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Original article

Diversity of diazotrophic bacteria in greenwater system of coastal aquaculture

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Abstract:

As atmospheric nitrogen (N_2) cannot be directly utilized, it must be reduced to ammonia (NH_3) by the community of nitrogen-fixing bacteria. The gene encoding *nifH* is largely unique to nitrogen fixing bacteria, as it converts atmospheric nitrogen into ammonia by producing dinitrogenase reductase enzyme. In the present study, water sample from greenwater system were analyzed using *nifH* gene as a biomarker. Metagenomic clone library was constructed for *nifH* genes. Clones exhibit 83-98% similarity at nucleotide level and 88-98% similarity at amino acid level with dominating cluster of gamma proteobacteria, other clusters of beta proteobacteria *(Dechloromonas aromatica* and *Zoogloea oryzae),* alpha proteobacteria *(Bradyrhizobium japonicum),* delta proteobacteria (*Desulfovibrio* sp.) and uncultured bacteria. Present study sheds light on the high level of species richness in the nitrogen fixing bacterial population in greenwater system of coastal aquaculture.

1. INTRODUCTION

In India, shrimp aquaculture is practiced extensively. Treatment of aquaculture water for purposes of its reuse is a sensible mean to support the further growth of aquaculture without excessive water demands. Hence, development of a simple, cost effective bioremediation technology in zero exchange and water reuse systems without much technical sophistication may be advantageous for saving water, reduced risk of contamination and better environmental control. Culturing in reservoirs, net cages or pens in shrimp culture ponds, acts as bioremediator or biomanipulator. The finfishes, mainly the grey mullet (*Mugil cephalus*), milkfish (*Chanos chanos*) and pearl spot, feed on the uneaten feed and algae in the green water system (Baliao et al., 1999; Baliao, 2000). The finfish cultured along with shrimp stocked in the pond served as bioremediator/biomanipulatore by secreting the slime so as to enhance the production of green water that helps in the condition of the water (Junelyn, 2004). This type of bioremediation technique practiced in the pond environment is commonly known as "greenwater culture system".

The finfish community belongs to euryhaline group of fishes that are grown in estuaries and creeks. These fishes have high tolerance level to survive in the poor quality water system by consuming detritus and algal growth. This limits ammonication in aquaculture waste treatment and increase the oxygen penetration to the sediments (Yearsley, 1999; Baliao, 2000), thus making them ideal candidate species for zero-water exchange system. However, the mechanism of how the system bioremediates in zero exchange and green water recirculation system is yet to be ascertained (Baliao et al., 1999; Baliao, 2000; Tookwinas, 2000; Krishnani et al., 2010c). Nitrogen is the most vital element in our atmosphere and as a component of many bio-molecules, it is essential for growth and development of all organisms (Kneip et al., 2007). As atmospheric nitrogen cannot be directly utilized, it must be reduced to ammonia by nitrogen-fixing bacteria present in the environment for the availability of living systems. This complex process is mediated by prokaryotic organisms in symbiotic relationships, associative relationships and under free-living conditions (Postgate, 1998). N_2-fixing microorganisms, such as unicellular cyanobacteria and endosymbionts in diatoms (e.g. *Richelia* spp.), could also be major sources of fixed N in oceanic waters (Martinez et al.,

1983; Mitsui et al., 1986; Waterbury et al., 1986; Waterbury et al., 1988). Cyanobacterial nitrogen fixation has also been reported in various other environments (Li and Wang, 1983; Venkataraman, 1986; Fontes et al., 1987; Berman-Frank et al., 2003; Diez et al., 2009; Ferreira et al., 2009).

The ability to fix atmospheric nitrogen via the nitrogenase enzyme complex is restricted to some bacteria (Kneip et al., 2007). The gene encoding *nifH* which is largely unique to nitrogen fixing bacteria which converts atmospheric nitrogen into ammonia by producing dinitrogenase reductase enzyme. The Nitrogenase enzyme complex that catalyzes nitrogen fixation, is composed of two proteins namely, dinitrogenase reductase (iron protein) and dinitrogenase (molybdenum iron protein) (Mehta et al., 2003) which produce 60 kDa dimer and 220 to 240 kDa tetramer respectively (Rubio and Ludden, 2005; Rajeswari and Mangai, 2009). The *nifH* gene encodes the iron protein and the *nifDK* genes encode the molybdenum iron protein (Mehta et al., 2003; Moisander et al., 2006).The *nifH* are highly conserved in nature (Zehr and Capone, 1996) among diverse microorganisms and phylogenetic tree of this gene largely resembles the 16S rRNA phylogenetic tree (Mitsui et al., 1986; Normand and Bousquet, 1989; Young, 1992). This conserved gene serves as a molecular marker to determine the biological nitrogen fixation in any environment (Zehr and Capone, 1996). Thus, the molecular approach of using *nifH* genes in diazotrophic communities is widely beneficial (Zhang et al., 2008).

The high level of conservation in the *nifH* gene and in other *nif* genes has prompted numerous studies on the genetic diversity of nitrogen-fixing organisms from different environments using oligonucleotide primers in polymerase chain reaction (Kirshtein et al., 1991; Ben-Porath and Zehr, 1994; Ueda et al., 1995). Chhug et al. (2010) has studied the diversity of bacterial community using *nifH* as a biomarker from Western Himalayan soils, revealed that diversity exist within the same geographical region. There was a need to examine nitrogen fixing bacteria in greenwater system of coastal aquaculture. This could be useful for determining the level of ammonia naturally fixed in the pond system, which in turns leads to nitrogen budget of feed formulation. Hence, the present study has been carried out aiming to investigate diazotrophic bacteria in greenwater system of coastal aquaculture through creation of meta-genomic clone libraries.

2. MATERIALS AND METHODS

2.1 Production and analysis of greenwater

In order to produce green water, finfish mullet-*M.cephalus* (Weight 97-177 g, Length 22.5 to 27.5 cm) were cultured in tanks (depth 1.5 m and diameter 1.8 m). Water quality parameters such as salinity, pH and total ammonia nitrogen (TAN) were measured using standard methods (APHA 1989; Strickland & Parson 1972).

2.2 DNA isolation from greenwater

Greenwater sample (10 L) was transferred to ultra-filtration unit to filter the bacteria onto the membrane (0.2µm). The membrane filter containing bacteria was used for genomic DNA isolation using modified phenol chloroform method. Genomic DNA extracted from greenwater was subsequently quantified by UV-spectrophotometer at 260nm. Purity was determined by measuring the 260/280 nm absorbance ratio.

2.3 PCR amplification of nifH genes

The polymerase chain reaction was performed on the samples along with negative control (water) with a 40 µl reaction mixture using Eppendorf thermal cycler (Master cycler gradient). The following composition was used for a single reaction: (1X) 40 µl: water 27.4 µl; buffer (10 x Tris with 15 mM $MgCl_2$)**:** 4 µl, 10mM dNTP (2.5 mM): 2 µl, forward primer (30 pM) 2 µl, reverse primer (30 pM) 2 µl, *Taq* (5U/µl) 0.2 µl, BSA (20 mg/ml) 0.4 µl, DNA template 2 µl. The amplification programs were as follows: one cycle consisting of 94ºC for 2 min, followed by 34 cycles consisting of denaturation (94 ºC for 40 sec), annealing (55 ºC for 40 sec) and elongation (72 ºC for 40 sec) and a final extension step consisting of 72ºC for 8 min. Aliquots (8 µl) of the amplification products were electrophoresed on 1% agarose gels by using standard electrophoresis procedures. A set of primers originally designed by Rosch et al., (2002); Rosch and Hermann, (2005) were used for the qualitative detection of nitrogen fixing bacteria.

nifHF : AAAGGYGGWATCGGYAARTCCACCAC
nifHRc: TGGGCYTTGTTYTCRCGGATYGGCAT

2.4 Cloning and sequence analysis

The amplified *nifH* gene was purified with a gel extraction kit (Himedia) by following the manufacture's instruction. The purified PCR products (407bp) were ligated by using the pDK101 as recommended by the manufacturer and were transformed into high efficiency competent cells. Clones were confirmed by using *NcoI* restriction endonuclease. Sequencing was done in an ABI 3100 Genetic Analyzer. Primary sequences were analyzed using the BLAST tool of www.ncbi.nlm.nih.gov. Translation was done using Expasy programme. Frame shifts have been taken into account. The deduced amino acid sequences were aligned using the Clustral W programme. Gaps and missing sequence information present in more than one sequence were excluded from calculation of distances and trees

yielding datasets of amino acids. The phylogenetic tree based on the alignment of various fragments of *nifH* genes were generated by using the maximum likelihood method from PHYLIP 3.57 programme packages. Bootstrap analyses of nucleotide and amino acid sequences were performed with the programme.

3. RESULTS AND DISCUSSION

3.1 Greenwater characteristics

Greenwater quality parameters such as salinity, pH and TAN were found to be in the range of 19-21 ppt, 7.7-8.1 and 0.12-0.28 mg/l respectively, which were well within the safe levels, prescribed for finfish and shellfish aquaculture. Low level of TAN could be attributed to the nitrification of ammonia in the system.

3.2 Detection of nitrogen fixing bacteria in greenwater

Nitrogen fixing bacteria have been detected in greenwater system using PCR amplification of *nifH* genes. Primers set originally designed by Rosch et al., (2002; 2005) were used in this study, which offered reliable amplification results and yielded PCR product of the expected length of 407 bp (Fig.1). Among the nitrogenise proteins, the *NifH* subunit is the most conserved. The primers nifHF and nifHRc allow PCR amplification of the target *nifH* gene segment in a wide range of N_2-fixing organisms. Amplification of *nifH* gene using direct method revealed optimum annealing temperature of 57ºC.

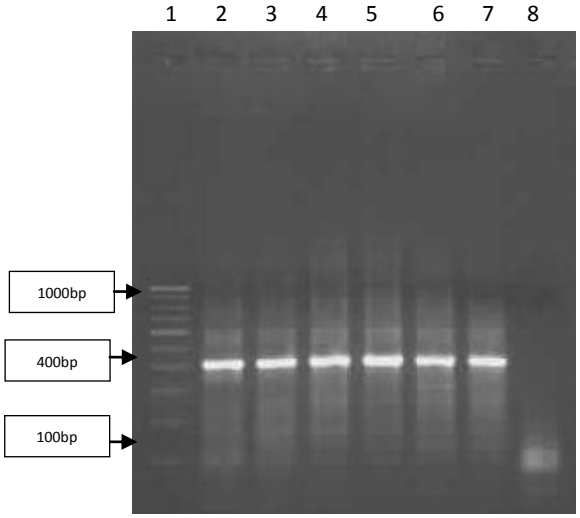

Figure 1. PCR amplification of 407 bp (*Lane 1:* 100 bp marker, *lane* 2, 3, 4, 5, 6, 7: 407 bp samples, lane 8: Negative control).

3.3 Characterization of nitrogen fixing bacteria in greenwater system

Diversity of the *nifH* gene was examined in greenwater coastal aquaculture system though creation of metagenomic clones libraries of *nifH* genes (407 bp). The high biodiversity in greenwater system of coastal aquaculture is reflected by present findings that 50 clones from greenwater yielded 17 different clones. The nucleotide sequences of *nifH* genes determined in this study have been deposited in the GenBank database. The accession numbers for the gene sequences are FJ609151 to FJ609167 (ACN21982 to ACN21998). The level of similarity between different pairs of *nifH* genes at nucleotide and amino acids levels ranged from 68-98% and 68-99% respectively. This revealed that sequence difference has been resulted from actual diversity in the sample and not a PCR artefact. For the *nifH* genes clone libraries, nucleotides and predicted amino acids sequences of the *nifH* genes identified in this study are compared with the sequences available in the GenBank. Percent similarity of amino acids predicted from 407 bp sequences of *nifH* genes is presented in Table-1. An alignment of the amino acids in parts of the *nifH* encoded proteins is presented in Figure 2. Residues conserved in *nifH* sequences are highlighted. A total of 69 of 135 amino acid residues were conserved in the environmental clones.

```
ACN21998          ----------KGGIGKSTTTQNLVAGLTELGKKVMIVGCDPKADSTRLILHSKAQNTIM 49
ABP79021          -MAMRQCAIYGKGGIGKSTTTQNLVAALAELGKKVMIVGCDPKADSTRLILHSKAQNTIM 59
ADM51801          -------------GIGKSTTTQNLVAGLAELGKKVMIVGCDPKADSTRLILHAKAQNTIM 47
AAF61009          -------------GIGKSTTTQNLVAGLAELGKKVMIVGCDPKADSTRLILHSKAQNTIM 47
ACN21997          ----------KGGIGKSTTTQNLVAGLAELGKKVMIVGCDPKADSTRLILHSKAQNTIM 49
AAF17302          -MAIRQCAIYGKGGIGKSTTTQNLVAALAEAGKKVMIIGCDPKADSTRLILHSKAQNTIM 59
AAD55588          ----------------KSTTTQNLVAALAEAGKKVMIIGCDPKADSTRLILHSKAQNTIM 44
ACN21990          ----------KGGIGKSTTTQNLVAALAEAGKSVMIVGCDPKADSTRLILHSKAQNTIM 49
ACN21987          ----------KGGIGKSTTTQNLVAALAEAGKKVMIVGCDPKADSTRLILHSKAQNTIM 49
ACN21983          ----------KGGIGKSTTTQNLVAALAEAGKKVMIVGCDPKADSTRLILHSKAQNTIM 49
AAA22140          -MAMRQCAIYGKGGIGKSTTTQNLVAALAEMGKKVMIVGCDPKADSTRLILHSKAQNTIM 59
AAF60986          -------------GIGKSTTTQNLVAALAEAGKKVMIVGCDPKADSTRLILHSKAQNTIM 47
ACN21995          ----------KGGIGKSTTTQNLVAALAEAGKKVMIVGCDPKADSTRLILHSKAQTTVM 49
ACN21988          ----------KGGIGKSTTTQNLVAALAEAGKKVMIVGCDPKADSTRLILHSKAQTTVM 49
ACN21985          ----------KGGIGKSTTTQNLVAGLAEAGNKIMIVGCDPKADSTRLILHSKAQDTIM 49
ADK92441          ---------YGKGGIGKSTTTQNLVAALAEAGKKIMIVGCDPKADSTRLILHAKAQETIM 51
ACN21993          ----------KGGIGKSTTTRNLVAALAEAGKKVMIVGCDPKADSTRLILHSKAQTTVM 49
ACN21984          ----------KGGIGKSTTTQNLVAALAEAGKKVMIVGCDPKADSTRLILHSKAQTTVM 49
AAF61029          -------------GIGKSTTTQNLVAALAESGKKVMIVGCDPKADSTRLILHSKAQTTVM 47
BAE80733          -------------GIGKCTTTQNLVAALAESGKKVMIVGCDPKADSTRLILHSKAQTTVM 47
ACN21992          ----------KGGIGKSTTSQNLVAALAESGKKVMIVGCDPKADSTRLILHSKAQTTVM 49
AAZ46164          MAKLRQCAIYGKGGIGKSTTTQNLVAALAESGKKVMIVGCDPKADSTRLILHSKAQTTVM 60
ADM63687          -------------GIGKSTTTQNLVAALAELGKKVMIVGCDPKADSTRLILHSKAQTTVM 47
AAF61010          -------------GIGKSTTTQNLVAGLAELGQKVMIVGCDPKADSTRLMLHSKAQTTVM 47
ACN21994          ----------KGGIGKSTTTQNLVAGLAALEKQVMIVGCDPKADSTRLMLHAKAQSTVM 49
ACN21986          ----------KGGIGKSTTTQNLVAGLAALEKQVMIVGCDPKADSTRLMLHAKAQSTVM 49
ACN21982          ----------KGGIGKSTTTQNLVAGLAALEKQVMIVGCDPKADSTRLMLHAKAQSTVM 49
ACN21989          ----------KGGIGKSTTTQNLVAGLASLGKNVMIVGCDPKADATRLILHSKSQETVM 49
ACN21991          ----------KGGIGKSTTTQNTVAGLAEMGKKVMVVGCDPKADSTRLLLGGLAQRTVL 49
ACT67982          ----------KGGIGKSTTTQNTVAGLAEMGRKVMVVGCDPKADSTRLLLGGLAQKSVL 49
ACN21996          ----------KGGIGKSTTTQNTVAGLAEMGKKVMVVGCDPKADSTRLLLGGLVQKTVL 49
YP_002953433      ---MRKIAIYGKGGIGKSTTTQNTVAGLAEMGKKVMVVGCDPKADSTRLLLHGLAQKTVL 57
                             *.**::* **.*:   ..:*::*******:***:* .  * :::

ACN21998          EM-AAEAGTVEDLELEDVLKTGYGDIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 108
ABP79021          EM-AAEAGTVEDLELEDVLKTGYGDIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 118
ADM51801          EM-AAEAGTVEDLELEDVLKTGYGDIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 106
AAF61009          EM-AAEAGTVEDLELEDVLKVGYGDIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 106
ACN21997          EM-AAEAGTVEDLELEDVLKVGYGDIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 108
AAF17302          EM-AAEAGTVEDIELEDVLKVGYGDVRCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 118
AAD55588          EM-AAEAGTVEDIELEDVLKIGYGDVRCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 103
ACN21990          EM-AAEAGTVEDLELEDVLKVGYGDVRCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 108
ACN21987          EM-AAEAGTVEDLELEDVLKIGYGDTKCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 108
ACN21983          EM-AAEAGTVEDLELEDVLKIGYGDVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 108
AAA22140          EMXAAEAGTVEDLELEDVLKVGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 119
AAF60986          EM-AAEAGTVEDLELEDVLKVGFGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 106
ACN21995          EM-AAEMGTVEDLELEDVLSVGFGDVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 108
ACN21988          EM-AAEMGTVEDLELEDVLSVGYGDVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYED 108
ACN21985          HL-AAEAGSVEDLELEDVLKEGFAGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 108
ADK92441          QM-AADAGSVEDLELEDVLKTGYAGIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 110
ACN21993          HL-AAEAGTVEDLELEDVMSVGYGDVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 108
ACN21984          HL-AAEAGSVEDLELEDVLAVGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 108
AAF61029          HL-AAEAGSVEDLELEDVLSVGFGDVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 106
BAE80733          HL-AAEAGSVEDLELEDVLSVGFGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 106
ACN21992          HL-AAEAGSVEDLELEDVLSVGFGGTKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 108
AAZ46164          HL-AAEAGSVEDLELEDVLSVGFGGIKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 119
ADM63687          HL-AAEAGTVEDLELEDVLSVGYGGIKCVESGGPEPGVGCAGRGVITAINFLEEESAYDE 106
AAF61010          HL-AAEAGTVEDLELDDVLAVGFGDVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 106
ACN21994          HL-AADAGSVEDLELEDVLRVGYGGVRCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 108
ACN21986          HL-AADAGSVEDLELEDVLRVGYGGVRCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 108
ACN21982          HL-AADAGSVEDLELEDVLRVGYGGVRCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 108
ACN21989          HL-AADAGSVEDLELEDVLQTGFGGVRCVESGGPEPGVGCAGRGVITAINFLEEEGAYEE 108
ACN21991          DT-LREEG--EDVELEDVRKEGYSGTMCTESGGPEPGVGCAGRGIITSINLLEQLGAYAD 106
ACT67982          DT-LREEG--EDVDLEDIRRTGFSGTVCVESGGPEPGVGCAGRGIITSINLLEQLGAYAD 106
ACN21996          DT-LREEG--EDVEIEDIVLEGFKGSLCTESGGPEPGVGCAGRGIITSINLLEQLGAFKE 106
YP_002953433      DT-LREEG--EDVELDDILKEGYGGTMCTESGGPEPGVGCAGRGIITSINLLEQLGAYEE 114
                  .    : *  **:::::*:   *: .  *.***************:**:**:**: .*: :


ACN21998          D--LDFVFYDVLGDVVCGGFAMPIRENKA------------------------------ 135
ABP79021          D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYVVCSGEMMAMYAANNICKGIVKYANSG 176
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ADM51801        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIV---------------------- 139
AAF61009        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIV---------------------- 139
ACN21997        D--LDFVFYDVLGDVVCGGFAMPIRKNKA---------------------------- 135
AAF17302        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNISKGICKYAATG 176
AAD55588        D--LDFVFYDVLGDVVCGGFAMPIRENKAEEIYIVVSGEMMAMYAANNISKGICKYATSG 161
ACN21990        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACN21987        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACN21983        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
AAA22140        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNISKGIVKYANSG 177
AAF60986        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIV---------------------- 139
ACN21995        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACN21988        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACN21985        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ADK92441        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVVSGEMMA---------------- 150
ACN21993        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACN21984        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
AAF61029        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIV---------------------- 139
BAE80733        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIV---------------------- 139
ACN21992        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
AAZ46164        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNIAKGIVKYANSG 177
ADM63687        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIV---------------------- 139
AAF61010        D--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIV---------------------- 139
ACN21994        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACN21986        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACN21982        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACN21989        D--LDFVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACN21991        SEDLDYVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
ACT67982        SEHLDYAFYDVLGDVVCGGFAMPIREGKAKEIYIVVSGEMMAMYAAN---------- 153
ACN21996        EKQIDYVFYDVLGDVVCGGFAMPIRENKA---------------------------- 135
YP_002953433    DKKLDYVFYDVLGDVVCGGFAMPIRDGKAEEIYIVCSGEMMAMYAANNICKGIVKYADTG 174
                .  :*:.******************..**
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Figure 2. Partial alignment of the predicted amino acids encoded by *nifH* gene

From the table and figure, it is evident that the environmental clones have 83-98% and 88–98% homology with that of *nifH* of dominating cluster of gamma proteobacteria, other clusters of beta proteobacteria *(Dechloromonas aromatic* and *Zoogloea oryzae)*, alpha proteobacteria *(Bradyrhizobium japonicum)* and

Table 1 Percent similarity of amino acid predicted from 407 bp sequence of *nifH* genes

Environmental clone	Accession number	Homology on amino acid levels	
DBTNF43	FJ609167- ACN21998	*Pseudomonas stutzeri* A1501- ABP79021	98%
DBTNF42	FJ609166- ACN21997	*Azotobacter chroococcum*- AAA22140	95%
DBTNF41	FJ609165- ACN21996	*Desulfovibrio magneticus* RS-1- YP_002953433	88%
DBTNF39	FJ609164- ACN21995	*Azomonas agilis*-AAF60986	97%
DBTNF38	FJ609163- ACN21994	Uncultured bacterium clone TSR12-5- ADM51801	89%
DBTNF33	FJ609162- ACN21993	Unidentified nitrogen-fixing bacteria clone B5- AAF61029	97%
DBTNF31	FJ609161- ACN21992	*Zoogloea oryzae*- BAE80733	98%
DBTNF30	FJ609160- ACN21991	*Bradyrhizobium japonicum*- ACL03220	91%
DBTNF19	FJ609159- ACN21990	*Vibrio diazotrophicus*- AAF17302	98%
DBTNF18	FJ609158- ACN21989	*Dechloromonas aromatica* RCB- AAZ46164	90%
DBTNF15	FJ609157- ACN21988	*Azotobacter chroococcum*- AAA22140	95%
DBTNF12	FJ609156- ACN21987	Uncultured nitrogen-fixing bacterium clone N040-AAF61009	97%
DBTNF10	FJ609155- ACN21986	Unidentified nitrogen-fixing bacteria clone HD2-2-AAF61010	91%
DBTNF5	FJ609154- ACN21985	*Ectothiorhodospira* sp. B7-7- ADK92441	93%
DBTNF4	FJ609153- ACN21984	*Azotobacter chroococcum*-AAA22140	93%
DBTNF2	FJ609152- ACN21983	*Vibrio natriegens*-AAD55588	98%
DBTNF1	FJ609151- ACN21982	Uncultured bacterium clone SVS5-6-ADM63687	90%

delta proteobacteria (*Desulfovibrio* sp.) and uncultured bacteria. Eight clones exhibited 93-98% similarity at amino acid level with dominating cluster of gammaproteobacteria, two clones exhibited 90-98% similarity with beta proteobacteria, one clone each exhibited 88% similarity with delta proteobacterium and 91% with alpha proteobacterium. Five clones exhibited 89-97% similarity with uncultured bacteria. Rhizosphere soils of chickpea (*Cicer aeritinum*) and wheat (*Triticum aestivum*) were analysed by Sarita et al., (2008) for the diversity of diazotrophic microorganisms by sequencing 41 *nifH* genotypes, which showed similarity to the corresponding genera of diazotrophs belonging to alpha, beta, gamma and delta proteobacteria.

3.4 Phylogenetic analysis of nifH genes

Phylogenetic tree based on predicted amino acids encoded by *nifH* as determined by maximum likelihood method is presented in Figure 3. Environmental clones DBTNF4, DBTNF15, DBT42 showed 81-85% similarity at nucleotide level and 93-95% similarity at amino acid level with gamma proteobacterium *Azotobacter chroococcum*. Zhang et al., (2008) and Lovell et al., (2001) have identified *A. chroococcum* contain 99% sequence similarity with SY8 clone of *nifH* from mangrove sediments and NIS11-4 from dead *Spartina* biomass, respectively. Rajeswari and Mangai, (2009) isolated 28 strains of *Azotobacter* sp. from Rameshwaram of Gulf of Mannar contain *nif*H gene and has the homology with uncultured bacteria. The nucleotide sequence on both sides of the *nifH* gene and part of the *nifD* gene encoding the molybdenum nitrogenase from *A. chroococcum* was determined by Jones et al. (1993).

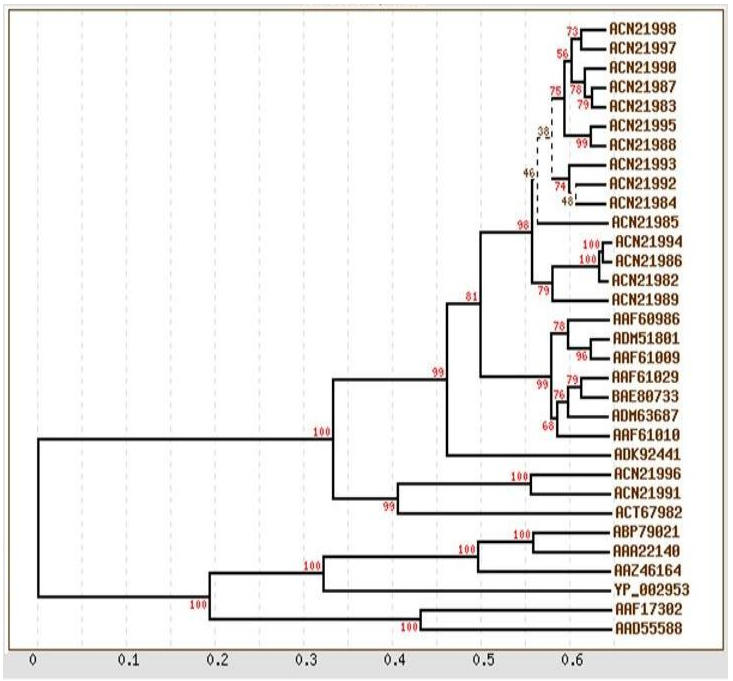

Figure 3. Phylogenetic tree based on predicted amino acids encoded by *nifH* as determined by maximum likelihood method.

Desnoues et al. (2003) have reported identification and sequence analysis of *nifH* gene of gamma proteobacterium *Pseudomonas stutzeri* A1501, which is a Gram-negative, highly motile, oxidase-positive, aerobic strain and it has 89% similarity with clone DBTNF43. The amino acid sequence of DBTNF43 exhibited 98% sequence similarity with the amino acid sequence of *P. stutzeri* A1501. The *nifH* clone NIS1-4 isolated by Lovell

et al. (2001) from salt marsh has 98% homology with *P. stutzeri*. It shows properties reported by Vermeiren et al. (1999), including denitrification in anaerobic conditions, a general feature of *P. stutzeri* strains. Nucleotide and amino acids identity values of environmental clone DBTNF41 with *D. magneticus* RS-1 are 84% and 88% respectively. The cultured and uncultured sequences of delta proteobacteria *Desulfovibrio* species in tropical and arctic site shows close phylogenetic relationship between their abundant sequence of *nifH* gene (Izquierdo and Nusslein, 2006).

Purple phototrophic bacteria *Ectothiorhodospira* belonging to gamma proteobacteria has the ability to deposit elemental sulphur outside the cell and able to fix the nitrogen reported by Tourova et al. (2007). Environmental clone DBTNF5 phylogenetically correlate to *Ectothiorhodospira* as evidenced from 88% similarity at nucleotide and 93% similarity at amino acid levels. This provides evidence of sulfate reduction in nitrogen fixing community in marine environment. *Azomonas agilis* is motile gram negative gamma proteobacterium showing 84% similarity at nucleotide with the clone DBTNF39. Lovell et al. (2001) recovered sequences, NIS1-2, NIS1-2, and SIW1-4 from salt marshes, which have 95-99% homology at nucleotide level with diazotrophic bacteria *A. agilis.* Clone DBTNF31 exhibits 88% homology at nucleotide and 98% homology at amino acid levels with nitrogen fixing beta proteobacterium *Z. oryzae*.

Izquierdo and Nusslein (2006) reported that *nifH* clones have 64-85% homology with *B. japonicum* from soil environment. The clones isolated by Rosch et al. (2002) from acid forest soil have 86% homology with *B. japonicum* identified. Clone DBTNF30 obtained in the present study showed 82% homology at nucleotide and 91% homology at amino acid level with alpha proteobacterium *B. japonicum.* Guerinot et al. (1982) have reported identification and production of nitrogenase enzyme of *Vibrio diazotrophicus*, which is gram negative, short rod, facultative anaerobic gamma proteobacterium. In the present study, clone DBTNF19 exhibited 98% homology at nucleotide and amino acid levels with *V. diazotrophicus.* Salinero et al. (2009) phylogenetically analyzed nitrogen fixation ability in *D. aromatica* RCB using rolling circular amplification. Clone DBTNF18 is phylogenetically related to beta proteobacterium *D.aromatica* RCB as evidenced from 81% similarity at nucleotide level and 90% similarity at amino acid level respectively.

Bird et al. (2005) reported matching of clone 01/11/20 with *V. natriegens* derived from Atlantic and Pacific oceans. Clone DBTF2 has 84% similarity at nucleotide and 98% similarity at amino acid levels with salt loving gamma proteobacterium *V. natriegens*. Clones DBTNF38, DBTNF33, DBTNF12, DBTNF10, DBTNF1 exhibited similarity with uncultured bacterial clones B5, SVS5-6, HD2-2, N040, TSR12 isolated by Davis (PhD thesis, 2010) from salt marsh in different environmental condition. In order to confirm further the nitrogenase function of clones, we searched for *nifH* signature motif in the clones using PROSTIE (http://prosite.expasy.org/). Interestingly, two signature motifs such as D-x-L-G-D-V-V-C-G-G-F-[AGSP]-x-P and E-x-G-G-P-x(2)-[GA]-x-G-C-[AG]-G were found to be commonly present in all clone sequences. These conserved patterns were previously reported as the binding sites for single 4Fe-4S iron sulfur cluster.

The biochemical importance indicates a need for development of reliable methods for identification of microorganisms in nature. Molecular detection systems based on the functional genes, which do not rely on traditional cultivation are much more promising in determining microbial populations in a aquaculture systems (Krishnani et al., 2009a; 2009b; 2010a; 2010b; 2010c; 2010d). In the present study, culture independent molecular technique has been used to monitor and recover diversity of nitrogen fixing bacterial populations in greenwater system of coastal environment. Sequencing of metagenomic clone library has provided sequences and the phylogenetic information of clones, which has led to realization that diazotrophic community in greenwater system of coastal aquaculture, is much more diverse. The findings of Bird et al. (2005) suggest that gamma proteobacteria are widespread and likely to be an important component of the heterotrophic diazotrophic microbial community of the tropical and subtropical oceans. Zehr et al. (1998) has also reported that gamma proteobacteria are major nitrogenase containing phylotypes in marine environment. The high biodiversity in greenwater system of coastal aquaculture is reflected by present findings that 50 clones from greenwater yielded 17 different clones belonging to clusters of alpha, beta, gamma, delta proteobacteria and uncultured bacteria. The dominant cluster for *nifH* diversity is gamma proteobacteria. Five clones have the homology with uncultured bacteria, which indicated high level of species richness in the N_2-fixing bacterial population in greenwater.

Cyanobacteria inhabit fresh, brackish, marine and hypersaline waters, as well as terrestrial environments. These pond systems are usually rich repositories of cyanobacteria, which can rapidly overtake an aquaculture pond and contribute to unstable conditions such as poor water quality linked with disease problems. Cyanobacteria can also cause off-flavor and objectionable odor in fish. A large portion of nutrients from fish feed in pond system is chemically or biologically transformed and then released into the water causing increase in

cyanobacteria. In the present study, *M. cephalus* were cultured in tanks containing brackishwater under yard condition, where water was not nutrient rich as fishes were fed with minimum required feed. Turker et al. (2003) reported that Nile Tilapia can act as a filter, which in turns can control cyanobacteria. The *nifH* phylogeny of present study revealed that only proteobacteria were present and there was a lack of cyanobacterial N_2 fixers in greenwater system. This may be attributed to nutrient deficient greenwater, where *M.cephalus* could have also controlled cyanobacteria.

Usually aquaculture ponds acquire high ammonia content from animal excreta through protein rich feed catabolism. In addition, diverse diazotrophs are occasionally significant contributor to the nitrogen budget (Hargreaves, 1998). Elevated level of ammonium is known to be a potent suppressor of nitrogenase activity. If the environment contains sufficient ammonia for the microbe's need, it does not make any nitrogenase. If ammonia is less than sufficient, microbes make only sufficient nitrogenase to satisfy its requirement for fixed nitrogen (Postgate, 1998).

In the present study, ammonia concentration in greenwater system was within safe levels prescribed for finfish and shellfish aquaculture and probably not sufficient to suppress nitrogenase activity. We initiated this work to identify the naturally occurring nitrogen fixing bacteria in such systems. This study focused only on diversity level and further study on the characterization of active species of these uncultured bacterial communities and effect of ammonia levels on suppression of nitrogenase activity will help to understand more on the biological nitrogen fixation in greenwater system of coastal aquaculture. The integration of greenwater bioaugmentation technology would allow meaningful interpretations regarding the biochemical processes in zero-water exchange system of coastal aquaculture.

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