CHAPTER-3
Genotypic characterization of vibrios

3.1. Introduction

3.1.1. Genotypic characters of vibrios

Traditionally, both detection and identification of marine vibrios have been depended on their growth on thiosulphate citrate bile salt sucrose (TCBS) selective medium and subsequent characterization by biochemical tests (Diggles et al., 2000). Taxonomy of *Vibrio* spp. is in the process of revision due to the increasing data obtained with molecular techniques, where different genes are examined or where whole genome is inspected. Since 1980s, the genus *Vibrio* has been subjected to an extensive taxonomic revision, with species of vibrios distributed into five phylogenetic robust clades corresponding to the genera *Vibrio*, *Photobacterium*, *Salinivibrio*, *Enterovibrio*, and *Grimontia* based on 16S rRNA gene sequences (Azam, 2001; Bang, 1978; Banin, 2001). Various DNA based methods have also been used to identify and type these organisms, including: pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting and enterobacterial repetitive intergenic consensus sequence (ERIC) PCR (Jiang et al., 2000 a, b; Rivera et al., 1995), fluorescent in situ hybridization (Hernandez and Olmos, 2006). Variations in the length and sequence of the 16S–23S intergenic spacer regions (IGSs) of rRNA (rrn) operons have been used to design species-specific PCR primers and/or probes for bacterial identification (Kong et al., 2002).

3.1.2. 16S rRNA

The ability to differentiate subtypes is important for the recognition of disease outbreaks, the determination of sources of infection, the detection of particularly virulent strains, host distribution and geographical origin of possible variants of a specific pathogen (Olive and Bean, 1999; Soll, 2000). The available molecular methods used for subtyping differ widely in their
ability to differentiate among strains (Soll, 2000). It is observed that the 16S rRNA is unable to resolve closely related species (Nagpal et al., 1998), such as the ones clustered in the *Vibrio* core group, namely *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. campbellii*, *V. natriegens* and the newly described *V. rotiferianus* (Gomez-Gil et al., 2003 a). The comparison of 16S rRNA gene sequences has been recognized as an invaluable tool for confirming bacterial species identity but not for differentiating among strains, since the sequence shows limited intraspecific variations (Drancourt et al., 2000). Nucleotide sequences, primers and probes of 16S rRNA gene have been determined for *Vibrio* species, which are not useful for clustering of the species of this genus.

Special emphasis has been paid to sequencing of the 16S rRNA gene, although other genes, such as those for 23S rRNA, 16S–23S intergenic spacer region (IGS), or the *gyrB* gene, have been employed for taxonomic positioning of various strains (Dorsch et al., 1992; Venkateswaran et al., 1998). The 23S rRNA gene and the 16S–23S intergenic spacer contains regions where the sequences vary significantly, hence these regions are more useful in phylogeny than the 16S gene. Comparison of the nucleotide sequence of 16S–23S intergenic spacer region of *V. cholerae*, indicated strain-to-strain variation and that the spacer region is effective for differentiation at intraspecific level (Heidelberg et al., 2000).

The identification of vibrios isolated from the aquaculture environment has been imprecise and is labour-intensive, requiring many biochemical and/or physiological tests (Vandenberghe et al., 2003). Several highly powerful molecular tools, e.g. amplified fragment length polymorphism (AFLP), (Rademaker et al., 2000; Gurtler and Mayall, 2001) and repetitive extragenic palindromic elements polymerase chain reaction (repPCR), have become readily available for the identification of bacteria, including vibrios (Thompson et al., 2001; Sawabe et al., 2003).
3.1.3. DNA-DNA Hybridization

DNA-DNA hybridization remains the “gold” standard for species delineation having at least 70% DNA-DNA similarity under stringent conditions (Stackebrandt et al., 2002). DNA-DNA hybridization experiments carried out in microplates in which DNA is non-covalently adsorbed and subsequently hybridized with photobiotin-labeled probe DNA (Willems et al., 2001) are much faster than the classic DNA-DNA hybridization techniques (e.g., initial renaturation, hydroxyapatite, and S1 nuclease). This technique can be performed in quadruplicate and with reciprocal reactions simultaneously, and has high correlation with classic techniques (Goris, 1998).

3.1.4. AFLP

Amplified fragment length polymorphism (AFLP) indices varies in the whole genome of different strains, hence considered as useful information in tracing short and long term evolution of bacterial isolates (Lan and Reeves, 2002). AFLP technique involves the digestion of total genomic DNA with two restriction enzymes, subsequent ligation of the restriction fragments with the halfsite-specific adaptors to all restriction enzymes; selective amplification of these fragments with two PCR primers that have corresponding adaptor and restriction site sequences at their target site, followed by the electrophoretic separation of the PCR products on polyacrylamide gels with selective detection of fragments that contain fluorescent labeled primer and computer-assisted numerical analysis of the banding patterns (Huys and Swings, 1999). AFLP fingerprinting has been carried out for isolates of *V.alginolyticus*, *V.cholerae*, *V.harveyi*, *V.vulnificus* and *P.damselae* (Vandenberghhe et al., 1999; Lan and Reeves, 2002; Arias et al., 1997 a,b; Thyssen at al., 2000). Thompson et al. (2001) carried out AFLP of vibrios using HindIII/TaqI as the RE combination, obtaining 102±24 bands with mean reproducibility at 91±3%. The analysis of the banding patterns revealed that *V.trachuri* and *V.shilonii* were highly related to *V.harveyi* and *V.mediterranei*
Chapter 3

respectively, indicating that they were synonyms. AFLP is a reliable fingerprinting identification and classification tool for vibrios. However, the variations in AFLP indices in the whole genome, including regions of unknown functions such as those coded by the pseudogenes or mobile genetic elements, limits its use as a phylogenetic marker.

3.1.5. RAPD

Molecular approaches that interrogate the whole genome appears to be a way forward to highlight what may be only minimal differences between strains. RAPD-DNA fingerprinting method has been widely used in the development of molecular diagnostic techniques for bacteria, because it allows a comparative analysis of genomes between different isolates of the same species by employing distinct molecular markers (Sudeesh et al., 2002). *V. harveyi, V. alginolyticus* and *V. parahaemolyticus* are characterized by RAPD-PCR techniques revealing greater genetic diversity among the three species. RAPD-PCR is a means of rapidly detecting polymorphisms for genetic mapping and strain identification (Welsh and McClelland, 1990). The method applies PCR with a single short oligonucleotide primer, randomly amplifying short fragments of genomic DNA, which are size-fractionated by agarose gel electrophoresis. The method has considerable appeal because it is generally faster and less expensive than any previous method for detecting DNA sequence variation. The fact that RAPDs survey numerous loci in the genome makes the method particularly attractive for analysis of genetic distance and phylogeny reconstruction. The amplification of a fragment depends strictly on the exact match between the oligonucleotide primer and a site on the genomic DNA, thus if one DNA sample amplifies a particular band and another DNA sample does not, it is assumed that a single nucleotide substitution in a primer site accounts for the difference (Clark and Lanigan, 1993). The recent advent of standardized and optimized reagents has greatly improved the accessibility and reproducibility of the RAPD method (Hytyiä et al., 1999). These improvements led to an increase in the number of successful applications of
the RAPD method for differentiating strains of marine bacterial pathogens (Romalde et al., 1999; 2002, Magariños et al., 2000; Ravelo et al., 2003).

RAPD analysis has several advantages including relative shorter time required to complete the analysis after standardization, prior knowledge of the organisms genome is not necessary, availability of series of primers for analysis, minimal operational cost, requirement of relatively small amount (approx 20ng) of high molecular weight DNA and simpler protocol allows strain differentiation based on the differences in nucleotide sequences in the entire genome (Gopalakrishnan and Mohindra, 2002). This technique has become increasingly important for discriminating strains of food-borne pathogens and to trace the routes of transmission and implementation of suitable control measure based on the results. Therefore, RAPD can be considered as powerful tool for the identification of strain variation or for population studies. Also this method is simple, fast and specific, that it could be very useful for typing and differentiating environmental vibrios, which are relatively difficult to identify using other techniques (Sudeesh et al., 2002). It has been shown that the rDNA sequence similarities between Vibrio spp. are so high that 16S rDNA genes cannot be used for differentiation at the species level (Kita-Tsukamoto et al., 1993). For this reason, screening with different gene-specific oligonucleotides is to be developed for V. harveyi.

3.1.6. Multi Locus Sequence Typing

In 1998, Maiden and co-workers proposed MLST a modified version of multilocus enzyme electrophoresis (MLEE) for studying the population biology and epidemiology of Neisseria meningitides. Essential genes are conserved in bacteria and other organisms but the sequences may vary, making them useful candidates for phylogenetic analysis. In addition, the genera within vibrios are defined on the basis of their shared sequence similarities in different loci. Species within the genus Vibrio share at least
85% gene sequence similarity in recA, rpoA, and pyrH (Thompson et al., 2005). DDH technique is time-consuming and can be performed in relatively few laboratories and, more importantly, the DDH data are not cumulative in online databases. Clearly, a reliable and straightforward alternative is the use of MLSA. MLSA based on the recA, rpoA, and pyrH genes of species form discrete clusters showed that the species have a cutoff level of 94% gene sequence similarity (Thompson et al., 2004). However, some groups of species, e.g., the Vibrio splendidus and Vibrio harveyi show variations on the basis of recA, gyrB, and gapA based MLSA. Thus, it is very important to evaluate additional genetic markers that can distinguish closely related species of vibrios. Some studies suggest that recombination might have occurred between different sister species, such as between V. cholerae and V. mimicus and between V. harveyi and V. campbellii, but it is not clear how prevalent and widespread this process is when all groups of vibrio species are analyzed simultaneously.

Accurate identification of vibrios at the family and genus levels is obtained by 16S rRNA gene sequencing, whereas identification at the species and strain levels requires the application of genomic analysis, including DNA-DNA hybridization, repetitive extragenic palindromic PCR (rep PCR), and amplified fragment length polymorphism (AFLP) analysis (Thompson et al., 2004). These techniques are essential for reliable species identification, because several vibrios have nearly identical 16S rRNA sequences and similar phenotypic features. The sequencing of housekeeping genes is emerging as an alternative to overcome this problem and may improve the current pragmatic definition of bacterial species (Fig. 3.1) (Sawbae et al., 2007). Different loci, e.g., 23S rRNA, gapA, gyrB, hsp60, and recA (Thompson et al., 2005; Gomez-Gil et al., 2004; Le Roux et al., 2004) have been used for phylogenetic studies and the identification of Vibrionaceae species. So far these genes (except for recA) have only been examined in a very limited number of species and strains. Alternative phylogenetic markers should fulfill several criteria, as put forward by
Zeigler (2003): (i) the genes must be widely distributed among genomes, (ii) the genes must be present as a single copy within a given genome, (iii) the individual gene sequences must be long enough to contain sufficient information but short enough to allow sequencing in a convenient way (900 to 2,250 nucleotides [nt]), and (iv) the sequences must predict whole genome relationships with acceptable precision and accuracy that correlate well with the 16S rRNA data and with whole genome similarities measured by DNA-DNA hybridization. A combination of in silico analyses and recent experimental studies of different bacteria, including Bacillus, Proteobacteria, lactic acid bacteria, Mycobacterium, and Mycoplasma, suggested that the RNA polymerase alpha subunit gene rpoA, recA, and the uridylate kinase gene (pyrH) fulfill these requisites and could therefore be used for identification purposes (Gevers et al., 2004; Thompson et al., 2005; Zeigler, 2003).

*V. harveyi* and *V. campbellii* are genetically related species with a DNA-DNA similarity value of 69% and a 16S rRNA similarity higher than 97% (Gomez-Gil et al., 2004). Also, *V. harveyi* and *V. carchariae* were synonymous, with *V. harveyi* having precedence as the senior synonym (Pedersen et al. 1998; Gauger and Gomez-Chiarri 2002). Information obtained from multilocus sequence analysis is, therefore, essential for the accurate and reliable identification of *Vibrio* species.
Fig:3.1 Concatenated split network tree based on nine gene loci: fisZ, gapA, gyrB, mreB, pyrH, recA, topA, and 16S rRNA gene sequences from 58 taxa were concatenated and reconstructed using the SplitsTree4 program with all nodes supported by 100 bootstrap replications. (Sawbae et al., 2007)
Fig: 3.2 Phylogenetic tree based on the neighbor-joining method, using the 16S rRNA, recA and rpoA concatenated gene sequences from the type strains of each species belonging to different families of vibrios. Felsenstein, (1985) method was employed for Bootstrap percentages after 1,000 simulations are shown. Bar, 1% estimated sequence divergence (Thompson et al., 2005).
3.1.7. Role of Housekeeping Genes in phylogenetic Analysis

Various housekeeping genes in particular, the recA gene essential for genetic recombination are used to demonstrate the divergence among interrelated Vibrio species and it had more discriminatory power than 16S rRNA gene in phylogenetic analysis of Vibrionaceae (Fig. 3.2) (Thompson et al., 2004). A 600bp sequence of hsp60 gene analyzed among 15 Vibrio species showed an identity of 71 to 82%, suggesting that this gene could also be a useful phylogenetic marker. The various genes involved in DNA replication are also well conserved, such as the gyrB gene coding for DNA gyraseB subunit. Based on phenotypic and 16S rRNA analysis, >99% similarity was shown by the strains of V.alginolyticus and V.parahaemolyticus, whereas the gyrB sequence established 86.8% identity between the two strains. Comparison of the gyrB sequence was useful for phylogenetic analysis of V.splendidus and its related species (Le Roux et al., 2004) and also PCR targeted to this gene for the identification of V.hollisae has been developed (Vuddhakul et al., 2000b).

Other molecular chronometers include the sodA gene coding for superoxide dismutase catalyzing dismutation of the superoxide radical to H2O2 and O2. This is conserved in prokaryotes and eukaryotes and is useful for identification of Vibrionaceae, and the gene targeted PCR is used for the identification of V.parahaemolyticus (Shyu and Lin, 1999). The lux genes of various luminescent bacteria, especially the luxA gene coding for luciferase showed 99% identity among V.choleare and 77% identity between V.cholerae and V.harveyi (Palmer and Colwell, 1991). The fur gene coding for a regulator of an iron uptake system is detected in many bacterial species (Colquhoun and Sorum, 2002). Although genetic differentiation of V.harveyi from related species is not easy (Gomez-Gil et al., 2004; Oakey et al., 2003), PCR based analysis of these chronometers from various Vibrio species shows that these genes can be effectively used for phylogenetic analysis of genus Vibrio.
Rationale of the study is to analyse the amplicons of eight genes (i.e., \textit{ftsZ}, \textit{gapA}, \textit{gyrB}, \textit{mreB}, \textit{pyrH}, \textit{recA}, \textit{topA}, and 16S rRNA), to determine the genotypic heterogeneity among the isolates.

3.2. Materials and Methods

3.2.1. DNA extraction using DNAZol (Invitrogen) from 158 isolates:

A single colony of the isolate was inoculated into LB broth and incubated for 18hrs. An aliquot of 2ml of the overnight grown culture was taken in a 2ml MCT and centrifuged at 10000xg for 10min at 4 °C. The supernatant decanted and the pellets were re-suspended in 1ml TNE (Tris-NaCl- EDTA buffer, pH-7.5). The above process was repeated twice. The pellets were re-suspended in 50µl TNE and added 20 µl (10mg/ml) Proteinase K, mixed gently and incubated at 37°C for 1hr. An aliquot of 1ml DNAZol was added and mixed thoroughly using cut-tips until the mass dissolved completely. The homogenate was centrifuged at 12,000xg for 10min at 4°C and transferred the supernatant into a fresh 1.5ml MCT. An aliquot of 0.5ml 100% ice cold ethanol was added, mixed by gentle inversion and incubated at room temperature for 5-10mins, or kept in -20°C for 30min. The homogenate was centrifuged at 12,000xg for 10 min at 4°C to form pellet. The supernatant (100% ethanol) was drained off and washed the pellet twice with 1ml 75% ethanol. The pellets were air-dried and dissolved in 200µl 8mM NaOH and 20µl Hepes buffer and incubated at 4°C. The DNA concentration was determined by running on 0.8 % agarose and the extracted DNA were stored at -20 °C in aliquots using 5mM Tris Cl (pH 8) until use.

3.2.2. Construction of Randomly amplified polymorphic DNA (RAPD) profile using a set of Operon primers

Standardization of RAPD-PCR was carried out with aid of a set of 20 RAPD primers using 11 type strains (Table-3.1) and one isolate from each cluster obtained upon phenotypic grouping. The primers screened were
obtained from Operon Technologies, USA. The 20 primers used for standardization and thereby for RAPD fingerprinting are as follows:

**Table-3.1: Details of the Operon primers used**

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Primer</th>
<th>Primer Code</th>
<th>Primer Sequence</th>
<th>Concentration (pmoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OPA-03</td>
<td>NP111</td>
<td>AGTCAGCCAC</td>
<td>5194</td>
</tr>
<tr>
<td>2</td>
<td>OPA-04</td>
<td>NP112</td>
<td>AATCGGGGCTC</td>
<td>5090</td>
</tr>
<tr>
<td>3</td>
<td>OPA-05</td>
<td>NP113</td>
<td>AGGGGTCTTTG</td>
<td>5194</td>
</tr>
<tr>
<td>4</td>
<td>OPA-06</td>
<td>NP114</td>
<td>GGTCCCTGAC</td>
<td>5743</td>
</tr>
<tr>
<td>5</td>
<td>OPA-07</td>
<td>NP115</td>
<td>GAAACGGGTTG</td>
<td>4627</td>
</tr>
<tr>
<td>6</td>
<td>OPA-08</td>
<td>NP116</td>
<td>GTGACGTAGG</td>
<td>4894</td>
</tr>
<tr>
<td>7</td>
<td>OPD-05</td>
<td>NP117</td>
<td>TGAGCGGACA</td>
<td>4801</td>
</tr>
<tr>
<td>8</td>
<td>OPD-06</td>
<td>NP118</td>
<td>ACCTGAACGG</td>
<td>4990</td>
</tr>
<tr>
<td>9</td>
<td>OPD-08</td>
<td>NP119</td>
<td>GTGTGCCCCA</td>
<td>5743</td>
</tr>
<tr>
<td>10</td>
<td>OPD-11</td>
<td>NP120</td>
<td>AGCGCCATTG</td>
<td>5302</td>
</tr>
<tr>
<td>11</td>
<td>OPD-15</td>
<td>NP121</td>
<td>CATCCGTGCT</td>
<td>5919</td>
</tr>
<tr>
<td>12</td>
<td>OPD-16</td>
<td>NP122</td>
<td>AGGGCGTAAG</td>
<td>4627</td>
</tr>
<tr>
<td>13</td>
<td>OPD-20</td>
<td>NP123</td>
<td>ACCCGGTCA</td>
<td>5616</td>
</tr>
<tr>
<td>14</td>
<td>OPAC-10</td>
<td>NP124</td>
<td>AGCAGCGAGG</td>
<td>4685</td>
</tr>
<tr>
<td>15</td>
<td>OPAH-01</td>
<td>NP125</td>
<td>TCCGCAACCA</td>
<td>5415</td>
</tr>
<tr>
<td>16</td>
<td>OPAH-02</td>
<td>NP126</td>
<td>CACTTCCGCT</td>
<td>4685</td>
</tr>
<tr>
<td>17</td>
<td>OPAH-03</td>
<td>NP127</td>
<td>GTTACTGCC</td>
<td>5656</td>
</tr>
<tr>
<td>18</td>
<td>OPAH-04</td>
<td>NP128</td>
<td>CTCCCCAGAC</td>
<td>5876</td>
</tr>
<tr>
<td>19</td>
<td>OPAH-05</td>
<td>NP129</td>
<td>TTGCAAGGCA</td>
<td>5090</td>
</tr>
<tr>
<td>20</td>
<td>OPAH-O6</td>
<td>NP130</td>
<td>GTAAGCCCCCT</td>
<td>5533</td>
</tr>
</tbody>
</table>

From the 20 primers screened, 7 primers (OPA-3, OPA-4, OPA-5, OPA-7, OPAC-10, OPD-16 and OPD-20) were selected based on the resolution of the distinct detectable bands. These 7 primers were used to construct the RAPD profile of the 158 isolates. The reaction mixture for
RAPD-PCR consisted of 1.0 μl Taq polymerase, 2.5 μl 10x Buffer, 2.0 μl dNTP mix, 0.5 μl Mg Cl₂, 1.5 μl Primer, 1 μl Template DNA and 16.5 μl MilliQ. Amplifications were performed on a thermal cycler, which was programmed for an initial denaturation cycle of 95°C for 4mins, followed by 45 cycles of denaturation at 94 °C for 1min, annealing at 36 °C for 1min and primer extension at 72 °C for 2mins. The program also included a final primer extension step at 72 °C for 10mins. The amplified products were analysed on 1.5% agarose gel electrophoresis carried out at a constant current of 60mA. Images of agarose gels were analyzed by manually transforming the scored DNA fragments obtained into binary data matrix by scoring as presence (1) or absence (0) for each isolate and comparing with the distinct bands at equivalent sites obtained by running 1kb and 100bp markers. Clustering and dendrogram construction by each bacterial isolate upon amplification using the chosen 7 primers based on similarity coefficient was carried out with the software NTSYS pc version 2.0. Further, population wise delineation of the 158 isolates with the seven selected primers was carried out using the software PopGene32. Accordingly, the isolates were grouped into 17 populations (clusters) as constructed examining the phenotypic characters (by way of numerical taxonomy) were processed using the software PopGene32. The amplicons represented as bands for each of the isolates were scored as binary data matrix and population wise analysis of correlation was carried out. Percentage similarity between each population was represented as dendrogram.

3.2.3. Amplification of Housekeeping genes:

The representative isolates (35 Nos. including the type stains) were selected from the dendrogram constructed based on phenotypic characterization. Genomic DNA was extracted using DNAzol method as described above in 3.3.1. The extracted DNA was stored at -20 °C in aliquots using 5mM Tris Cl (pH 8) until use. The genomic DNA of the 35
isolates was amplified for the already reported housekeeping gene markers (Table-3.2). Following amplification, the banding pattern was analyzed by running on 1% agarose gel. Subsequent to agarose gel electrophoresis, the molecular weight of the bands was analysed using Quantity1 software. The bands were scored as 0 or 1 for absence and presence respectively to the corresponding positions of 100bp maker. The scored data was processed in the PopGene32 software and a dendrogram was constructed.

Table- 3.2: Details of Housekeeping Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>PRIMER SEQUENCE</th>
</tr>
</thead>
</table>
| gapA (glyceraldehydes-3-phosphodehydrogenase) | AACTCACGGTCGCTTTTCAAC  
CGTTGTCGTACCAAGATAC |
| ftsZ (Cell division protein)          | GCTGTTGAACACATGGTGACCG  
GCACCAGCAAGATCGATATC |
| topA (topoisomerase I)              | GAGATCATCGGTGGTGATG  
GAAGGACGAATCGCTTCTG |
| mreB (rod shaping protein gene B-subunit) | ACTTCGTGGCATGTTTTC  
CCGTGCATATCGATCATTTC |
| gyrB (gyraseB)                   | GAAGTTATCATGACGGTGACTTC  
CCTTACGACGAGTCATTTC |
| pyrH (uridylate kinase)           | GATCGTATGGCTCAAGAAG  
TAGGCATTTTGTGGTCACG |
| recA (recombinaseA)               | GTCTACCAATGGTGCTTATC  
GCCATTGTAGCTGTACCAAG |
| 16S rRNA                        | GAGTTTGATCCTGGCTCA  
ACGGCTACCTTGTTACGACTT |

16S rRNA gene sequence analysis

This was carried out to confirm the identity of the representative isolates of the phena generated by numerical taxonomy (Fig. 2.1). PCR products of representative isolates (25 nos) were sequenced (SciGenom Labs Pvt Ltd, Cochin, India) and the 16SrRNA gene sequences obtained were matched with the (http://www.ncbi.nih.gov/sites/entrez?db=nuccore) Genbank database using the BLAST search algorithm (Altschul et al., 1990). These sequences were deposited with GenBank to obtain accession numbers.
3.3. Results

3.3.1. RAPD profiling

All 158 isolates were subjected to RAPD-PCR with the 7 selected Operon primers and scored (1) for the presence and (0) for absence of distinct bands at equivalent sites obtained by running 1kb and 100bp markers. The scores obtained with each primer were processed to determine the divergence pattern of the isolates using NTSYS pc.2.0. Amplification of the isolates with the 7 selected primers gave 13, 11, 15, 14, 12, 13 and 14 loci respectively. All the loci obtained with the selected 7 primers were analysed, yielding 27, 26, 46, 48, 41, 44 and 44 clusters respectively (Figs-3.3 to 3.16). The clusters obtained with each primer where compared with the source of isolation and represented in Table-3.3. Dendrograms obtained suggests that there existed a wide heterogenicity among the isolates of vibrios, exhibiting a correlation coefficient ≥0.62 (62%S). Highest extend of heterogeneity was exhibited by isolates of *V.harveyi*, followed by *V.vulnificus, V.mediterranei, V.alginolyticus, V.cholerae* and *V.nereis*. Since, the isolates were widely diverging; analysis of banding pattern of each phena with all the 7 selected primers in total was carried out to determine if there was any relation between banding pattern and the source of isolation. The banding pattern shown by each bacterial phenon (population) upon amplification, the total loci were processed using PopGene32 (Fig. 3.17). Dendrogram obtained showed that all the isolates grouped into 8 Clusters and were interrelated at ≥76%. Phenon1 & 2 having the isolates of *V.harveyi* was correlated at 99.1%, suggesting a high degree of homogeneity; hence these isolates in these two phena could be members of the same species. These two phena joined with the isolates of *V.mediterranei* in phena 3 & 4 at 91.6%S and to the 5th phenon of *V.harveyi* at 89.1%S. At 84.5%S this cluster (Cluster-1) joined with Cluster-2 having the isolates grouped to phena 6, 7, and 8. Phena 7 and 8 comprising isolates of *V.vulnificus* at 97.4%S, joined with isolates of *V.fluvialis* of phenon6 at 86%S. The first cluster of *V.harveyi* showed 84.2%S, 82%S, 81%S and
Chapter 3

79.8%S to the phena representing the isolates of *V.nereis* in Clusters 3 & 4, *V.parahaemolyticus* in Cluster-5 and *V.splendidus* in Cluster-6 respectively. At 86.8%S the isolate and type strain of *V.proteolyticus* (Cluster-7), grouped under phena 14 and 15 were correlated. At 80.6%S isolates of *V.alginolyticus* (phenon16) and of *V.cholerae* (phenon17) in cluster-8 were related and this cluster showed 76%S to the first cluster of *V.harveyi*.

![Fig: 3.3 Amplicons obtained using the primer OPA-3](image)

A- V1 to V12, 1Kb Marker, V13 to V28, 1Kb Marker  
B- V29 to V38, 1Kb Marker, V39 to V47  
C- V48 to V62, 1Kb Marker, V63 to V67  
D- V68 to V75, 1Kb Marker, V76 to V86  
E- 100bp Marker, V87 to V96, 100bp Marker  
F- V97 to V104, 1Kb Marker, V105 to V114  
G- V115 to V123, 1Kb Marker, V124 to V132  
H- V133 to V137, 100bp Marker, V138 to V142  
I- 100bp Marker, V143 to V150  
J- 100bp Marker, V151 to V158
Fig: 3.4 Dendrogram of the Vibrio spp. based on RAPD profile using the primer OPA-3

Clusters- 1, 2, 3, 4, 5

Clusters- 6, 7, 8, 9

Clusters- 10, 11, 12

Clusters- 13, 14, 15, 16, 17

Clusters- 18, 19, 20, 21, 22, 23

Clusters- 24, 25, 26, 27

Clusters- 1, 2, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18 - Isolates of V. harveyi; Clusters- 19, 20 - Isolates of V. parahaemolyticus; Clusters- 3, 21, 22, 23, 24, 27 - Isolates of V.mediterranei; Clusters- 23, 27 - Isolates of V.proteolyticus; Clusters- 9, 24, 25, 28 - Isolates of V.alginolyticus; Clusters- 8, 18 - Isolates of V.fluvialis; Clusters- 5, 26 - Isolates of V.cholerae; Clusters- 3, 8, 19 - Isolates of V.vulnificus; Clusters- 5, 20 - Isolates of V. splendidus
Fig: 3.5 Amplicons obtained using the primer OPA-4
A- V1 to V9, 1Kb Marker, V10 to V19
B- V20 to V28, 1Kb Marker, V29 to V37
C- V38 to V46, 1Kb Marker, V47 to V55
D- V56 to V64, 1Kb Marker, V65 to V74
E- V75 to V83, 1Kb Marker, V84 to V93
F- V94 to V102, 1Kb Marker, V103 to V111
G- V112 to V120, 1Kb Marker, V121 to V129
H- 100bp Marker, V130 to V137
I- 1Kb Marker, 100bp Marker, V138 to V147, 1Kb Marker, 100bp Marker, V148 to V158
Fig: 3.6 Dendrogram of the *Vibrio* spp. based on RAPD profile using the primer OPA-4

Clusters- 1, 2, 4, 6, 7, 8, 9, 10, 11, 17, 22, 23, 25- Isolates of *V. harveyi*; Clusters- 21 - Isolates of *V. parahaemolyticus*; Clusters- 18, 19, 24 - Isolates of *V. mediterranei*; Clusters- 23 - Isolates of *V. proteolyticus*; Clusters- 14 - Isolates of *V. alginolyticus*; Clusters- 15, 16 - Isolates of *V. nereis*; Clusters- 17, 20 - Isolates of *V. cholerae*; Clusters- 3, 17, 20 - Isolates of *V. vulnificus*; Clusters- 12- Isolates of *V. splendidus*
Fig: 3.7 Amplicons obtained using the primer OPA-5

A- 100bp Marker, V1 to V9
B- V10 to V17, 1Kb Marker, V18 to V27
C- V28 to V36, 1Kb Marker, V37 to V46
D- 1Kb Marker, V47 to V60
E- V61 to V69, 1Kb Marker, V70 to V78
F- V79 to V87, 1Kb Marker, V88 to V96
G- 100bp Marker, V97 to V115
H- 100bp Marker, V116 to V134
I- V135 to V141, 1Kb Marker, V142-V148
J- V149 to V153, 100bp Marker, V154 to V158
Fig: 3.8 Dendrogram of the *Vibrio* spp. based on RAPD profile using the primer OPA-5

Clusters- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 22, 25, 26, 29, 30, 31, 32, 33, 34, 35, 43, 44- Isolates of *V. harveyi*; Clusters- 14- Isolates of *V. parahaemolyticus*; Clusters- 14, 20 - Isolates of *V. mediterranei*; Clusters- 24, 42 - Isolates of *V. proteolyticus*; Clusters- 23, 45, 46 - Isolates of *V. alginolyticus*; Clusters- 40- Isolates of *V. nereis*; Clusters- 27- Isolates of *V. fluvialis*; Clusters- 28, 36, 37, 38- Isolates of *V. cholerae*; Clusters- 8, 39- Isolates of *V. vulnificus*; Clusters- 21, 41 - Isolates of *V. splendidus*
Fig: 3.9 Amplicons obtained using the primer OPA-7

A- 1Kb Marker, V1 to V8
B- V9 to V16, 1Kb Marker, V17 to V27
C- V28 to V36, 1Kb Marker, V37 to V46
D- V47 to V51 1Kb Marker, V52 to V56, 1Kb Marker, V57 to V60
E- V61 to V66, 1Kb Marker, V67 to V70
F- 1Kb Marker, 100bp Marker, V71 to V81, 1Kb Marker, 100bp Marker, V82 to V91
G- 100bp Marker, V92 to V104
H- 100bp Marker, V105 to V116
I- 1Kb Marker, V117 to V142
J- V143 to V148, 1Kb Marker, V149 to V158
Fig: 3.10 Dendrogram of the *Vibrio* spp. based on RAPD profile using the primer OPA-7

Clusters- 12, 13, 14, 15, 21, 22, 23, 24, 25, 27, 30, 33, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48 - Isolates of *V. harveyi*; Clusters- 5, 31 - Isolates of *V. parahaemolyticus*; Clusters- 6, 29, 32 - Isolates of *V. mediterranei*; Clusters- 35 - Isolates of *V. proteolyticus*; Clusters- 1, 26, 34 - Isolates of *V. alginolyticus*; Clusters- 9, 18, 20, 33 - Isolates of *V. nereis*; Clusters- 11, 16 - Isolates of *V. fluvialis*; Clusters- 7, 8, 10, 28 - Isolates of *V. cholerae*; Clusters- 2, 3, 4- Isolates of *V. vulnificus*; Clusters-17, 19 Isolates of *V. splendidus*
Fig: 3.11 Amplicons obtained using the primer OPAC-10

A- 100bp Marker, V1 to V12, 1Kb Marker
B- 100bp Marker, V13 to V24
C- 100bp Marker, V25 to V35, 1Kb Marker
D- V36 to V60
E- V61 to V85
F- V86 to V89, 1Kb Marker, V90 to V96
G- V97 to V105, 1Kb Marker, V106 to V114
H- V115 to V120, 1Kb Marker, V121 to V132
I- V133 to V141, 100bp Marker, V142 to V148
J- 100bp Marker, V149 to V158
Fig: 3.12 Dendrogram of the *Vibrio* spp. based on RAPD profile using the primer OPAC-10

Clusters- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 34, 35, 36, 40- Isolates of *V. harveyi*; Clusters- 37 - Isolates of *V. parahaemolyticus*; Clusters- 11, 12, 13, 14, 38 - Isolates of *V.mediterranei*; Clusters – 7, 27- Isolates of *V.proteolyticus*; Clusters- 39, 42- Isolates of *V.alginolyticus*; Clusters- 28, 32 - Isolates of *V.nereis*; Clusters- 31- Isolates of *V.fluvialis*; Clusters-25, 41 - Isolates of *V. cholerae*; Clusters- 29, 33- Isolates of *V.vulnificus*; Clusters-20, 40 - Isolates of *V. splendidus*
**Fig: 3.13 Amplicons obtained using the primer OPD-16**

A- V1 to V9, 1Kb Marker, V10-V19  
B- V20- V23, 100bp Marker, V24 to V30  
C- V31 to V38 100bp Marker, V39 to V41  
D- 1Kb Marker, 100bp Marker, V42 to V65  
E- V66 to V89, 100bp Marker  
F- V90 to V100, 1Kb Marker  
G- V101 to V111, 1Kb Marker  
H- V121 to V130, 1Kb Marker, V131 to V139  
I- V140 to V145, 100bp Marker, V147 to V158, 100bp Marker
Fig: 3.14 Dendrogram of the *Vibrio* spp. based on RAPD profile using the OPD-16

Clusters- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 27, 28, 35- Isolates of *V. harveyi*; Clusters- 31 - Isolates of *V. parahaemolyticus*; Clusters- 18, 30, 35, 36 - Isolates of *V. mediterranei*; Clusters- 34- Isolates of *V. proteolyticus*; Clusters- 40, 42, 45, 46- Isolates of *V. alginolyticus*; Clusters- 30, 39- Isolates of *V. nereis*; Clusters- 29- Isolates of *V. fluvialis*; Clusters- 44- Isolates of *V. cholerae*; Clusters- 30, 32, 33, 37, 38- Isolates of *V. vulnificus*; Clusters- 20, 26 - Isolates of *V. splendidus*
Fig: 3.15 Amplicons obtained using the primer OPD-20

A- V1 to V9, 1Kb Marker, V10-V18
B- V19- V27, 1Kb Marker, V28 to V36
C- V37 to V40, 1Kb Marker, V41 to V49
D- V50 to V58, 1Kb Marker, V59 to V68
E- V69 to V77, 1Kb Marker, V78 to V87
F- V88 to V96, 1Kb Marker, V96 to V106
G- V107 to V115, 1Kb Marker, V116 to V124
H- 100bp Marker, V125 to V132
I- 1Kb Marker, V133 to V142, 100bp Marker
J- V143 to V158, 1Kb Marker
Fig: 3.16 Dendrogram of the *Vibrio* spp. based on RAPD profile using the OPD-20

Clusters- 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 27, 28, 35- Isolates of *V. harveyi*; Clusters- 31- Isolates of *V. parahaemolyticus*; Clusters- 18, 35 - Isolates of *V. mediterranei*; Clusters- 34- Isolates of *V. proteolyticus*; Clusters- 32, 33, 36, 37, 38- Isolates of *V. alginolyticus*; Clusters- 30, 35, 39- Isolates of *V. nereis*; Clusters- 29- Isolates of *V. fluvialis*; Clusters- 44- Isolates of *V. cholerae*; Clusters- 26, 29- Isolates of *V. vulnificus*; Clusters-26, 32- Isolates of *V. splendidus*
Table 3.3: Cluster analysis based on phenotypic characterization and RAPD profiling, correlated with the source where from the strains were isolated

<table>
<thead>
<tr>
<th>Phenon</th>
<th>OPA-3</th>
<th>OPA-4</th>
<th>OPA-5</th>
<th>OPA-7</th>
<th>OPAC-10</th>
<th>OPD-16</th>
<th>OPD-20</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 V. harveyi</td>
<td>1,2,4,6,10,11,13,14,15,16,17</td>
<td>1,2,4,6,7,8,9,10,11,17,22,23,25</td>
<td>1,2,3,4,5,6,7,9,10,1,12,13,13,16,17,18,19,22,23,24,26,34,35,38,39,40,41,42,43,44,45,46,47,48</td>
<td>12,13,14,15,21,22,23,24,25,27,33,36,37,38,39,40,41,42,43,44,45,46,47,48</td>
<td>1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,26,34,35,38,39,40,41,42,43,44,45,46,47,48</td>
<td>1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,26,34,35,38,39,40,41,42,43,44,45,46,47,48</td>
<td>1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,26,34,35,38,39,40,41,42,43,44,45,46,47,48</td>
<td>Mass Mortality &amp; morbidity, PL, Boca Maya, nauplii from Kakinada, Andhra Pradesh</td>
</tr>
<tr>
<td>P2 V. harveyi</td>
<td>1,10,12,13</td>
<td>11,23</td>
<td>3,5,7</td>
<td>22</td>
<td>2,8,11</td>
<td>5,7,15</td>
<td>7,15</td>
<td>Mass Mortality &amp; morbidity, PL, Boca Maya, Andhra Pradesh</td>
</tr>
<tr>
<td>P3 V. harveyi</td>
<td>6,7,8,18</td>
<td>25</td>
<td>7,11,22</td>
<td>25,26,30</td>
<td>23,26,40</td>
<td>11,14,28</td>
<td>13,14,23,25,28</td>
<td>PL, mass mortality, Kodungallur, Kerala &amp; LMG 4044- dead amphipod (Talorchestia sp.), Woods Hole, Massachusetts, United States</td>
</tr>
<tr>
<td>P4 V. parahaemolytica</td>
<td>19, 20</td>
<td>21</td>
<td>14</td>
<td>.51</td>
<td>37</td>
<td>31</td>
<td>31</td>
<td>PL, mass mortality, Ashikode, Kerala &amp; LMG 2850- patient suffering from &quot;Shirasuki&quot; food poisoning, Japan</td>
</tr>
<tr>
<td>P5 V. mediterranei</td>
<td>21,22,23,24</td>
<td>5,18</td>
<td>20</td>
<td>29,32</td>
<td>11,12,13,38</td>
<td>18,36</td>
<td>18,35</td>
<td>Post Larvae, mass mortality, Ashikode, Kerala &amp; LMG 11258- Coastal</td>
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<td>marine plankton, Valencia, Spain</td>
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<tr>
<td>P6</td>
<td>V.\textit{mediterranei}</td>
<td>3,27</td>
<td>18,24,19</td>
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<td>6</td>
<td>14</td>
<td>30,35</td>
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<td>P7</td>
<td>V.\textit{proteolyticus}</td>
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<td>P8</td>
<td>V.\textit{proteolyticus}</td>
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<td>26</td>
<td>24</td>
<td>35</td>
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<tr>
<td>P9</td>
<td>V.\textit{alginogeticus}</td>
<td>6, 9</td>
<td>14</td>
<td>23,43,46</td>
<td>1,26,34</td>
<td>39,42</td>
<td>40,41,42,43</td>
<td>32,33,40, 41,42,43</td>
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<tr>
<td>P10</td>
<td>V.\textit{nereis}</td>
<td>24,25, 28</td>
<td>15,16</td>
<td>40</td>
<td>9,18,33</td>
<td>32</td>
<td>30</td>
<td>30,35</td>
</tr>
<tr>
<td>P11</td>
<td>V.\textit{nereis}</td>
<td>9</td>
<td>16</td>
<td>40</td>
<td>20</td>
<td>28</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>P12</td>
<td>V.\textit{floridensis}</td>
<td>8,18</td>
<td>14</td>
<td>27</td>
<td>11,16</td>
<td>31</td>
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</tbody>
</table>
Comparisons of the clusters of the isolates obtained based on both phenotypic and genotypic characters showed that there exists a further divergence of the isolates formerly grouped together as a single cluster based on the phenotypic characters alone.

<table>
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<tr>
<th></th>
<th>Clusters</th>
<th>26,5</th>
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<th>7,8,10,28</th>
<th>25,41</th>
<th>44</th>
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<tr>
<td>P13</td>
<td><em>V. cholerae</em></td>
<td>26,5</td>
<td>13</td>
<td>28,36,37,38</td>
<td>7,8,10,28</td>
<td>25,41</td>
<td>44</td>
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<tr>
<td>P14</td>
<td><em>V. vulnificus</em></td>
<td>3,8,19</td>
<td>3,17,20</td>
<td>3,8,9</td>
<td>2,3,4</td>
<td>29,33</td>
<td>30,32,33,37,38</td>
<td>30,33,36,37,38</td>
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<tr>
<td></td>
<td></td>
<td>3,8,19</td>
<td>3,17,20</td>
<td>3,8,9</td>
<td>2,3,4</td>
<td>29,33</td>
<td>30,32,33,37,38</td>
<td>30,33,36,37,38</td>
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<tr>
<td>P15</td>
<td><em>V. vulnificus</em></td>
<td>19</td>
<td>17</td>
<td>39</td>
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<td>P16</td>
<td><em>V. splendidus</em></td>
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<td>12</td>
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<td>41</td>
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<td>20</td>
<td>25,32</td>
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</table>
Fig-3.17 Construction of RAPD profile based on the population clusters obtained on phenotypic characterization of the 17 phena with all 7 Operon primers
Chapter 3

3.3.2. Banding pattern analysis of housekeeping genes

Thirty five representative isolates when amplified with 8 housekeeping genes and processed in NTSYSpc software yielded 8 dendrograms. The clusters obtained with each primer and the similarities between interrelated clusters are described below.

\textit{ftsZ}:

Amplification of the 35 isolates with \textit{ftsZ} gene primer yielded 10 clusters $\geq 92\%$S. Cluster-1 consisted of the lone isolate of \textit{V.harveyi} (V3), exhibited $\geq 92\%$S to 8 isolates of \textit{V.harveyi} grouped together as cluster-2 at 100\%S. Cluster-2 also exhibited $\geq 92\%$S to cluster-3 which contained one isolate of \textit{V.harveyi} (V11), one isolate of \textit{V.vulnificus} (V34) and the type strain of one isolate of \textit{V.harveyi}. Cluster-3 exhibited $\geq 92\%$S to cluster-4 containing isolates of \textit{V.alginolyticus} and \textit{V.proteolyticus} sharing 100\%S. Cluster-4 exhibited $\geq 92\%$S to cluster-5 containing isolate and type strain of \textit{V.cholerae}. Cluster-5 exhibited $\geq 92\%$S to cluster-6 containing type strains of \textit{V.fluvialis} and one isolate each of \textit{V.nereis} and \textit{V.parahaemolyticus}, sharing 100\%S. Cluster-6 exhibited $\geq 92\%$S to cluster-7 containing a lone type strain of \textit{V.nereis}. Cluster-8 at 100\%S consisted of isolates of \textit{V.splendidus}, exhibited $\geq 92\%$S to cluster-7. Cluster-8 which exhibited $\geq 92\%$S to cluster-9 contained isolates of \textit{V.fluvialis}, \textit{V.mediterranei}, \textit{V.parahaemolyticus}, \textit{V.nereis} and \textit{V.vulnificus} sharing 100\%S between them. Cluster-10 containing lone isolate of \textit{V.harveyi}, which remained as an outgroup, joining with the other 34 isolates $\geq 64\%$S.

\textit{gapA}:

Amplification of the 35 isolates with \textit{gapA} gene primer yielded 7 clusters $\geq 93\%$S. Cluster-1 containing the isolates of \textit{V.harveyi} joined at 93\%S to cluster-2 containing isolates of \textit{V.harveyi} and one isolate of \textit{V.alginolyticus} (V13). Cluster-2 exhibited 93\%S to the lone isolate (type strain of \textit{V.harveyi}) in cluster-3. Cluster-3 exhibited 93\%S to cluster-4
Chapter 3

containing type strain of *V.alginolyticus* (V14). Cluster-4 exhibited 93%S to cluster-5 containing type strain of *V.alginolyticus* (V15), isolates of *V.cholerae, V.fluvialis, V.mediterranei, V.proteolyticus* and *V.splendidus*, related at 100%S. Cluster-5 exhibited 93%S to cluster-6 containing type strains of *V.cholerae* and *V.mediterranei* sharing 100%S to isolates of *V.nereis* and *V.parahaemolyticus*. Cluster-6 exhibited 93%S to cluster-7 containing type strain of *V.parahaemolyticus* which shared 100%S with the type strain of *V.vulnificus*.

topA:

Amplification of the 35 isolates with *topA* gene primer yielded 10 clusters at ≥ 91%S. The isolates of *V.harveyi* in clusters1 & 2 were related at 95%S to each other and to cluster-3 containing isolates of *V.alginolyticus, V.cholerae, V.fluvialis, V.mediterranei, V.nereis, V.parahaemolyticus* and *V.proteolyticus*. Cluster-3 was related to cluster-4 at 95% S, where as cluster-4 with the isolate of *V.splendidus* shared 95%S to cluster-5 *V.splendidus*. Cluster-5 exhibited 95%S to isolates of *V.harveyi* in cluster-6 containing one isolate each of *V.harveyi, V.fluvialis, V.mediterranei, isolates of V.cholerae and V.vulnificus* and type strains of *V.alginolyticus* and *V.parahaemolyticus*. Cluster-6 showed 95%S to cluster-7 containing isolate of *V.harveyi*, which in turn exhibited 95%S to lone isolate of *V.harveyi* in cluster-8. Clusters-7&8 joined at 92%S to isolates in clusters-1 to 6. Clusters- 9 &10 contained isolates of *V.harveyi* interrelated at 95%S, these two clusters were related with the isolates in other clusters at ≥91%S.

recA:

All the 35 isolates showed wide range of heterogeneity, but are interrelated ≥90%S. Cluster1 consisted of the isolate of *V.harveyi* (V3) exhibiting >96%S to type strain of *V.nereis* in Cluster-2. Cluster-3&4 grouped with it the remaining isolates of *V.harveyi* at >96%S. Cluster-5 contained the isolates of *V.mediterranei, V.nereis, V.vulnificus* and type strain of *V.splendidus*, sharing 100%S, also exhibiting >96%S to a lone
isolate of *V. vulnificus* in cluster-6. Isolates of *V. nereis* in cluster-7 exhibited 100%S to isolate of *V. proteolyticus* and >93%S to type strain of *V. parahaemolyticus* (Cluster-8). Cluster-8 >93%S joins to isolates in cluster-9 which included strains of *V. alginolyticus, V. cholerae, V. fluvialis, V. splendidus* and type strain of *V. proteolyticus*. Cluster-10 & 11 are formed up of the isolate of *V. alginolyticus* showing >96%S and joined with the above six clusters at >91%S. The lone isolate of *V. parahaemolyticus* (V27) in cluster-12 joined with the above seven clusters at 90%S.

**merB:**

Thirty five isolates amplified exhibited ≥90%S and were represented as 7 clusters. Cluster-1 consisted of one isolate of *V. harveyi* (V1) which exhibits 98%S to other isolates of *V. harveyi* in cluster-2 sharing 100%S between them. Clusters 1& 2 joined with the other clusters ≥93%S. Cluster-3 was consisted of the type strain and six isolates of *V. harveyi* joined with cluster-4 which contained isolates of *V. alginolyticus, V. cholerae, V. fluvialis, V. mediterranei, V. nereis, V. parahaemolyticus* and *V. proteolyticus* at 94%S. Cluster-4 joined with cluster-5 at 95%S containing isolates of *V. splendidus* and *V. vulnificus*. Cluster-5 joined with cluster-6 at 95%S, in which is grouped the isolate of *V. alginolyticus*, which shared 100%S to the type strains of *V. proteolyticus* and *V. vulnificus*. Cluster-7 contained the type strain of *V. parahaemolyticus* (V28), joining with the isolates in the above six clusters ≥90%S.

**gyrB:**

35 isolates amplified were grouped into 4 clusters, which were inter-related ≥93%S. Cluster-1 at 100%S contained isolates of *V. harveyi, V. splendidus, V. vulnificus* and type strain of *V. nereis*, joining with cluster-2 at 93%S. Cluster-2 contained one isolate of *V. harveyi* (V2) which shares 100%S to type strains of *V. parahaemolyticus* and *V. proteolyticus*. At 93%S cluster-3 containing isolates of *V. alginolyticus, V. cholerae, V. fluvialis, V. mediterranei* and *V. nereis* joined with cluster-2. Cluster-3 exhibited
93% S to cluster-4 containing isolates and type strain of \textit{V.} \textit{harveyi}, isolate of \textit{V.} \textit{alginolyticus} and \textit{V.} \textit{nereis} and type strain of \textit{V.} \textit{vulnificus}.

**pyrH:**

Thirty five isolates were grouped into 4 main clusters inter-related at $\geq 93\%$ S. Cluster-1 at 100\% S contained isolates and type strain of \textit{V.} \textit{harveyi}, type strain of \textit{V.} \textit{mediterranei} and isolates of \textit{V.} \textit{alginolyticus} and \textit{V.} \textit{nereis}. This cluster joined with cluster-2 at 93\% S, which contained isolates of \textit{V.} \textit{harveyi}, type strain of \textit{V.} \textit{fluvialis}, isolates of \textit{V.} \textit{mediterranei} and \textit{V.} \textit{vulnificus} related at 100\% S. Cluster-2 joined with cluster-3 at 93\% S, containing isolates of \textit{V.} \textit{alginolyticus} and \textit{V.} \textit{cholerae}. Cluster-3 also exhibited 93\% S to cluster-4 containing isolates of \textit{V.} \textit{parahaemolyticus}, \textit{V.} \textit{proteolyticus}, \textit{V.} \textit{splendidus}, \textit{V.} \textit{vulnificus} and type strain of \textit{V.} \textit{nereis}.

**16S rRNA:**

Thirty five isolates were grouped into 9 clusters inter-related at $\geq 95\%$ S. Cluster-1 has a lone isolate of \textit{V.} \textit{harveyi} exhibiting 95\% S to cluster-2 having four isolates of \textit{V.} \textit{harveyi} sharing 100\% S. Cluster-2 showed 95\% S to cluster-3 having two isolates of \textit{V.} \textit{harveyi}, which exhibited 95\% S to three isolates of \textit{V.} \textit{harveyi} in cluster-4. Cluster-4 exhibits 95\% S to cluster-5, containing one isolate and type strain of \textit{V.} \textit{harveyi}. Cluster-5 at 95\% S showed similarity to cluster-6 containing type strain of isolates of \textit{V.} \textit{alginolyticus} and \textit{V.} \textit{fluvialis}, which exhibited 95\% S to cluster-7. Cluster-7 at 95\% S was related to cluster-8 which had the isolates of \textit{V.} \textit{mediterranei}, \textit{V.} \textit{vulnificus} and \textit{V.} \textit{nereis}. This cluster joined with cluster-9 at 95\% S, which contained isolates of \textit{V.} \textit{nereis} \textit{V.} \textit{parahaemolyticus}, \textit{V.} \textit{proteolyticus}, \textit{V.} \textit{splendidus} and \textit{V.} \textit{vulnificus}, inter-related at 100\% S.

The 16SrRNA sequences of the 25 selected vibrio isolates from the clusters obtained on phenotypic characterization were compared with the GenBank database using the BLAST algorithm. The wild isolates of vibrios which were phenotypically characterized and clustered exhibited 95 to 100\% similarity (Table 3.5) to \textit{vibrio} strains deposited in the GenBank database and were assigned with accession numbers (Appendix-1). On
comparing the results of phenotypic and genotypic characterisation, all the representative isolated identified through numerical taxonomy could be confirmed of their identity based on 16S rRNA sequence analysis. However, isolates of *V. harveyi* also shared 95%S to 100%S to NCBI deposits of *V. rotiferanus* (LMG21460), and *V. natriegens* (ATCC 14048). Similarly the isolate of *V. parahaemolyticus* (MCCB 133) showed 95%S to NCBI deposits of *V. parahaemolyticus* (ATCC 17802) and 96%S to *V. natriegens* (ATCC 14048). The isolate of *V. alginolyticus* (MCCB 112) which was deposited with Genbank as *Vibrio* sp., shared 96%S with NCBI deposits of *V. natriegens* (ATCC 14048) and 97%S to *V. alginolyticus* NCBI deposits (ATCC 17749).

![Fig: 3.18 Amplicons from 35 isolates of vibrios using *fts*Z gene primer](image1)

![Fig: 3.19 Amplicons from 35 isolates of vibrios using *gapA* gene primer](image2)
Fig: 3.20 Amplicons from 35 isolates of vibrios using *topA* gene primer

Fig: 3.21 Amplicons from 35 isolates of vibrios using *recA* gene primer

Fig: 3.22 Amplicons from 35 isolates of vibrios using *merB* gene primer
Chapter 3

Fig: 3.23 Amplicons from 35 isolates of vibrios using \textit{gyrB} gene primer

Fig: 3.24 Amplicons from 35 isolates of vibrios using \textit{pyrH} gene primer

Fig: 3.25 Amplicons from 35 isolates of vibrios using 16SrRNA gene primer

Lane1-1kb Marker, Lane2-Vh3, Lane3-Vh28, Lane4-Vh36, Lane5-Vh45, Lane6-Vh54, Lane7-Vh57, Lane8-Vh64, Lane9-Vh71, Lane10-Vh76, Lane11-Vh81, Lane12-Vh88, Lane13-VhL (LMG 4044), Lane14-Va3, Lane15-VaL (LMG 4409), Lane16-VaM (MTCC 4439), Lane17-Vc12, Lane18-Vc35, Lane19-VcM (MTCC 3906), Lane20-Vf26, Lane21-VfL (LMG 11654), Lane22-Vm18, Lane23-Vm26, Lane24-VmL (LMG 11258), Lane25-Vn30, Lane26-Vn32, Lane27-VnL (LMG 3895), Lane28-Vpa6, Lane29-VpaL (LMG 2850), Lane30-Vpr4, Lane31-VprL (LMG 3772), Lane32-Vsp3, Lane33-VspL (LMG 19031), Lane34-Vv9, Lane35-VvL (LMG 13545)

148
Fig: 3.26 Relatedness of the isolates based on amplification with \textit{ftsZ} gene primer

Isolates V1 to V12 - (\textit{V.harveyi} - Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15 - (\textit{V.alginolyticus} - Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18 - (\textit{V.cholerae} - Vc12, Vc35, VcM (MTCC 3906)),
V19 to V20 - (\textit{V.fluvialis} - Vf26, VfL(LMG 11654)),
V21 to V23 - (\textit{V.mediterranei} - Vm18, Vm26, VmL(LMG 11258)),
V24 to V26 - (\textit{V.nereis} - Vn30, Vn32, VnL(LMG 3895)),
V27 to V28 - (\textit{V.parahaemolyticus} - Vpa6, VpaL(LMG 2850)),
V29 to V30 - (\textit{V.proteolyticus} - Vpr4, VprL(LMG 3772)),
V31 to V32 - (\textit{V.splendidus} - Vsp3, VspL (LMG 19031)),
V33 to V35 - (\textit{V.vulnificus} - Vv9, Vv23, VvL (LMG 13545))
Fig: 3.27 Relatedness of the isolates based on amplification with \textit{gapA} gene primer

Isolates V1 to V12 - (\textit{V.harveyi} - Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15 - (\textit{V.alginolyticus} - Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18 - (\textit{V.cholerae} - Vc12, Vc35, VcM (MTCC 3906)),
V19 to V20 - (\textit{V.fluvialis} - Vf26, VfL(LMG 11654)),
V21 to V23 - (\textit{V.mediterranei} - Vm18, Vm26, VmL(LMG 11258)),
V24 to V26 - (\textit{V.nereis} - Vn30, Vn32, VnL(LMG 3895)),
V27 to V28 - (\textit{V.parahaemolyticus} - Vpa6, VpaL(LMG 2850)),
V29 to V30 - (\textit{V.proteolyticus} - Vpr4, VprL(LMG 3772)),
V31 to V32 - (\textit{V.splendidus} - Vsp3, VspL (LMG 19031)),
V33 to V35 - (\textit{V.vulnificus} - Vv9, Vv23, VvL (LMG 13545))
Fig: 3.28 Relatedness of the isolates based on amplification with *topA* gene primer

Isolates V1 to V12 - (*V. harveyi* - Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15 - (*V. alginolyticus* - Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18 - (*V. cholerae* - Vc12, Vc35, VcM (MTCC 3906)),
V19 to V20 - (*V. fluvialis* - Vf26, VfL(LMG 11654))
V21 to V23 - (*V. mediterranei* - Vm18, Vm26, VmL(LMG 11258)),
V24 to V26 - (*V. nereis* - Vn30, Vn32, VnL(LMG 3895))
V27 to V28 - (*V. parahaemolyticus* - Vpa6, VpaL(LMG 2850)),
V29 to V30 - (*V. proteolyticus* - Vpr4, VprL(LMG 3772)),
V31 to V32 - (*V. splendidus* - Vsp3, VspL (LMG 19031)),
V33 to V35 - (*V. vulnificus* - Vv9, Vv23, VvL (LMG 13545))
Fig: 3.29 Relatedness of the isolates based on amplification with *recA* gene primer

Isolates V1 to V12 - (*V. harveyi* - Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),

V13 to V15 - (*V. alginolyticus* - Va3, VaL (LMG 4409), VaM (MTCC 4439)),

V16 to V18 - (*V. cholerae* - Vc12, Vc35, VcM (MTCC 3906)),

V19 to V20 - (*V. fluvialis* - Vf26, VfL (LMG 11654)),

V21 to V23 - (*V. mediterranei* - Vm18, Vm26, VmL (LMG 11258)),

V24 to V26 - (*V. nereis* - Vn30, Vn32, VnL (LMG 3895)),

V27 to V28 - (*V. parahaemolyticus* - Vpa6, VpaL (LMG 2850)),

V29 to V30 - (*V. proteolyticus* - Vpr4, VprL (LMG 3772)),

V31 to V32 - (*V. splendidus* - Vsp3, VspL (LMG 19031)),

V33 to V35 - (*V. vulnificus* - Vv9, Vv23, VvL (LMG 13545))
Isolates V1 to V12 - (V.harveyi- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (V.alginolyticus- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (V.cholerae- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (V.fluvialis- Vf26, VfL(LMG 11654))
V21 to V23- (V.mediterranei- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (V.nereis-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (V.parahaemolyticus- Vpa6, VpaL(LMG 2850)),
V29 to V30- (V.proteolyticus-Vpr4, VprL(LMG 3772)),
V31 to V32- (V.splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (V.vulnificus-Vv9, Vv23, VvL (LMG 13545))
Chapter 3

Fig: 3.31 Relatedness of the isolates based on amplification with gyrB gene primer

Isolates V1 to V12 - (V.harveyi- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (V.alginolyticus- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (V.cholerae- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (V.fluvialis- Vf26, VfL(LMG 11654))
V21 to V23- (V.mediterranei- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (V.nereis-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (V.parahaemolyticus- Vpa6, VpaL(LMG 2850)),
V29 to V30- (V.proteolyticus-Vpr4, VprL(LMG 3772)),
V31 to V32- (V.splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (V.vulnificus-Vv9, Vv23, VvL (LMG 13545))
Fig: 3.32 Relatedness of the isolates based on amplification with \textit{pyrH} gene primer

Isolates V1 to V12 - (\textit{V.harveyi} - Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),

V13 to V15 - (\textit{V.alginolyticus} - Va3, VaL (LMG 4409), VaM (MTCC 4439)),

V16 to V18 - (\textit{V.cholerae} - Vc12, Vc35, VcM (MTCC 3906)),

V19 to V20 - (\textit{V.fluvialis} - Vf26, VfL (LMG 11654))

V21 to V23 - (\textit{V.mediterranei} - Vm18, Vm26, VmL (LMG 11258)),

V24 to V26 - (\textit{V.nereis} - Vn30, Vn32, VnL (LMG 3895))

V27 to V28 - (\textit{V.parahaemolyticus} - Vpa6, VpaL (LMG 2850)),

V29 to V30 - (\textit{V.proteolyticus} - Vpr4, VprL (LMG 3772)),

V31 to V32 - (\textit{V.splendidus} - Vsp3, VspL (LMG 19031)),

V33 to V35 - (\textit{V.vulnificus} - Vv9, Vv23, VvL (LMG 13545))
Fig: 3.33 Relatedness of the isolates based on amplification with 16S gene primer

Isolates V1 to V12 - (V.harveyi- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (V.alginolyticus- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (V.cholerae- Vc12, Vc35, VcM (MTCC 3906)),
V19 to V20- (V.fluvialis- Vf26, VfL(LMG 11654))
V21 to V23- (V.mediterranei- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (V.nereis-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (V.parahaemolyticus- Vpa6, VpaL(LMG 2850)),
V29 to V30- (V.proteolyticus-Vpr4, VprL(LMG 3772)),
V31 to V32- (V.splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (V.vulnificus-Vv9, Vv23, VvL (LMG 13545))
Analysis of the isolates based on amplification of all 8 housekeeping genes:

The dendrogram obtained with the combination of all 8 housekeeping genes showed that the representative isolates were grouped into three core groups. Core group 1 was with isolates of *V. harveyi* belonging to Phena 1 & 2 correlated at 89%S (Fig- 3.34). These 2 phena showed 82.2%S to isolates of *V. parahaemolyticus* (phenon 3) and 79%S to phenon 17 having the isolate and type strain of *V. harveyi*. The second core group consisted of the isolates of *V. mediterranei* (phenon 4), joining to the type strain of *V. nereis* at 92.9%S. This cluster showed 92.3%S to isolates of *V. cholerae* (phenon 6), 91.2%S to isolates of *V. nereis* (phenon 7), 90.8%S to isolates of *V. mediterranei* (phenon 8) and to *V. fluvialis* (phenon 9) grouped at 94.6%S. The third core group consisted of the isolates of *V. proteolyticus* (phenon 10) joined with the isolates of *V. alginolyticus* (phenon 11) at 98.2%S and to the type strain of *V. proteolyticus* (phenon 12) at 94.7%S. This cluster joined with the isolate and type strain of *V. splendidus* (phenon 13 & 14) at 88.5%S. 100%S existed between the isolate and type strain of *V. vulnificus*; this group showed 83.1%S to core groups 2 & 3. At 80.36%S third core group exhibited similarity to the first core group. Core group 2 was related with third core group at 84.5%S.
Table 3.4 Molecular weight of the amplicons given by 35 isolates of vibrios with the 8 housekeeping gene primers

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Code</th>
<th>ftsZ</th>
<th>gapA</th>
<th>topA</th>
<th>recA</th>
<th>merB</th>
<th>gyrB</th>
<th>pyrH</th>
<th>16SrRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vh3 V1</td>
<td>700</td>
<td>700</td>
<td>1070.76</td>
<td>1037.14</td>
<td>1322.49, 1124.82, 961.26</td>
<td>883.84</td>
<td>526.74</td>
<td>1635.17</td>
<td></td>
</tr>
<tr>
<td>Vh28 V2</td>
<td>669.84</td>
<td>709.41</td>
<td>1058.63</td>
<td>974</td>
<td>415.16</td>
<td>837.07</td>
<td>545.35</td>
<td>1713.66</td>
<td></td>
</tr>
<tr>
<td>Vh36 V3</td>
<td>660.08</td>
<td>821.22</td>
<td>694.88</td>
<td>961.26</td>
<td>422.95</td>
<td>883.84</td>
<td>550.11</td>
<td>1719.2</td>
<td></td>
</tr>
<tr>
<td>Vh45 V4</td>
<td>674.77</td>
<td>700</td>
<td>700</td>
<td>974</td>
<td>434.91</td>
<td>915.94</td>
<td>554.9</td>
<td>1795.91</td>
<td></td>
</tr>
<tr>
<td>Vh54 V5</td>
<td>674.77</td>
<td>821.22</td>
<td>1095.44, 527.75</td>
<td>980.44</td>
<td>443.07</td>
<td>932.17</td>
<td>569.74</td>
<td>1762.55</td>
<td></td>
</tr>
<tr>
<td>Vh57 V6</td>
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<td>837.5</td>
<td>987.68</td>
<td>980.44</td>
<td>451.39</td>
<td>932.17</td>
<td>554.9</td>
<td>1729.8</td>
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</tr>
<tr>
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<td>837.5</td>
<td>987.68</td>
<td>980.44</td>
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<td>569.74</td>
<td>1705.91</td>
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</tr>
<tr>
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<td>700</td>
<td>987.68</td>
<td>974</td>
<td>455.61</td>
<td>932.17</td>
<td>550.11</td>
<td>1829.91</td>
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<td>Vh76 V9</td>
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<td>700</td>
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<td>924.02</td>
<td>545.35</td>
<td>1812.83</td>
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<td>981.58</td>
<td>974, 800</td>
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<td>948.88</td>
<td>535.96</td>
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<td>709.41</td>
<td>969.49, 560.8</td>
<td>974</td>
<td>464.16</td>
<td>915.94</td>
<td>531.33</td>
<td>1882.11</td>
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<td>VhL V12</td>
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<td>1000</td>
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<td>711.08</td>
<td>573.77</td>
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<td>508.76</td>
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<td>953.24</td>
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<td>983.92</td>
<td>491.49</td>
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<td>962.41</td>
<td>722.34</td>
<td>600</td>
<td>1082.55</td>
<td>983.92</td>
<td>462.83</td>
<td>1927.45</td>
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<tr>
<td>Vc35 V17</td>
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<td>967.03</td>
<td>745.4</td>
<td>600</td>
<td>1091.11</td>
<td>983.92</td>
<td>443.39</td>
<td>1927.45</td>
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<tr>
<td>VcM V18</td>
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<td>1009.16</td>
<td>757.2</td>
<td>628.4</td>
<td>1073.95</td>
<td>983.92</td>
<td>421.14</td>
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<tr>
<td>Vf26 V19</td>
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<td>967.03</td>
<td>728.04</td>
<td>623.57</td>
<td>1082.55</td>
<td>975.98</td>
<td>406.93</td>
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<td>VfL V20</td>
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<td>769.19</td>
<td>648.07</td>
<td>1073.95</td>
<td>975.98</td>
<td>552.74</td>
<td>1935.81</td>
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<td>Vm18 V21</td>
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<td>981.03</td>
<td>751.28</td>
<td>638.16</td>
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<td>960.29</td>
<td>552.74</td>
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<td>990.47</td>
<td>745.4</td>
<td>668.37</td>
<td>1073.95</td>
<td>968.1</td>
<td>568.06</td>
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<tr>
<td>VmL V23</td>
<td>939.64</td>
<td>1018.4</td>
<td>769.19</td>
<td>684</td>
<td>1065.47</td>
<td>960.29</td>
<td>530.04</td>
<td>1517</td>
<td></td>
</tr>
<tr>
<td>Vn30 V24</td>
<td>921.81</td>
<td>1000</td>
<td>769.19</td>
<td>948.68, 689.29</td>
<td>1091.11</td>
<td>968.1</td>
<td>533.92</td>
<td>1517</td>
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Fig: 3.34 Analysis of Housekeeping genes using Popgene of the 17 Phena obtained
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3.4. Discussion:

The current taxonomy of vibrios is based mainly on genomic data, as this approach establishes highly informative measure of intra and interspecific genomic relatedness between strains; enabling reproducible and stable classification frame. Application of various techniques, including RAPD profiling, House Keeping gene profiling and 16S rRNA gene sequence analysis indicated the occurrence of several species within the family Vibrionaceae.

RAPD-PCR, using arbitrary primers to detect polymorphism has been used in discrimination of microbes both at inter and intrapecies level. RAPD-PCR and pathogenicity testing of *P. monodon* revealed that non-luminescent, sucrose fermenting biotypes of *V. harveyi* could be important aetiological agents of vibriosis (Alavandi *et al*., 2006). Bramha Chari *et al.* (2006) demonstrated the rapid detection of marine luminous and non-luminous *V. harveyi* isolates for molecular epidemiology. Main drawback of using RAPD fingerprinting for subtyping of microbial populations is the reproducibility of the same banding pattern. However, in this study of the 20 selected Operon primers, 7 primers exhibited distinct and reproducible banding pattern ranging from 100-4500 base pair. Similar results were observed by Somarny *et al.* (2002) with RAPD-PCR of 25 isolates of two different *Vibrio* species (*V. cholerae* and *V. harveyi*) with 20 different primers and observed that 14 oligonucleotide primers yielded clear and reproducible bands corresponding to amplified products ranging in size from 250 – 6,000 nucleotide base pairs. Somarny *et al.* (2002) suggested that *V. harveyi* isolates could be grouped into one cluster, whereas *V. cholerae* isolates were grouped into another clusters on the analysis of dendrogram produced from RAPD fingerprint analysis. However, in the present study, the isolates of *V. harveyi* which initially were grouped into three distinct phena based on phenotypic characterization, exhibited further genetic diversification into many clusters upon amplification with each of the 7 selected primers. Maiti *et al.*, (2009) showed that diversity existed among *V. harveyi* isolates
Chapter 3

based on the analysis of RAPD profiles obtained with primers CRA25 and PM3 individually. Cluster analysis carried out by Maiti et al. (2009) based on combined similarity matrix grouped all strains into 15 clusters, indicating a genetically heterogeneous group of *V. harveyi* to be prevalent along the Indian coast. Similar observations were obtained with the selected 7 Operon primers and the results were in agreement with previous studies reporting the presence of a large number of heterogenic genotypes within *V. harveyi* (Hernandez and Olmos, 2004; Alavandi et al., 2006).

The study conducted by Somary et al. (2002) showed that one amplicon of size 800 bp was shared by almost all *V. harveyi* isolates and with PM-3, two bands of sizes 700 bp and 850 bp were common to nearly all strains. Pujalte et al. (2003) reported that most of the *V. harveyi* isolates in their study amplified a common band of 800bp when subjected to RAPD-PCR using Primer M13. Similar results were obtained from the present study, where 600, 400 and 200bp bands were found to be shared by most of the *Vibrio* isolates which were subjected to fingerprinting with 7 selected primers. The sharing of common bands indicated the presence of a highly conserved genomic region in diverse *Vibrio* strains. This assumes significance as amplification of common fragments by RAPD-PCR with a particular primer has been shown to be useful in genetic amplifications and hybridization assays for diagnostic purpose (Dalla et al., 2002). Further, these highly conserved fragments could be ideal for identifying strains that are atypical or which may be difficult to identify by phenotypic tests.

To determine whether correlation existed between the RAPD type and source of the isolates, the data with all the 7 primers corresponding to the loci obtained were processed using PopGene software. Interestingly, vibrios isolated from post larvae with necrosis from Trichur, Kerala, identified as *V.alginolyticus* and *V.cholerae* on the basis of phenotypic characterization were grouped together exhibiting 80.6%S., hence clustered together into the same core group (*V.cholerae* core group). Also the isolates
obtained from Azhikode, Kerala, during mass mortality of post larvae were closely related, especially the isolates of *V. parahaemolyticus*, *V. splendidus* and *V. proteolyticus*, suggesting that there existed a relation between the RAPD pattern and source of isolation. Bramhachari *et al.* (2006) analysed several samples isolated from the same location and found that the isolates shared similar RAPD pattern, but no correlation was obtained between a given RAPD type and the geographical location or the source of the isolates. In this study isolates belonging to *V. mediterraneii* were grouped into the cluster of *V. harveyi* and isolates of *V. fluvialis* into *V. vulnificus*. Though the isolates were obtained from different sources, they exhibited high relatedness, above 91%, 86% and 84.5% S respectively, suggesting that the isolates had some genes in common that remained conserved. These results suggested that the isolates analysed had unique bands representing in the fingerprinting pattern which could be used in the recognition of genera and species. This technique is simple and rapid and could also be useful in molecular epidemiology for tracing the route of infection and for implementing suitable control measures for the pathogen (Maiti *et al.*, 2009). Further studies are required to clearly establish an association between particular RAPD pattern to virulence and disease, which could have important implication in the discrimination of pathogenic strains from the non-pathogenic forms.

Examination of various genomic loci is more stable in species discrimination rather than the analysis of the 16S rRNA which screens only 5 to 10% of the total bacterial genomic content. Garg *et al.* (2003) analysed sequence of *dnaE, lap, recA, pgm, gyrB, cat, chi, rstR* and *gmd* genes and concluded that homologous recombination may have occurred; leading to cohesion of the species. In this study based on the analysis of the amplicons size obtained using the eight housekeeping genes, the 35 isolates of vibrios could be clustered into three core- groups at ≥79%S. The banding pattern exhibited by all eight housekeeping genes was distinct enabling the
clustering of the isolates except for the gene \textit{ftsZ}. The primer of \textit{ftsZ} gene, which coded for cell division protein failed to amplify for isolates and type strains of \textit{V.alginolyticus} and \textit{V.proteolyticus}, suggesting that \textit{ftsZ} was not a good phylogenetic marker. However, the MLSA carried out by Sawbae et al. (2007) on 78 isolates, showed that \textit{ftsZ} gene which was selected as one of the housekeeping genes enabled effective clustering of the isolates and >85% sequence homogeneity using ClustalX program. According to Thompson et al., (2005), the genus \textit{Vibrio} is heterogeneous and polyphyletic, with \textit{V.fischeri}, \textit{V. logei}, and \textit{V. wodanis} grouping closer to genus \textit{Photobacterium}. Also \textit{V. halioticoli}, \textit{V. harveyi}, \textit{V.splendidus}, and \textit{V. tubiashii}-related species form groups within the genus \textit{Vibrio}. Similar results were obtained from this study with the isolates of \textit{V.parahaemolyltics} grouped along with \textit{V.harveyi} core group, the isolates of \textit{V.mediterraneii}, \textit{V.nereis}, \textit{V.cholerae} and \textit{V.fluvials} were clustered together as core group-2 ( \textit{V.cholerae} core group), although the isolates were obtained from different sources. Also the isolates of \textit{V.proteolytics}, \textit{V.alginolyticus}, \textit{V.splendidus} and \textit{V.vulnificus} were grouped together as the core group 3. Interestingly the 5 isolates of \textit{V.harveyi} obtained from Kodungallor, Kerala and the type strain of \textit{V.harveyi} (LMG 4044) diverged widely from the other \textit{V.harveyi} isolates obtained from Kakinada, Andhra Pradesh. Similarly the isolates of \textit{V.alginolyticus} which occupied a major position in \textit{V.cholerae} core group based on phenotypic characterisation and RADP fingerprinting, occupied a position away from \textit{V.cholerae} group and was clustered along with the isolates grouped into core group 3.

Analysis of 16S rRNA sequence of 25 wild isolates of vibrios suggests that heterogeneity exist at the inter and intra species level, especially considering the isolates of \textit{V.harveyi}, \textit{V.parahaemolyltics} and \textit{V.alginolyticus}, which could be the result of horizontal gene transfer or plasmid exchange or the high degree of mobility of \textit{Vibrio} genetic elements suggesting the possibility of conflicting histories (Thompson et al. 2004a, b,
Thompson et al. 2005, Thompson et al. 2007, Thompson et al. 2009). These three isolates could be clustered phenotypically and identified genotypically at \( \geq 95\% \) as \( V.\text{harveyi} \), \( V.\text{parahaemolyticus} \) and \( V.\text{alginolyticus} \), hence we consider the wild isolates clustered into these three phena as members of \( V.\text{harveyi} \), \( V.\text{parahaemolyticus} \) and \( V.\text{alginolyticus} \). Further studies are needed to determine the exact cause/s of inter-relatedness of isolates, enabling their clustering under respective clade or core group.

Swabae et al. (2007) estimated a process of recombination existing based on the rate of amino acid substitutions in housekeeping protein genes which resulted in radiation of different sister species. High correlation between pair wise similarity of \( rpoA \), \( atpA \), \( recA \) and 16S rRNA, which are in agreement with polyphasic taxonomic studies, suggests that these genes may be used as an alternative phylogenetic identification markers. Thompson et al., (2005) differentiated families of Vibrionaceae, Photobacteriaceae, Enterovibrionaceae and Salinivibrionaceae on the basis of each genetic locus of the housekeeping genes, with each species clearly forming separate clusters with 98, 94, and 94% \( rpoA \), \( recA \), and \( pyrH \) gene sequence similarity respectively. Further studies are needs to be carried out on sequence analysis of the amplicons obtained to determine the exact cause for divergence of the isolates from their respective clade or core group.

Sequence of vibrio genome and their phylogenetic comparison suggested that consistent phylogenies for each chromosome, gene organization and phylogeny of the respective origins confirmed their shared history (Kirkup et al., 2010). The gene content of a conserved region is useful to infer phylogeny and chromosome specific genes and provide an estimate of the history of the whole chromosome. MLSA schemes devise include analysis of a numbers of genes, rather than examining a single gene for estimating the phylogenetic relatedness. Hence separate MLSA schemes are not required for determining the interrelatedness between species. These
Chapter 3

genes have potential primer sequences that are hypothetically capable of creating phylogenetic trees with the highest resolution and consistent signal.

From the present study we confirm phenotypic characterization as an important tool for the identification of the wild isolates of vibrios. Identification of the isolates using 16S rRNA gene alone which screens only 5 to 10% of the total bacterial genomic content (Thompson et al. 2004a, b, Thompson et al. 2005, Thompson et al. 2007, Thompson et al. 2009), without studying the phenotypic profile of the wild strains may lead to erroneous identification, hence a detailed investigation of the phenotypic profile of the isolates is a prerequisite for identifying wild strains rather than completely depending on genotypic characterization such as analysis of 16S rRNA gene. Analysis of the amplicon size obtained is in accordance with the study of Thompson et al. (2005), suggesting that recA, topA, and pyrH genetic loci could be used for species variations.

3.5. Conclusion

The isolates of vibrios studied diverged widely from the ones which were grouped together as a cluster, based on phenotypic characterisation, suggesting the presence of a large number of heterogenic genotypes within the isolates. However, the presence of conserved regions suggests that the isolates shared the same phylogenetic lineage. The RAPD profile suggested that the isolates analyzed having unique bands could be used in the recognition of genera and species. A detailed investigation of the phenotypic profile of the isolates is important for identifying wild strains rather than completely depending on genotypic characterization such as analysis of 16S rRNA gene alone. Similarity at \( \geq 95\% \) with the isolates deposited in GenBank database was exhibited by 25 wild isolates of vibrios based on 16S rRNA gene sequence analysis. Of the eight different housekeeping gene markers only pyrH recA, topA, and genes could be used as powerful markers for the identification of vibrios.