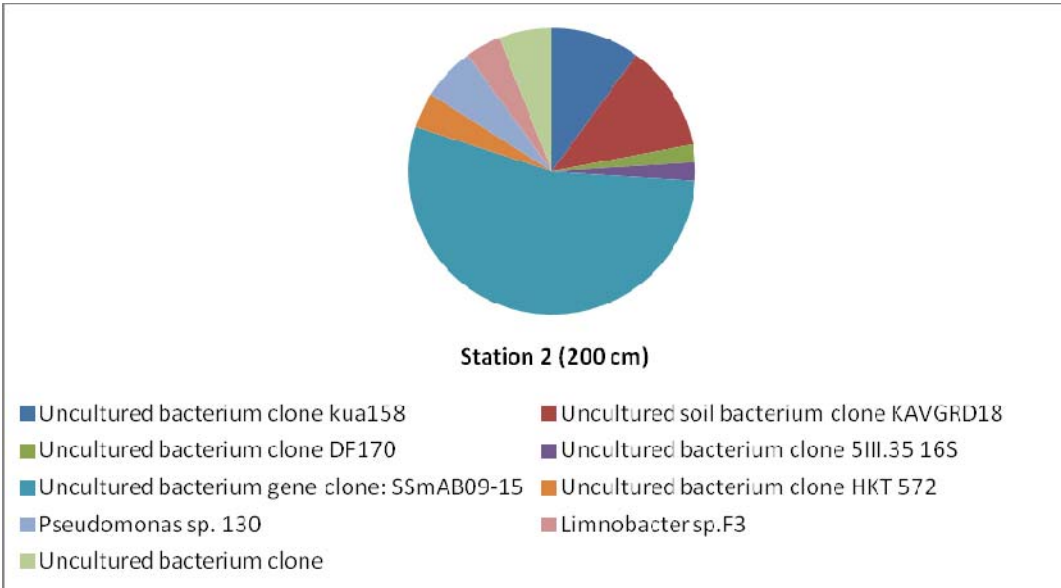


Fig. 5 Percentage of the 58 clones retrieved from deep sea sediment at 200 cm clustering in various taxonomic groups of bacteria.

Table 1: Phylogenetic groups identified using a 95% cutoff value for the 64 clones obtained from Afanasiy-Nikitin seamount deep sea sediment (150 cm depth)

Phylogroup	Accession number	Most closely related organism	Closest organism isolated from	Ac. No of closest organism	Sequence identity (%)	% clones
1	KF280210	Uncultured <i>Shigella</i> sp. clone C257	Potassium mine soil	JF833729.1	99	15
2	KF280211	<i>Bacillus pumilus</i> strain	Coral <i>Acropora digitifera</i>	GU297609.1	99	11
3	KF280212	Uncultured bacterium clone	Thermally hydrolysed waste activated sludge	HM107079.1	100	2
4	KF280213	Uncultured bacterium clone HWB1012-3-95	Lake sediments	HM243764.1	100	4
5	KF280214	Uncultured bacterium clone ORI	Gas hydrate potential area	JX000659.1	99	2
6	KF280215	<i>Pseudomonas</i> sp. HC4-14	Arctic cyanobacterial mats	JF313000.1	100	2
7	KF280216	Uncultured bacterium clone D-44 16S	Dianchi Lake, China	HQ860670.1	99	11
8	KF280217	Uncultured bacterium clone kub066	Volcanic sites, Germany	HM150425.1	99	19
9	KF280218	Uncultured <i>Dolosigranulum</i> sp.	Digestive gland of Sydney rock oysters	FM995175.1	99	4
10	KF280219	Uncultured gamma proteobacterium clone TK-SH5	Anoxic hypolimnion	DQ463708	99	2
11	KF280220	Uncultured bacterium clone P3-B9	Hydrothermal chimney	FR852992	99	2
12	KF280221	Uncultured bacterium clone 172	Mud volcanos in Xinjiang	EU531778.1	99	2
13	KF280222	Uncultured <i>shigella</i> sp. clone WCFC84	Saffron cormosphere	JX852677	99	2
14	KF280223	Uncultured bacterium clone E4	Sulfide-fed microbial fuel cell	FJ347716.1	99	2
15	KF280224	Iron-reducing enrichment clone CI-A2	Sediments of the polluted estuary	DQ676994.1	99	6
16	KF280225	<i>Bacillus</i> sp. A2116	Microbial communities involved in mineral-weathering	JX266337.1	99	8
17	KF280226	<i>Bacillus</i> sp. S1142	Deep-sea surface sediment	AB736322.1	99	2
18	KF280227	<i>Bacillus</i> sp. LS289	Bacteria Associated with the Marine Sponge	FJ937943.1	99	2
19	KF280228	<i>Bacillus</i> sp. MBEP31	Deep-sea Bacteria	AB733569.1	99	2

Table 2: Phylogenetic groups identified using a 95% cutoff value for the 58 clones obtained from Afanasiy-Nikitin seamount deep sea sediment (200 cm depth)

Phylogroup	Accession number	Most closely related organism	Closest organism isolated from	Ac. No of closest organism	Sequence identity (%)	% clones
1	KF280229	Uncultured bacterium clone kua158	Volcanic sites , Germany	HM150324.1	100	10
2	KF280230	Uncultured soil bacterium clone KAVGRD18	Iron-ore mine sites , India	HQ404634.1	99	12
3	KF280231	Uncultured bacterium clone DF170	Human pathogen	HQ894922.1	99	2
4	KF280232	Uncultured bacterium clone 5III.35 16S	Lake sediments, Antarctica	JX948730.1	99	2
5	KF280233	Uncultured bacterium gene clone: SSmAB09-15	Deep-sea hydrothermal field of the Suiyo Seamount	AB176368.1	99	54
6	KF373534	Uncultured bacterium clone HKT 572	Effluent treatment plant in petrochemical industries	DQ022177.2	99	4
7	KF280234	<i>Pseudomonas</i> sp. 130	Deep sea bacterium	AM409194.1	99	6
8	KF280235	<i>Limnobacter</i> sp.F3	Phe-degrading bacterium	EU697075.1	100	4
9	KF280236	Uncultured bacterium clone	Iron snow in an acidic lake Germany	HE604013.1	100	6