8. Culture-independent microbial diversity with special reference to actinobacteria

8.1. Introduction

DNA sequencing is one of the most significant platforms to study the biotic systems. The most commonly performed sequencing is Sanger’s di-deoxy chain termination method (Sanger et al., 1977). Although 16S rRNA sequencing of microorganisms has been the gold standard method for characterization of microbial communities, culture independent analysis of sequences derived from the environmental genomic DNA samples has increased our understanding of microbial diversity, function and process (Stahl et al., 1984; Hugenholtz and Pace, 1996).

Many alternative DNA sequencing principles have been developed. Among them, only a few methods have shown great possibilities for the complete study of microorganisms and they are sequencing by hybridization (Drmanac et al., 1988; Khrapko et al., 1989), parallel signature sequencing based on ligation and cleavage (Brenner et al., 2000) and pyrosequencing (Ronaghi et al., 1996; Ronaghi et al., 1998). The pyrosequencing technology is a novel DNA sequencing technology, developed at the Royal Institute of Technology (KTH), and is the first alternative to the conventional Sanger method for de novo DNA sequencing.

In recent times, scientific developments of pyrosequencing have facilitated the rapid characterization of microbial communities by their faster and greater sequence depth analyses. Pyrosequencing has a wide variety of applications such as genotyping, detection of single-nucleotide polymorphism and microbial identification (Marsh, 2007).
The high-throughput sequencing technologies are widespread and commercialized during the recent times; 454 Life Sciences was the one company that commercially developed pyrosequencing for metagenomes; thus, we are using 454 sequencing. The term 454 sequencing refers to high-throughput sequencing platforms for metagenomes that are based on pyrosequencing chemistry.

In the present study, three different environmental samples (Mangrove, Coral reef and Beach) collected from the Neil island, were used to study and compare the culture-independent microbial diversities.

8.2. Materials and methods

8.2.1. Extraction of DNA

Sediment samples were collected from three different environments \textit{viz}. station 4 - mangrove (Lat. 11°46’45.7″N & Long. 93’01’ 22.3″E), station 2 - coral reef (Lat. 11°46’25.6″N & Long. 93’00’ 45.9″E) and station 3 - beach (Lat. 11°50’16.2″N & Long. 93’02’ 21.3″E) of the Neil island (Chapter 4). DNA from these samples was extracted using the method of Zhou \textit{et al.} (1996) with slight modification; 0.5 g of sediment was extracted with 1.3 ml of extraction buffer (100 mM TrisHCl, pH 8.0; 100 mM EDTA, pH 8.0; 1.5 M NaCl, 100 mM sodium phosphate, pH 8.0 and 1% CTAB). After proper mixing, 13 µl of proteinase K (10 mg/ml) was added.

All eppendorf tubes were incubated horizontally at 37°C with shaking for 45 min. After that, 160 µl of 20% SDS was added and vortexed for 30 sec with further incubation at 60°C for 2 h. The sample in each eppendorf was mixed thoroughly after every 15 min interval. The samples were centrifuged at 5000 x g for 10 min. The supernatant was transferred into a new vial. The remaining pellets were treated three times with 400 µl of
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extraction buffer and 60 µl of SDS (20%). The treated pellets were kept at 60°C for 15 min with intermittent shaking after every 5 min. The supernatants collected from all the four extractions were mixed with equal quantity of chloroform and isoamyl alcohol (24:1) and extracted three to four times. Aqueous layer was separated and precipitated with 0.6 volume of isopropanol. After centrifugation at 12,000 x g for 15 min, the pellet was washed with 70% ethanol, dried at room temperature and 70% ethanol, air dried and dissolved in 50 µl of MilliQ water and stored at -20°C.

8.2.2. Pyrosequencing and data Analysis

DNA was purified by electrophoresis in a 1% (wt./vol.) low-melting-point agarose gel. DNA fragments were excised and recovered from the agarose using Quiagen gel extraction kit. Polymerase chain reaction (PCR) was performed using the universal bacterial primers 939 Forward primer (5’-adaptor-tag-TTGACGGGGCCCGCAAG-3’) and 1492 Reverse primer (5’-adaptor-TACCTTGTTACGACTT-3’) covering the base pair (V6–V9 region) of the 16S rRNA gene (Dowd et al., 2008).

The amplicon library was generated through one-step PCR consisting of hot start mixture and high fidelity Taq polymerase for 30 cycles and pyrosequencing was performed using 454 Roche FLX instrument following the titanium protocols at the Research and Testing Laboratory. The sequence reads were screened and filtered for quality and length using the programme, MOTHUR (Schloss et al., 2009). The chimeric sequences were removed by using chimera check and chimera slayer (Zajec et al., 2012). High quality sequence reads were aligned against the RDP trainset bacterial database and cluster linkage analysis and other ecological metrics were calculated using the Ribosomal Database Project (RDP).
OTUs and rarefaction curves were created from the aligned sequence reads by complete cluster linkage tool and also used to determine richness, diversity (Shannon–Weaver and Chao1) and evenness at each dissimilarity level by Shannon index calculator tools of RDP. The taxonomic assignments were given using the RDP classifier program (Cole et al., 2004) with a bootstrap score of 80%. For analysing the actinobacterial taxa, obtained actinobacterial sequences upon classification were analyzed by BLAST analysis against NCBI database and the results are represented, using the MEGAN 5 analysis tool (Tamura et al., 2007).

8.2.3. Bacterial and actinobacterial classification

Bacterial populations were classified at their appropriate taxonomic levels by BLASTN analysis performed through the web tool VITCOMIC (Mori et al., 2010). The taxonomic assignments were given on the basis of the BLAST average at different similarity levels i.e. 80, 85, 90, 95 and 100% from the known or well characterized 16S rRNA gene sequence database. The overall taxonomic composition of the sample was represented by the vitcomic plot of dots in circle (Sundarakrishnan et al., 2012).

8.3. Results

8.3.1. Station 1 (Mangrove)

8.3.1.1. Pyrosequencing data

A total of 6,120 sequence reads were obtained from the Genome sequencer FLX system. The sequence has been deposited in the GenBank Sequence Read Archive with the accession number SRX361077. The sequences were screened for the quality reads, ambiguous base, and homopolymers and the primer sequence was also trimmed by using
MOTHUR (http://www.mothur.org), which gave 5,150 high quality sequence reads with the average read length of 560 bp.

8.3.1.2. Bacterial and actinobacterial diversity of mangrove sediments

Richness of the bacterial and actinobacterial community present in the sample was estimated by rarefaction curves. The members of the sample or number of organisms assigned at each phylogenetic level depend on the number of sequences analyzed. The rarefaction curve was used to analyze whether complete diversity of the sample was obtained despite the number of sequences. The rarefaction curves based on the OTU values are good indications of the diversity within the sample as various percentages of dissimilarity level are known to differ at different taxonomic levels. A distance level of 3% will be able to differentiate at species level, whereas a distance level of 5% will be able to differentiate at genus level and a distance level of 10%, at family/class level (Schloss and Handelsman, 2004).

Rarefaction curves (Fig. 8.1) indicate that the bacterial and actinobacterial richness of the mangrove sediment sample is almost completely revealed i.e. very close to the true microbial diversity of the sample. A total of 683 OTUs by clustering at 3% dissimilarity level (0.03 distance units) and 427 OTUs by clustering at 5% dissimilarity level (0.05 distance units) were observed. For the total microbial communities clustered at 3% dissimilarity level, the number of OTUs obtained was very close to the number of OTUs estimated and the statistical estimates of the species richness like Chao1 and ACE diversity indices (Table 8.1). The 99% of Good’s coverage indicated the level of coverage of the 16S rRNA sequences identified in these groups, representing the majority of the bacterial sequences present in the tested sample.
Fig. 8.1. Rarefaction curves representing the richness of the pyrosequencing reads with distance values (dissimilarity level) of 0 (unique), 3 (0.03) and 5% (0.05).

Table 8.1. Phylotype coverage and diversity estimation of the pyrosequencing analysis.

<table>
<thead>
<tr>
<th>Distance units</th>
<th>Reads</th>
<th>Coverage</th>
<th>OTUs</th>
<th>Chao1</th>
<th>ACE</th>
<th>Shannon</th>
<th>Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>5150</td>
<td>99%</td>
<td>2602</td>
<td>5.57613</td>
<td>6.00571</td>
<td>7.3952</td>
<td>0.94038</td>
</tr>
<tr>
<td>0.03</td>
<td>5150</td>
<td>99%</td>
<td>683</td>
<td>744.32</td>
<td>777.2963</td>
<td>5.53844</td>
<td>0.84861</td>
</tr>
<tr>
<td>0.05</td>
<td>5150</td>
<td>99%</td>
<td>427</td>
<td>440</td>
<td>454.8551</td>
<td>4.99043</td>
<td>0.82394</td>
</tr>
</tbody>
</table>
The sequences revealed nineteen phyla, where the majority of the sequences were assigned to the Phylum Proteobacteria (39%), Actinobacteria (36%), Gammatimonadetes (21%), Planctomycetes (6%), Acidobacteria (4%), Firmicutes (4%) and Nitrospirae (2%). Verrucomicrobia, Nitrospinae, Thermodesulfobacteria, Lentisphaerae, Deinococcus-Thermus, Cyanobacteria, Bacteroidetes, Aquificae, Dictyoglombi, Chlorobi, Thermatogae and Chloroflexi were the other phyla identified with relatively low abundance (<1%).

Phylogenetic diversity of 5,150 reads of the sediment sample was computed by MEGAN, upon the classification of the sequence reads by using RDP classifier tool against RDP database. At the genus level, 90 different abundant genera were found in the mangrove sediments (Figs.8.2. a,b,c). In that, there were 64 bacterial genera: Akkermansia, Alkalilimnicola, Alkaliphilus, Anaerocellum, Anaeromyxobacter, Anaplasma, Bacillus, Bradyrhizobium, Brevibacillus, Campylobacter, Carboxydothermus, Caulobacter, Chlorobium, Chromohalobacter, Clostridium, Coprothermobacter, Coxiella, Dehalococcoides, Desulfatibacillum, Desulfotalea, Desulfotomaculum, Desulfovibrio, Dictyogluomus, Ehrlichia, Gemmatimonas, Geobacillus, Geobacter, Halorhodospira, Halothermobacter, Magnetococcus, Magnetospirillum, Maricaulis, Mesorhizobium, Methylacidiphilum, Methylobacterium, Moorella, Myxococcus, Nitratiruptor, Nitrobas, Novosphingobium, Oceanobacillus, Oligotropha, Paenibacillus, Pelvibaculum, Pelobacter, Pelotomaculum, Persephonella, Pseudoalteromonas, Pseudomonas, Rhodopirellula, Shigella, Sorangium, Sphingomonas, Sphingopyxis, Staphylococcus, Sulfurhydrogenibium, Syntrophus, Thermoanaerobacter,
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*Thermosipho*, *Thermosynechococcus*, *Thioalkalivibrio*, *Vibrio*, *Wolinella* and *Zymomonas*.

In addition, 26 actinobacterial genera were found in the mangrove sediments: *Acidothermus*, *Actinomadura*, *Actinomyces*, *Arthrobacter*, *Beutenbergia*, *Clavibacter*, *Corynebacterium*, *Frankia*, *Iamia*, *Illumatobacter*, *Kineococcus*, *Kocuria*, *Leifsonia*, *Micrococcus*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Propionibacterium*, *Rhodococcus*, *Rubrobacter*, *Saccharopolyspora*, *Salinispora*, *Streptomyces*, *Thermobifida* and *Tropheryma*.

The total BLAST score was calculated against each sequence of the reference database (http://mg.bio.titech.ac.jp/vitcomic/) to identify the nearest relative of the sample sequence in order to classify at species level (Figs. 8.3. a,b). The abundant actinobacterial species were *Actinomadura livida*, *Actinomadura pelletieri*, *Actinomyces odontolyticus*, *Arthrobacter oxydans*, *Corynebacterium mucifaciens*, *Iamia majanohamensis*, *Illumatobacter fluminis*, *Micromonospora coxensis*, *Mycobacterium peregrinum*, *Nocardia ninae*, *Propionibacterium acnes*, *Streptomyces coelicolor*, *Streptomyces diastatochromogenes*, *Streptomyces panacagri* and *Streptomyces scabrisporlus*. In the Figs. 8.3.a,b species names in the reference database are placed in circles with ordered phylogenetic relatedness.

Physical distances between the nearest species in the plot indicate the genetic distances of 16S rRNA genes between them. The circles indicate the boundaries of BLAST average similarities (inner most circle starting at 80%, followed by 85%, 90%, 95% and 100% identity to the database sequence). Each dot represents the average similarity of each sequence against the nearest relative species in the reference dataset.
The size of these dots indicates the relative abundance of sequences in the sample. The VITCOMIC plot contains four categories of dot size that indicate the relative abundance of the sample sequence.
Fig. 8.2. a. Phylogenetic diversity of 5,150 reads of the mangrove sediment sample by MEGAN. The classification for the sequence reads were obtained by using Ribosomal Database Project Classifier tool against the RDP database. Each circle represents a bacterial taxon in the RDP taxonomy database and the size of the circle represents the number of reads assigned to the particular taxon.
Fig. 8.2. b. Phylogenetic diversity of 5, 150 reads of the mangrove sample computed by MEGAN. The classification for the sequence reads were obtained by using Ribosomal Database Project Classifier tool against the RDP database. Each circle represents an actinobacterial taxon in the RDP taxonomy database and the size of the circle represents the number of reads assigned to the particular taxon.
Fig. 8.2. c. Graphical representation of 5,150 reads of the mangrove sediment sample, representing bacterial and actinobacterial taxa in the RDP taxonomy database and the number of reads assigned to the particular taxon.
Fig. 8.3. a. Vitcomic map representing the species diversity of the sample, based on the comparison of the reads against the NCBI nonredundant database using the blastn tool. Large circles indicate boundaries of BLAST average similarities, inner most circle (iv) 80–85%, followed by (iii) 85–90%, (ii) 90–95%, (i) 95–100% similarity of the database sequence.
Fig. 8.3.b. High-resolution view of the region containing the predominant Phylum actinobacteria. Larger size dots indicate that the relative abundance of the particular taxon is more than 10% of the sample sequence.
8.3.2. Station 2 (Coral reef)

8.3.2.1. Pyrosequencing data

A total of 2,580 sequence reads were obtained from the Genome sequencer FLX system. The sequence has been deposited in the GenBank Sequence Read Archive with the accession number SRX350583. The sequences were screened for the quality reads, ambiguous base, and homopolymers and the primer sequence was also trimmed, using MOTHUR (http://www.mothur.org), which gave 2,339 high quality sequence reads with the average read length of 480 bp.

8.3.2.2. Bacterial and actinobacterial diversity of coral reef sediments

Richness of the bacterial and actinobacterial community present in the sediment sample was estimated by rarefaction curves. The members of the sample or number of organisms assigned at each phylogenetic level depend on the number of sequences analyzed. The rarefaction curve was used to analyze whether complete diversity of the sample was obtained despite the number of sequences. The rarefaction curves based on the OTU values are good indications of the diversity within the sample as various percentages of dissimilarity level are known to differ at different taxonomic levels. A distance level of 3% will be able to differentiate at species level, whereas a distance level of 5% will be able to differentiate at genus level and a distance level of 10%, at family/class level (Schloss and Handelsman, 2004).

Rarefaction curves (Fig. 8.4) indicate that the bacterial and actinobacterial richness of the coral reef sediment sample is almost completely revealed i.e. very close to the true microbial diversity of the sample. A total of 316 OTUs by clustering at 3% dissimilarity level (0.03 distance units) and 188 OTUs by clustering at 5% dissimilarity?
level (0.05 distance units) were observed. For the total microbial communities clustered at 3% dissimilarity level, the number of OTUs obtained was very close to the number of OTUs estimated and the statistical estimates of the species richness like Chao1 and ACE diversity indices were obtained (Table 8.2). The 99% of Good’s coverage indicates the level of coverage of the 16S rRNA sequences identified in these groups, representing the majority of the bacterial sequences present in the tested sample.

Fig. 8.4. Rarefaction curves representing the richness of the pyrosequencing reads with distance values (dissimilarity level) of 0 (unique), 3 (0.03) and 5% (0.05).
Table 8.2. Phyloype coverage and diversity estimation of the pyrosequencing analysis.

<table>
<thead>
<tr>
<th>Distance units</th>
<th>Reads</th>
<th>Coverage</th>
<th>OTUs</th>
<th>Chao1</th>
<th>ACE</th>
<th>Shannon</th>
<th>Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>2339</td>
<td>99%</td>
<td>1113</td>
<td>2,639.49</td>
<td>2,351.98</td>
<td>6.47382</td>
<td>0.92288</td>
</tr>
<tr>
<td>0.03</td>
<td>2339</td>
<td>99%</td>
<td>316</td>
<td>373.1475</td>
<td>348.9828</td>
<td>4.91179</td>
<td>0.85337</td>
</tr>
<tr>
<td>0.05</td>
<td>2339</td>
<td>99%</td>
<td>188</td>
<td>198.8</td>
<td>192.1521</td>
<td>4.38352</td>
<td>0.83712</td>
</tr>
</tbody>
</table>

The sequences revealed fourteen phyla, where the majority of the sequences were assigned to the Phylum Actinobacteria (58%), Proteobacteria (20%), Planctomycetes (8%), Firmicutes (4%), Nitrospirae (4%) and Gammatimonadetes (3%). Acidobacteria, Verrucomicrobia, Thermodesulfobacteria, Lentisphaerae, Thermotogae, Chlorobi, Chloroflexi and Aquificae were the other phyla identified with relatively low abundance (<1%).

Phylogenetic diversity of 2,339 reads of the sediment sample was computed by MEGAN, upon the classification of the sequence reads by using RDP classifier tool against RDP database. At the genus level, 52 different abundant genera were found in the coral reef sediments (Figs. 8.5. a,b,c). In this, 30 bacterial genera were found: Alkalilimnicola, Alkaliphilus, Anaerocellum, Anaeromyxobacter, Bartonella, Brucella, Caldicellulosiruptor, Carboxydothermus, Chlorobaculum, Chlorobium, Chromohalobacter, Coxiella, Desulfatibacillum, Desulfotalea, Desulfovibrio, Gemmatimonas, Geobacillus, Magnetospirillum, Methylobacterium, Moorella, Myxococcus, Nitrosococcus, Paenibacillus, Pelobacter, Sorangium, Syntrophobacter, Thermoanaerobacter, Thermosipho, Thermotoga and Thioalkalivibrio. In addition, 22 actinobacterial genera were found in the coral reef sediments: Acidothermus, Actinomadura, Arthrobacter, Beutenbergia, Bifidobacterium, Clavibacter,
Corynebacterium, Kineococcus, Kocuria, Leifsonia, Illumatobacter, Iamia, Micrococcus, Mycobacterium, Nocardia, Nocardioides, Propionibacterium, Rhodococcus, Saccharopolyspora, Streptomyces, Thermobifida and Tropheryma.

The total BLAST score was calculated against each sequence of the reference database (http://mg.bio.titech.ac.jp/vitcomic/) to identify the nearest relative of the sample sequence in order to classify at species level (Figs. 8.6. a,b). The abundant actinobacterial species were, Actinomadura pelletieri, Iamia majanohamensis, Illumatobacter fluminis, Mycobacterium hassiacum, Mycobacterium hasiacum, Nocardioides aestuarii, Propionibacterium acnes, Streptomyces mutabilis and Streptomyces sampsonii. In the Figs. 8.6. a,b, species names in the reference database are placed in circles with ordered phylogenetic relatedness.

Physical distances between the nearest species in the plot indicate the genetic distances of 16S rRNA genes between them. The circles indicate the boundaries of BLAST average similarities (inner most circle starting at 80%, followed by 85%, 90%, 95% and 100% identity to the database sequence). Each dot represents the average similarity of each sequence against the nearest relative species in the reference dataset. The size of these dots indicates the relative abundance of sequences in the sample. The VITCOMIC plot contains four categories of dot size that indicate the relative abundance of the sample sequence.
Fig. 8.5.a. Phylogenetic diversity of 2,339 reads of the coral reef sediment sample computed by MEGAN. The classification for the sequence reads were obtained by using Ribosomal Database Project Classifier tool against the RDP database. Each circle represents a bacterial taxon in the RDP taxonomy database and the size of the circle represents the number of reads assigned to the particular taxon.
Fig. 8.5.b. Phylogenetic diversity of coral reef sample computed by MEGAN. The classification for the sequence reads were obtained by using Ribosomal Database Project Classifier tool against the RDP database. Each circle represents an actinobacterial taxon in the RDP taxonomy database and the size of the circle represents the number of reads assigned to the particular taxon.
Fig. 8.5.c. Graphical representation of 2, 339 reads of the coral reef sediment sample, representing bacterial and actinobacterial taxa in the RDP taxonomy database and the number of reads assigned to the particular taxon.
Fig. 8.6.a. Vitcomic map representing the species diversity of the sample, based on the comparison of the reads against the NCBI nonredundant database using the blastn tool. Large circles indicate boundaries of BLAST average similarities, inner most circle (iv) 80–85%, followed by (iii) 85–90%, (ii) 90–95%, (i) 95–100% similarity of the database sequence.
Fig. 8.6.b. High-resolution view of the region containing the predominant Phylum actinobacteria. Larger size dots indicate that the relative abundance of the particular taxon is more than 10% of the sample sequence.
8.3.3. Station 3 (Beach)

8.3.3.1. Pyrosequencing data

A total of 2,215 sequence reads were obtained from the Genome sequencer FLX system. The sequence has been deposited in the GenBank Sequence Read Archive with the accession number SRX350583. The sequences were screened for the quality reads, ambiguous base, and homopolymers and the primer sequence was also trimmed by using MOTHUR (http://www.mothur.org), which gave 2,014 high quality sequence reads with the average read length of 440 bp.

8.3.3.2. Bacterial and actinobacterial diversity of beach sediments

Richness of the bacterial and actinobacterial community present in the sediment sample was estimated by rarefaction curves. The members of the sample or number of organisms assigned at each phylogenetic level depend on the number of sequences analyzed. The rarefaction curve was used to analyze whether complete diversity of the sample was obtained despite the number of sequences. The rarefaction curves based on the OTU values are good indications of the diversity within the sample as various percentages of dissimilarity level are known to differ at different taxonomic levels. A distance level of 3% will be able to differentiate at species level, whereas a distance level of 5% will be able to differentiate at genus level and a distance level of 10%, at family/class level (Schloss and Handelsman, 2004).

Rarefaction curves (Fig.8.7) indicate that the bacterial and actinobacterial richness of the beach sediment sample is almost completely revealed i.e. very close to the true microbial diversity of the sample. A total of 273 OTUs by clustering at 3% dissimilarity level (0.03 distance units) and 186 OTUs by clustering at 5% dissimilarity level (0.05
distance units) were observed. For the total microbial communities clustered at 3% dissimilarity level, the number of OTUs obtained was very close to the number of OTUs estimated and the statistical estimates of the species richness like Chao1 and ACE diversity indices were obtained (Table 8.3). The 99% of Good’s coverage indicates the level of coverage of the 16S rRNA sequences identified in these groups, representing the majority of the bacterial sequences present in the tested sample.

Fig.8.7. Rarefaction curves representing the richness of the pyrosequencing reads with distance values (dissimilarity level) of 0 (unique), 3 (0.03) and 5 % (0.05).
Table 8.3. Phylotype coverage and diversity estimation of the pyrosequencing analysis.

<table>
<thead>
<tr>
<th>Distance units</th>
<th>Reads</th>
<th>Coverage</th>
<th>OTUs</th>
<th>Chao1</th>
<th>ACE</th>
<th>Shannon</th>
<th>Evenness</th>
</tr>
</thead>
<tbody>
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<td>983</td>
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<td>2,168.35</td>
<td>6.3785</td>
<td>0.92568</td>
</tr>
<tr>
<td>0.03</td>
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<td>99%</td>
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<td>288.7424</td>
<td>4.69927</td>
<td>0.83774</td>
</tr>
<tr>
<td>0.05</td>
<td>2014</td>
<td>99%</td>
<td>186</td>
<td>205.6875</td>
<td>194.602</td>
<td>4.31736</td>
<td>0.82617</td>
</tr>
</tbody>
</table>

The sequences revealed ten phyla, where the majority of the sequences were assigned to the Phylum Actinobacteria (23%), Proteobacteria (22%), Planctomycetes (11%), Firmicutes (10%) Nitrospirae (8%) and Acidobacteria (2%). Cyanobacteria, Thermodesulfobacteria, Lentisphaerae and Deinococcus-Thermus were the other phyla identified with relatively low abundance (<1%).

Phylogenetic diversity of 2,014 reads of the sediment sample was computed by MEGAN, upon the classification of the sequence reads by using RDP classifier tool against RDP database. At the genus level, 49 different abundant genera were found in the beach sediments (Figs. 8.8. a,b,c). Among them, 29 bacterial genera were found: Alkaliphilus, Anaerocellum, Anaeromyxobacter, Aquifex, Bacillus, Caldicellulosiruptor, Carboxydothermus, Caulobacter, Desulfotalea, Desulfovibrio, Dictyoglomus, Ehrlichia, Gemmatimonas, Geobacter, Gloeobacter, Methylacidiphilum, Myxococcus, Natranaerobius, Novosphingobium, Paenibacillus, Pelobacter, Persephonella, Rhodopirellula, Sorangium, Syntrophobacter, Thermoanaerobacter, Thermospirula, Thermosynechococcus and Thioalkalivibrio. Further, 20 actinobacterial genera were found in the beach sediments: Acidothermus, Actinomadura, Arthrobacter, Corynebacterium, Kineococcus, Kocuria, Leifsonia, Illumatobacter, Iamia, Micrococcus,
Mycobacterium, Nocardia, Nocardioides, Propionibacterium, Rhodococcus, Rubrobacter, Saccharopolyspora, Streptomyces, Thermobifida and Tropheryma.

The total BLAST score was calculated against each sequence of the reference database (http://mg.bio.titech.ac.jp/vitcomic/) to identify the nearest relative of the sample sequence in order to classify at species level (Figs. 8.9. a,b). The abundant actinobacterial species were Iamia majanohamensis, Illumatobacter fluminis, Mycobacterium engbaekii, Mycobacterium psychrotolerans, Streptomyces aureofaciens, Streptomyces longisporus, Streptomyces paradoxus, Streptomyces pseudogriseolus, Streptomyces sampsonii, Streptomyces sodiiiphilus, Streptomyces thermospinosisporus and Streptomyces xinjiangensis In the Figs. 8.9. a,b species names in the reference database are placed in circles with ordered phylogenetic relatedness.

Physical distances between the nearest species in the plot indicate the genetic distances of 16S rRNA genes between them. The circles indicate the boundaries of BLAST average similarities (inner most circle starting at 80%, followed by 85%, 90%, 95% and 100% identity to the database sequence). Each dot represents the average similarity of each sequence against the nearest relative species in the reference dataset. The size of these dots indicates the relative abundance of sequences in the sample. The VITCOMIC plot contains four categories of dot size that indicate the relative abundance of the sample sequence.
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Fig. 8.8.a. Phylogenetic diversity of 2,014 reads of the beach sediment sample computed by MEGAN. The classification for the sequence reads were obtained by using Ribosomal Database Project Classifier tool against the RDP database. Each circle represents a bacterial taxon in the RDP taxonomy database and the size of the circle represents the number of reads assigned to the particular taxon.
Fig. 8.8.b. Phylogenetic diversity of the Beach sample computed by MEGAN. The classification for the sequence reads were obtained by using Ribosomal Database Project Classifier tool against the RDP database. Each circle represents an actinobacterial taxon in the RDP taxonomy database and the size of the circle represents the number of reads assigned to the particular taxon.
Fig. 8.8.c. Graphical representation of 2,014 reads of the beach sediment sample, representing bacterial and actinobacterial taxa in the RDP taxonomy database and the number of reads assigned to the particular taxon.
Fig. 8.9.a. Vitcomic map representing the species diversity of the sample, based on the comparison of the reads against the NCBI nonredundant database using the blastn tool. Large circles indicate boundaries of BLAST average similarities, innermost circle (iv) 80–85 %, followed by (iii) 85–90 %, (ii) 90–95 %, (i) 95–100 % similarity of the database sequence.
Fig. 8.9.b. High-resolution view of the region containing the predominant Phylum actinobacteria. Larger size dots indicate that the relative abundance of the particular taxon is more than 10% of the sample sequence.
8.4. Discussion

Present study is an attempt to analyze the bacterial diversity (with special reference to actinobacteria) and bacterial community structure of the three different (Mangrove, Coral reef and Beach) environmental sediment samples of the Neil island, through bTEFAP pyrosequencing analysis. Accuracy of the bacterial diversity of the particular environment is dependent on the size and number of sequences used for the analysis. Length of the sequences would strongly affect the phylogenetic affiliation (Andersson et al., 2008; Schloss, 2010; Briggs et al., 2012). Accuracy of the taxonomic assignment of the microorganisms also depends on the targeted 16S rRNA gene sequence as the required length varies with different hypervariable regions. Different hypervariable regions have different efficacies with respect to species calls in different genera (Tekere et al., 2012). V6 hypervariable region is widely used as it has been reported to have good discriminating power (Aarthi, 2013). In this study, V6–V9 variable region with an average read length of 560, 480 and 440 bp was used, which is long enough to classify the reads up to the genus level.

The obtained sequences and the observed OTUs represented a near saturation of rarefaction curves. In addition, Good’s coverage revealed that 99% of the phylotypes identified in the three different marine sediment samples could represent the majority of the sequences present in the sample. Sundarkrishnan et al. (2012) estimated the OTU values in the pelagic sediments of the Andaman Sea. Similarly, Aarthi (2013) also used coverage value to estimate the microbial diversity of the Barantang mud volcano, of the Andamans.
Proteobacteria could be dominant or nearly dominant in various marine sediments (Stackebrandt et al., 1993; Zhou et al., 1997). In the present study, Proteobacteria and Actinobacteria were dominant in the three environments. Gray and Herwig (1996) also detected six major lineages of the domain bacteria and reported the dominance of the Proteobacteria and gram positive bacteria in a marine sediment sample.

Culture-independent studies of marine samples have revealed that members of the Phylum Actinobacteria could be frequently observed in the marine bacterial community (Giovannoni and Stingl, 2005). Actinobacteria were also cultivated from the marine sediments (Magarvey et al., 2004; Jensen et al., 2005; Maldonado et al., 2005a; Gontang et al., 2007) and there are a lot of evidences to show that actinobacteria occur in the marine environment (Moran et al., 1995; Chen et al., 2000; Mincer et al., 2005; Chen et al., 2010; Everest et al., 2011). Description of three marine species viz. Salinibacterium amurkyense, Serinicoccus marinus and Salinispora arenicola (Han et al., 2003; Yi et al., 2004; Maldonado et al., 2005b) also provide us with a strong support for the occurrence of marine-specific actinobacteria.

Present study reports the occurrence of the following actinobacterial genera: Acidothermus, Actinomadura, Actinomyces, Arthrobacter, Beutenbergia, Bifidobacterium, Clavibacter, Corynebacterium, Frankia, Iamia, Illumatobacter, Kineococcus, Kocuria, Leifsonia, Micrococcus, Micromonospora, Mycobacterium, Nocardia, Nocardoides, Nitrosococcus, Propionibacterium, Rhodococcus, Rubrobacter, Saccharopolyspora, Salinispora, Streptomyces, Thermobifida and Tropheryma. Among the three stations, mangrove environment possessed the higher actinobacterial diversity followed by the coral reef and beach habitats.
The genus *Acidothermus* has thermophilic species and generally it grows between 37°C to 70°C. Mohagheghi *et al.* (1986) first isolated this genus from hot springs in Yellowstone nation park. In the present study, *Acidothermus* occurred in all the three coastal ecosystems (Mangrove, Coral reef and Beach) of the Neil island.

*Actinomadura* presently contains more than 28 validly described species (Athalye *et al.*, 1985; Ochi *et al.*, 1991; Zhang *et al.*, 2001 and Lu *et al.*, 2003) and it has both marine and terrestrial forms. In the present study, *Actinomadura* occurred in all the three ecosystems. Recently, He *et al.* (2012) have isolated *Actinomadura* from the mangrove sediments of the in Dugong Creek, Little Andaman, India.

*Actinomyces* species are commensals and normal inhabitants of the oropharynx, gastrointestinal track and female genital track (Smith *et al.*, 2005) and this genus occurred in the mangrove sediments of the Neil island.

More than 52 species of *Arthrobacter* have been isolated from various sources such as soil, cheese, clinical specimens, paintings, seals, alpine ice cave, fish and waste water reservoir sediments (Hou *et al.*, 1998; Osorio *et al.*, 1999; Collins *et al.*, 2002; Reddy *et al.*, 2002; Margesin *et al.*, 2004; Heyrmann *et al.*, 2005; Irlinger *et al.*, 2005; Roh *et al.*, 2008). However, some *Arthrobacter* species have been isolated from the marine environment. For example, *Arthrobacter antarcticus* has been isolated from Antarctic marine sediment. *A. gambptoemesis* and *A. kerguelensis* were also isolated from the marine sediments of Antarctica. In the present study, *Arthrobacter* was present in all the three habitats of the Neil island.

*Beutenbergia* and *Clavibacter* were present in both the mangrove and coral reef sediments. To our knowledge, this is the first report of their occurrence in both the
mangrove and coral reef environments. Groth et al. (1999) have isolated Beutenbergia caernea from the lime stone cave, Guangxi (China).

The human gut is colonized with a wide variety of microorganisms, including species, such as those belonging to the bacterial genus Bifidobacterium. It was first described by Tisser in 1900, and more than 32 species have been assigned to this genus (Scardovi, 1986, Biavati and Mattarelli, 1991). Zinedine and Faid (2007) isolated Bifidobacterium from different sources (fecal matter of newborns, bovine meat and traditional fermented milk). Presently, Bifidobacterium was noted in the coral ecosystem.

In the present study, Corynebacterium was present in the all the three ecosystems and this genus is one of the largest genera within the actinobacteria. More than 50 species have been identified (Funke et al., 1997; Collins et al., 2004). Most of the species of Corynebacterium are present in the dairy products, soil, sewage and sediments (Renaud et al., 2007; Yassin and Siering, 2008; Funke et al., 2009; Yassin, 2009). However, some Corynebacterium species have been isolated from the marine environment and they may occur as part of the indigenous flora of the marine animals: C. caspium was isolated from seals (Collins et al., 2004). C. sphensicorum was recovered from wild penguins (Goyache et al., 2003), C. marins was found in the mucus of the coral Fugia ganulose (Ben-Dov et al., 2009). Corynebacterium has been reported by Karuppiam (2011) also from the coral reef sediments of the Gulf of Mannar, Biosphere Reserve.

Frankia is found as a nitrogen-fixing symbiont in the root nodules of angiosperm plants and it can also survive as free-living soil microbe (Udwary et al., 2011). This was present in the mangrove sediments of the Neil island.
The genus *Kineococcus* was first reported by Yokata *et al.* (1993) with the species of *K. antarcticus* and recently three more species have been added to the genus: *K. radiotolerance* (Philips *et al*., 2002), *K. marinus* (Lee, 2006) and *K. xinjiangensis* (Liu *et al*., 2009). *K. antarcticus* and *K. marinus* are recorded from marine samples. In the present study, *Kineococcus* was isolated from all the three coastal ecosystems.

The genus *Kocuria* isolated in the present study, occurred all the three stations. It was first proposed by Stackebrandt *et al.* (1995) to accommodate the phylogenetically distinct actinobacteria, formerly classified in the genus *Micrococcus*. The genus currently comprises six species and have been validly published *K. kristinae*, *K. paulstris*, *K. polaris*, *K. rhizophila*, *K. rosea* and *K. varians* (Stackebrandt *et al*., 1995; Kovacs *et al*., 1999; Reddy *et al*., 2003; Kim *et al*., 2004). No complete information is currently available on the ecological and industrial importance of the species *Kocuria* or *Micrococcus* group (Takarada *et al*., 2008).

The genus *Leifsonia*, isolated from all the three stations of the Neil island was originally described by Evtushenko *et al.*, (2000) and the description was amended by Reddy *et al.* (2008). *Leifsonia* can survive as both psychrotolerant (0-4°C) and mesophile (20-45°C).

Fujinami *et al.* (2013) opined that the phylogenetic analysis of the genus *Illumatobacter* has revealed branches near the presumed root of the class actinobacteria, and thus may represent a new taxon outside the known family Acidimicrobiaceae, although the family accommodating this genus has not been decided yet. No complete or draft genome information is currently available for the genera *Illumatobacter* and *Iamia*. Zhang *et al.* (2013) have reported the presence of *Illumatobacter* from King’s Bay.
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*Illumatobacter* was also reported from the estuarine sediments of Japan (Matsumoto et al., 2009). Similarly, *Iamia* was reported from the sea cucumber, *Holothuria edulis*, from the coast of Japan. Present study has shown that *Illumatobacter* and *Iamia* were the major components of the three coastal ecosystems of the Neil island. Karuppiah (2011) has also reported *Illumatobacter* and *Iamia* from the coral reef environment of the Gulf of Mannar. Recently, Aarthi (2013) has reported both *Illumatobacter* and *Iamia* from the mud volcanic samples of the Baratang island, the Andamans.

*Micromonospora* has been reported to inhabit soil, water and marine environment (Ludemann and Brodsky, 1963; Kawamoto, 1989). *M. endolithica* has been isolated from the extreme Antarctic sand stone environment. In the present study, *Micromonospora* occurred only in the mangrove sediments. Similarly, Huang *et al.* (2008) reported *M. rifamycinica* from the mangrove sediments and Xie *et al.* (2012) isolated *M. haikouensis* from the mangrove sediments of Halkou, China. So, *Micromonospora* could be a common inhabitant of the mangrove sediments.

*Mycobacterium*, *Nocardia* and *Nocardioides* are commonly found in all the marine environments. In the present study, these three genera occurred in all the three marine sediments. Poongodi *et al.* (2012) have isolated *Mycobacterium* from the coral reef environment of the Gulf of Mannar. Some reports are also available on *Nocardioides marinus*, isolated from the Korean sea water (Choi *et al.*, 2007).

The first marine ammonia-oxidizing strain isolated from the seawater was *Nitrosococcus*, and *N. oceani* and *N. halophilus* have been reported from the marine, saline environments. *N. oceani* has been detected by immunofluorescence and by PCR from the saline lake of the Antarctica (Voytek *et al.*, 1999) and also from various other
marine systems (O’ Mullan and Ward, 2005; Ward and Carlucci, 1985). It is worth mentioning that there are only a few reports on the occurrence of *Nitrosococcus* in the marine environment and none from the fresh water ecosystems. In the present study, *Nitrosococcus* has occurred in the coral reef sediments.

Genus *Propionibacterium* consists of members of the normal microbial flora of human skin and mouth (Clayton et al., 2006). In the present study, *Propionibacterium* has been found in all the three environments.

*Rhodococcus* consists of a diverse group of bacteria commonly present in many environments, from soil to seawater. In the present study, it occurred in all the three coastal sediments. Similarly, Li et al. (2012b) isolated *R. nanhaiensis* from the marine sediments of the South China sea. *Rhodococcus* sp. has also been reported from the deep sea sediments of the Pacific Ocean (Peng et al., 2008).

*Rubrobacter* is a thermophilic organism mostly present in the higher temperature environment. In the present study, *Rubrobacter*, occurred in the mangrove and beach sediments. Mangrove and beach temperatures were relatively higher and this could be the reason for the presence of *Rubrobacter* here. Abdelmohsen et al. (2010) have reported *Rubrobacter* from a marine sponge. To our knowledge, this is the first report on the occurrence of *Rubrobacter* in the mangrove and beach environment.

Presently noticed genus, *Saccharopolyspora* was originally proposed by Lacey and Goodfellow in 1975 and assigned to the family Pseudonocardiaeeae (Embley et al., 1988; Warwick et al., 1994). *Saccharopolyspora* is a potentially rich source of natural products. Erythromycin, produced by *S. erythraea*, is currently an important antibiotic
which is commercially available (Zhang et al., 2008). In the present study, *Saccharopolyspora* was found to be present in all the three coastal sediments.

Many workers have reported the presence of indigenous *Streptomyces* populations from the marine environment (chapter 7). In the present study, *Streptomyces* occurred in all the three stations.

Maldanado et al. (2005b) reported *Salinispora* from the marine sediments and the *Salinispora* strains were found to have a cosmopolitan distribution in tropical and subtropical marine sediments (Jensen and Mafnas, 2006; Freel et al., 2012; Ahmed et al., 2013). In the present study, *Salinispora* was found only in the mangrove sediments. Occurrence of *Salinispora* in the mangrove environment of the Neil island may be due to the accumulation of rich organic content and higher salinity level in the mangrove sediments.

*Thermobifida*, a thermophilic microbe, occurred in all three coastal sediments. Yang et al. (2008) isolated *T. halotolerans* form salt mine samples.

*Tropheryma (T. whipple)*, widely known to be associated with whipple disease, is characterized by various clinical signs such as diarrhea, weight loss, lymphadenopathy and polyarthritis (Boushia et al., 2010). In the present study, this genus occurred in the mangrove, coral reef and beach sediments, signally the impact of anthropogenic activity here.

Thus, the molecular information of the marine actinobacteria, obtained from the present study would help facilitate the rational selection of culture media to target specific groups and species of marine actinobacteria for culturing them in the laboratory and screening them for bioactive natural products for drug discovery and other uses.