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ORIGINAL RESEARCH

Diversity of denitrifying bacteria in the greenwater system of coastal aquaculture

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Abstract Greenwater technology is based on integrating finfishes inside pens for zero water exchange system of shrimp aquaculture. Nitrogen transformation could be improved by rearing euryhaline finfishes like grey mullet, milkfish which have a broad diet and tolerate poor water quality. The abundance of four denitrifying functional genes coding for *nitrate reductase* (*narG* and *napA*), *nitrite reductase* (*nirS*), *nitric oxide reductase* (*qnorB*) and *nitrous oxide reductase* (*nosZ*) has been examined in the greenwater system through a metagenomic approach. Phylogeny revealed homology of *narG* clones with uncultured environmental clones, whereas *napA* clone sequences were found to have homology with cultured (*Stappia aggregata*) and uncultured microorganisms. The *nirS* clones show uniqueness with *Marinobacter hydrocarbonoclasticus, Aromatoleum aromaticum,* and *Ruegeria pomeroyi*. The *qnorB* gene has been reported for the first time from culture systems along the Indian coast and clone exhibited 84–87 % identity with different uncultured bacteria. The *nosZ* clones are closely affiliated with *S. aggregate* and alpha bacterium. This study revealed denitrifying diversity from a greenwater system which could eventually be used in planning the future strategy for comprehending nitrogen fluxes, greenhouse gases and their mitigation in coastal aquaculture systems.

Keywords Greenwater · Coastal aquaculture · Denitrification · Nitrogen flux · Functional gene · Diversity

Introduction

Nitrates are a by-product of excess feed and decayed organic wastes from animals in aquaculture ponds and can be toxic to animals at higher concentrations. Denitrification is a reductive process which converts nitrate (NO_3) into atmospheric nitrogen (N_2) through nitrite (NO_2), nitric oxide (NO), and nitrous oxide (N_2O). These intermediate products can be used as alternative electron acceptors for energy production in anaerobic condition (Stres and Murovec 2008). Nitric oxide and nitrous oxide attract the modern scientist as they are 310

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times more potent greenhouse gases than carbon dioxide (Fernandez et al. 2008). In the denitrification pathway, different functional genes are involved that encode for enzyme to use nitrogenous metabolites by targeting conserved regions. Complete denitrification needs the sequential action of four enzymes: *nitrate reductase* (*narG* and *napA*), *nitrite reductase* (*nirK/nirS*), *nitric oxide reductase* (*nor*), and *nitrous oxide reductase* (*nosZ*) (Chen et al. 2011). They can be used to remove excess nitrogen from wastewater treatment plants (Throback et al. 2004). Cultivation-independent functional gene-based approaches have an important role to play in examining the entire diversity (Palumbo et al. 2004). The use of functional genes involved in denitrification is a more direct method of detecting or identifying denitrifying bacteria (Wolsing and Prieme 2004). Analysis of functional diversity and its dynamics in the environment could help to understand the microbial ecology and biogeochemistry of aquatic systems (Taroncher-Oldenburg et al. 2003).

Previous studies have focused mainly on the composition and activities of soil microbiota in aquaculture. A few studies have employed molecular tools to understand the diversity of archaeal and bacterial community structures in coastal aquaculture. The composition of nitrate reducing community has been explored from various habitats. Studies conducted by Sanford et al. (2012) have expanded the current understanding of the nitrogen cycle. Denitrification in aquatic environment leads to nitrate removal from the aquatic system; therefore, it is necessary to understand functional diversity of denitrifier communities in the system (Huang et al. 2011).

Herbivores finfish mainly the grey mullet (*Mugil cephalus*) and milkfish (*Chanos chanos*) are propagated as bioremediators in fish cages in shrimp growing ponds. This is an innovative bioaugmentation technique called as greenwater technology, which is proven most functional among all others for environmental management in coastal aquaculture (Baliao et al. 1999; Baliao 2000). We have previously examined the diversity of sulfur oxidizing bacteria, nitrogen fixing bacteria and nitrite oxidizing bacteria in greenwater system (Krishnani et al. 2010; Kathiravan and Krishnani 2012; Kathiravan et al. 2012). In the present study, functional genes: *narG, napA*, *nirS*, *qnorB,* and *nosZ* are used as molecular markers to identify denitrifying population in the greenwater system of coastal aquaculture.

Methods

Sampling and analysis

The greenwater technology experiment with milkfish-*C. chanos* was conducted in a 0.5 ha shrimp pond at Karapadagai (10°60'84"N, 79°79'19") village in Nagapattinam Dist., Tamil Nadu, India. 100-g soil and 200-ml water samples each were collected from four corners and a center of the pond and pooled in order to prepare a composite soil sample (0.5 kg) and water sample (1 l). Soil pH (Cyberscan, Canada), electrical conductivity (Elico, India), available phosphorous, total nitrogen (APHA 1989) and organic carbon were determined using the chromic acid digestion method (Walkley and Black 1934).

Metagenomic DNA isolation

Composite water samples (1 l) collected from the pond were transferred to an ultra-filtration unit to filter the bacteria onto a membrane (0.2 μm). The filter membrane containing bacteria was used for metagenomic DNA isolation using modified CTAB-phenol chloroform method (Krishnani et al. 2009a). The DNA was extracted from composite soil using the PowerSoil DNA isolation kit (PowerSoil, Mobio Laboratories Inc., CA, USA), according to the manufacturer's instructions. Metagenomic DNA from composite soil and water samples were pooled to obtain the absolute diversity of the pond. Purity was determined by measuring the 260/280 nm absorbance ratio. The DNA was stored at −20 °C for further molecular analyses.

PCR amplification of functional genes

Polymerase chain reaction was performed with a negative control of 40-μl reaction mixture using the mastercycler (Eppendorf Mastercycler gradient, Germany). The denitrifying functional genes were amplified using the primer pairs shown in Table 1. Two microliters of pooled metagenomic DNA were added to a 40-μl

Table 1 Oligonucleotides used for PCR amplification and sequencing of functional genes

Genes	Primer	References
narG	narG1960f-5′TAYGTSGGSCARGARAA 3′	Philippot et al. (2002)
	narG2650r-5′TTYTCRTACCABGTBGC 3′	
napA	V67F-5′TAYTTYYTNHSNAARATHATGTAYGG 3′	Flanagan et al. (1999)
	V67R-5′DATNGGRTGCATYTCNGCCATRTT 3′	
nirS	NirS1F-5′CCTAYTGGCCGCCRCART 3′	Braker et al. (1998)
	NirS6R-5′CGTTGAACTTRCCGGT 3′	
qnorB	qnorB2F-5′ GGNCAYCARGGNTAYGA 3′	Braker and Tiedje (2003)
	qnorB7R-5′ GGNGGRTTDATCADGAANCC 3′	
nosZ	Nos1527F-5′CGCTGTTCHTCGACAGYCA 3′	Scala and Kerkhof (1998)
	NosZ-F-5′CG(CT)TGTTCMTCGACAGCCAG 3′	Kloos et al. (2001)
	NosZR-5′CATGTGCAGNGCRTGGCAGAA 3′	

PCR mixture containing 1X PCR buffer (INVITROGEN, USA), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 30 pM of each primer, and 1 unit of Taq polymerase (INVITROGEN, USA). The reaction was initially denatured at 94 °C for 2 min; followed by 34 cycles of 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 40 s; followed by a final extension step at 72 °C for 8 min. The PCR products were analyzed on 1.5 % (w/v) ethidium bromide-stained agarose gel to ensure the correct size fragment were amplified.

Cloning and sequence analysis

The amplified functional genes were purified with a gel extraction kit (Himedia, India) according to manufacturer's instructions. The purified PCR products were ligated using the pDK101 as recommended by the manufacturer and were transformed into high efficiency competent cells. Clones were confirmed using *NcoI* restriction endonuclease. Sequencing was carried out in an ABI 3100 Genetic Analyzer (Applied Biosystems, USA). Nucleotide sequences were translated into protein sequences using the Translate tool on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (http://us.expasy.org/tools/dna.html). The nucleotide and protein sequences were compared with the entries in GenBank using BlastN and BlastP, respectively (Altschul et al. 1990). The phylogenetic tree based on the alignment of predicted amino acids was generated using the Maximum Composite Likelihood from MEGA 4 (Tamura et al. 2007). Bootstrap analyses of amino acid sequences were also performed using this program. Signature motif of *qnorB* was identified by using PROSTIE (http://www.prosite.expasy.org/). The richness of denitrifying bacterial community in greenwater system has been analyzed by EcoSim (Gotelli and Entsminger 2012).

Results and discussions

Soil characteristics of greenwater system

Soil quality parameters such as pH, electrical conductivity (EC), available phosphorous, total nitrogen, and organic carbon were found to be in the range of 8.29–8.40, 10.86–15.92 dS/m, 0.0288–0.0682 ppm, 8.4–10.12 ppm and 0.19–0.31 %, respectively, which are well within the safe levels prescribed for finfish and shellfish aquaculture.

Detection of denitrifying functional genes

Denitrifying bacterial community was detected in greenwater-bioaugmentation system using PCR amplification of functional genes such as *narG napA, nirS, qnorB* and *nosZ* gene. The primers used in this study amplified well and yielded PCR products of the expected 650 bp (Fig. 1a), 414 bp (Fig. 1b), 890 bp (Fig. 1c), 660 bp (Fig. 1d) and 700 bp (Fig. 1e) for *narG, napA, nirS, qnorB,* and *nosZ,* respectively.

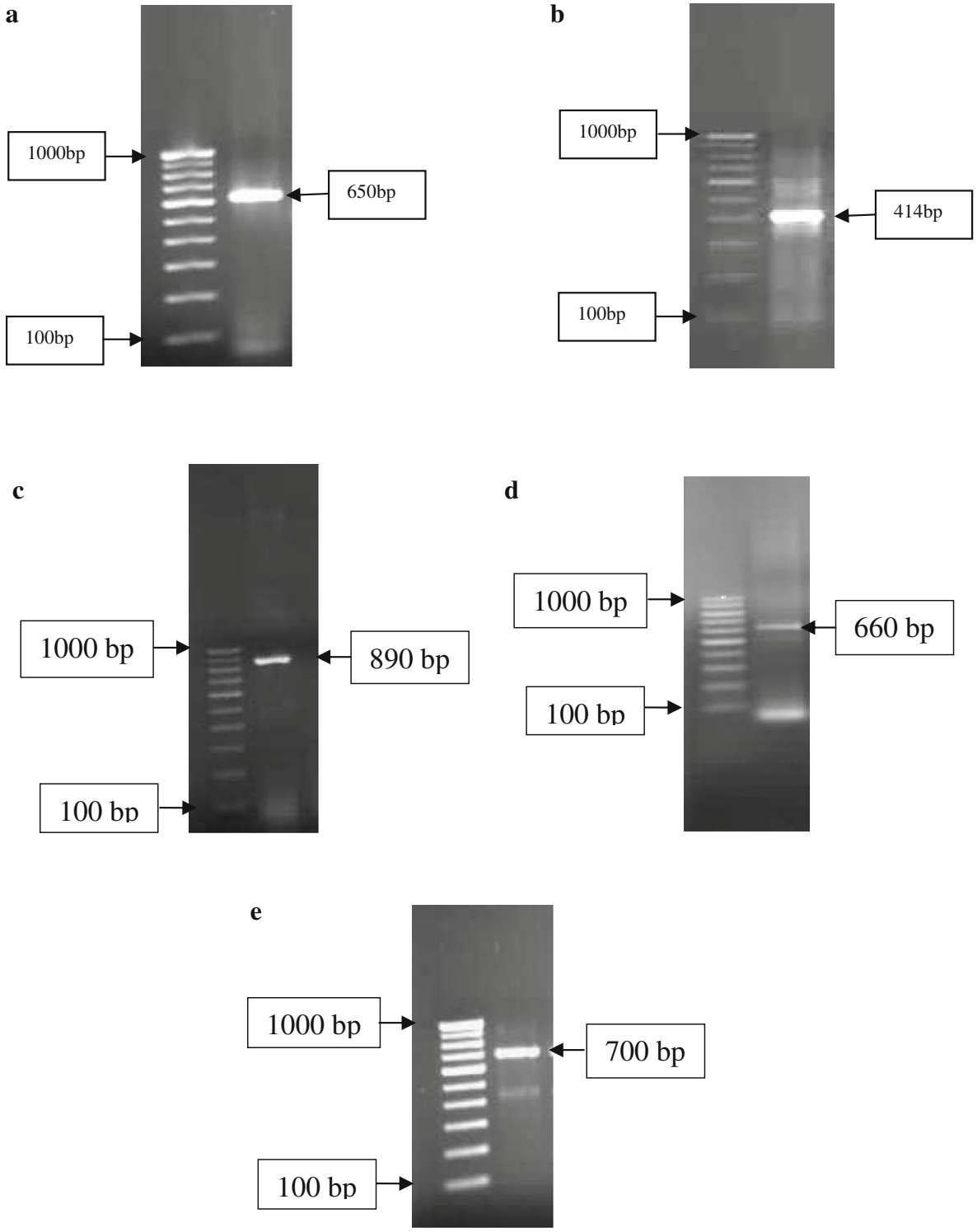

Fig. 1 PCR amplification of functional genes. **a** 650 bp fragment of narG gene; **b** 414 bp fragment of napA gene; **c** 890 bp fragment of nirS gene; **d** 660 bp fragment of qnorB gene; **e** 700 bp fragment of nosZ gene

Nucleotide sequence accession numbers

The nucleotide sequences of denitrifying functional genes determined in this study have been deposited in the GenBank database. The accession numbers for the *narG, napA, nirS* and *nosZ* gene sequences are FJ905006 to

Table 2 Percent similarity of amino acid predicted from denitrifying functional genes

Environmental clone	Accession number	Homology on amino acid levels
narG gene		
V18DBT21	FJ905013-ACQ65795	u.bacterium(ABF20959) 80 %
V18DBT17	FJ905012-ACQ65794	u.bacterium (ACH90505) 81 %
V18DBT14	FJ905011-ACQ65793	u.bacterium(CAL92046) 78 %
V18DBT9	FJ905010-ACQ65792	u.bacterium (ACH90505) 81 %
V18DBT24	FJ905009-ACQ65791	u.bacterium(CAL92058) 97 %
V18DBT23	FJ905008-ACQ65790	u.bacterium(CAL92058) 96 %
V18DBT18b	FJ905007-ACQ65789	u.bacterium(CAL92058) 97 %
V18DBT4	FJ905006-ACQ65788	u.bacterium(CAL63724) 95 %
napA gene		
GSV13DBT14	FJ905004-ACQ65787	*S.aggregata*(ZP_01549680) 99 %
GSV13DBT4	FJ905003-ACQ65786	u.bacterium(CAL63635) 85 %
GSV13DBT11	FJ905002-ACQ65785	u.bacterium(CAL63627) 87 %
GSV13DBT20	FJ905001-ACQ65784	u.bacterium(CAL63659) 89 %
GSV13DBT21	FJ905000-ACQ65783	u.bacterium(CAL63637) 93 %
GSV13DBT24	FJ904999-ACQ65782	u.bacterium(ABW17415) 88 %
GSV13DBT23	FJ904998-ACQ65781	u.bacterium(ABW17415) 88 %
GSV13DBT19	FJ904997-ACQ65780	u.bacterium(CAL63637) 93 %
GSV13DBT12	FJ904996-ACQ65779	*S.aggregata*(ZP_01549680) 99 %
GSV13DBT7	FJ904995-ACQ65778	u.bacterium(ABW17415) 86 %
GSV13DBT3	FJ904994-ACQ65777	u.bacterium(ABW17382) 85 %
GSV13DBT2	FJ904993-ACQ65776	u.bacterium(CAL63637) 93 %
nirS gene		
DBT26	GU553355-ADD54535	*M.hydrocarbonoclasticus*(ACN97382) 99 %
DBT2GW17	GU553357-ADD54537	*R.pomeroyi*_(YP_165049) 86 %
DBT9GW17	GU553358-ADD54538	*A.aromaticum*(YP_157499) 85 %
DBT13GW17	GU553359-ADD54539	*A.aromaticum*(YP_157499) 88 %
qnorB gene		
DBT20AA10	GU553356-ADD54536	u.bacterium (AEH50055) 87 %
		u.bacterium (AEH49944) 86 %
		u.bacterium (CAD45402) 84 %
		u.bacterium (AEH49905) 86 %
		u.bacterium (AEH50007) 86 %
		u.bacterium (AEH50064) 86 %
		u.bacterium (AEH49888) 84 %
		u.bacterium (CAD45392) 85 %
		u.bacterium (CAD45394) 85 %
nosZ gene		
DBTNZ6	GU122973-ACY92298	*S. aggregata* (ZP_01549649) 99 %
DBTNZ7	GU122967-ACY92292	u.bacterium(ACJ02316) 83 %
DBTNZ51	GU122968-ACY92293	*Rhodobacterales*_sp (ZP_05078392) 98 %
DBTNZ52	GU122969-ACY92294	a.proteobacterium(AFC41135) 100 %
DBTNZ56	GU122970-ACY92295	*S. aggregata* (ZP_01549649) 100 %
DBTNZ58	GU122972-ACY92297	a.proteobacterium(AFC41135) 99 %
DBTNZ67	GU122971-ACY92296	*S. aggregata* (ZP_01549649) 99 %

FJ905013, FJ904993 to FJ905004, GU553355 to GU553359, and GU122967 to GU122973, respectively. The accession number of the *qnorB* gene sequence is GU553356.

Diversity of denitrifying functional genes

Determination of the physiological characteristics of unknown groups of denitrifiers and the development of approaches for identifying active denitrifiers are crucial (Philippot 2005). Predicted amino acid sequences of functional genes were compared with sequences available in the GenBank. Percent similarity of amino acids predicted from gene sequences is depicted in Table 2. Phylogenetic tree based on predicted amino acids encoded by *narG* with bootstrap support of 100 % is presented in Fig. 2. Eight distinct clones were yielded from 28 clones. Five clones (V1DBT4, V1DBT14, V1DBT18b, V1DBT23 and V1DBT24) were most closely related to sequences from estuarine sediments (GenBank: CAL63724, CAL92046, CAL92071, CAL92086, CAL92058, and CAL92131) (Smith et al. 2007) with the identity ranging 78–97 %. The Clone V1DBT21 isolate was closely related to *narG* sequence (GenBank: ABF20959). A further two clones (V1DBT9 and V1DBT17) were related to *narG* sequence (GenBank: ACH90505) derived from sediments of the river (Reyna et al. 2010).

Phylogenetic tree based on predicted amino acids encoded by *napA* was shown in Fig. 3. Comparison with the GenBank database using BLASTN search revealed that sequences showed homology to *napA* sequences from uncultured environmental clones. The Clones GSV13DBT3 and GSV13DBT7 showed 85–86 % similarity at the amino acid level with the clones GenBank: ABW17382 and ABW1741 isolated from coral reef sediments. Clones GSV13DBT12 and GSV13DBT14 have 99 % amino acid homology with *nitrate reductase* gene of *Stappia aggregata* IAM 12614 strain (King 2006). Clones GSV13DBT23 and GSV13DBT24 exhibited 88 % similarity with clone GenBank: ABW17415. The remaining six *napA* clones (GSV13DBT2, GSV13DBT4, GSV13DBT11, GSV13DBT19, GSV13DBT20, and GSV13DBT21) were closely related with the clones from estuarine sediment (Smith et al. 2007).

Phylogenetic tree based on predicted amino acids encoded by *nirS* is presented in Fig. 4. Clone DBT26 matched 99 % with *Marinobacter hydrocarbonoclasticus* strain DSM 8798 (GenBank: ACN97382) at amino

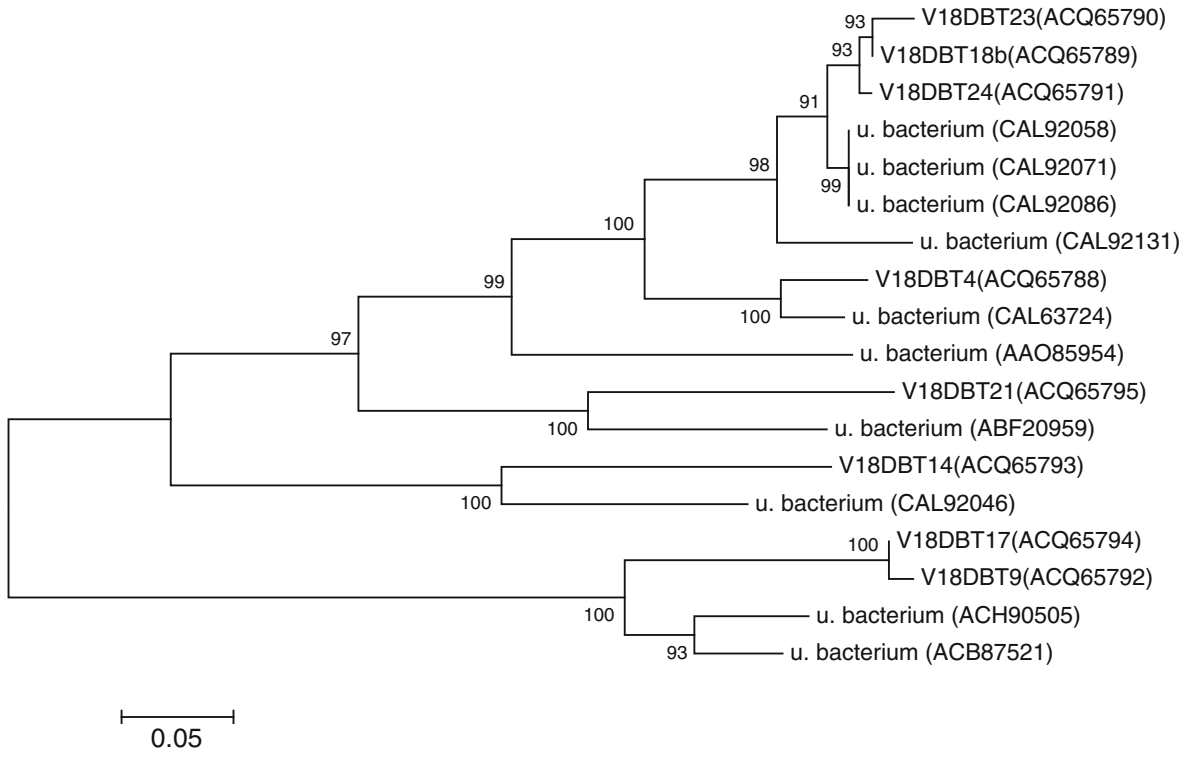

Fig. 2 Phylogenetic tree based on predicted amino acids encoded by narG as determined by maximum likelihood method

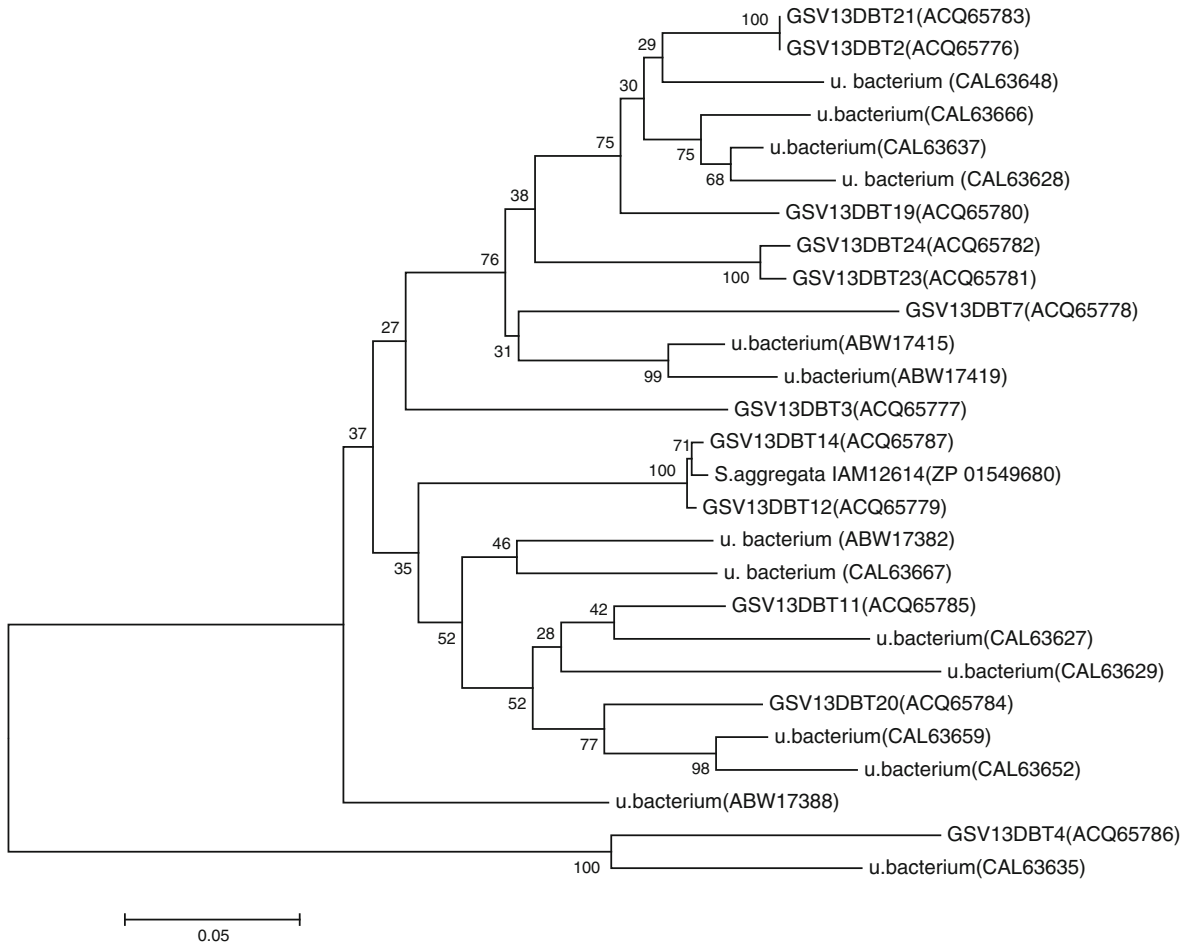

Fig. 3 Phylogenetic tree based on predicted amino acids encoded by napA as determined by maximum likelihood method

acid level (Gonzalez-Domenech et al. 2010). Tiedje (1988) has reported *M. hydrocarbonoclasticus,* which is a true denitrifier as it can reduce nitrate into nitric oxide (N_2O) to the extent of 90 % in the presence of acetylene. Clones DBT13GW17 and DBT9GW17 show 88 and 87 % similarity at amino acid levels with *nirS* gene from *Aromatoleum aromaticum* EbN1 (GenBank: YP_157499) (Kuhner et al. 2005) and *Azoarcus*_sp.KH32C (GenBank: BAL25833), respectively. Clone DBT2GW17 exhibits 86 % similarity with *Ruegeria pomeroyi* DSS-3 (GenBank: YP_165049) (Moran et al. 2007). Prieme et al. (2002) amplified *nirS* genes from marsh soil and most of the clones were not found in cultivated denitrifiers. Krishnani (2010) has reported a *nirS* clone in coastal soil showing similarity with uncultured bacteria from coastal aquaculture.

The *qnorB* clone DBT20AA10 is not correlated to any known cultivable denitrifying isolate. It has a maximum similarity with uncultured bacterial clones retrieved from the Taoyuan Agro-ecosystem Research Station of the Chinese Academy of Sciences, China (Chen et al. 2011). The homology level of clone with uncultured clones ranged 84–87 % at the amino acid level (Table 2). This shows that the retrieved clone is diverse from other *nitric oxide reductase* present in the GenBank. Phylogenetic tree based on predicted amino acids encoded by *qnorB* is presented in Fig. 5. One signature motif such as [YWG]-[LIVFYWTA](2)-[VGS]-H-[LNP]-x-V-x(44,47)-H-H was found in clone sequence. This confirmed that the clone belongs to *nitric oxide reductase*.

Phylogenetic tree based on predicted amino acids encoded by *nosZ* is presented in Fig. 6. The biodiversity in greenwater-bioaugmentation system of coastal aquaculture is reflected in the finding that 23 *nosZ* clones from greenwater yielded seven different clones. The Clones DBTZ6, DBTNZ56, and DBTZ67 show 99–100 % similarity at amino acid level with *nosZ* gene from *S. aggregata* IAM 12614 (GenBank: ZP_01549649) (King 2006). The Clones DBTNZ7, DBTNZ52, DBTNZ58 have 99–100 % sequence

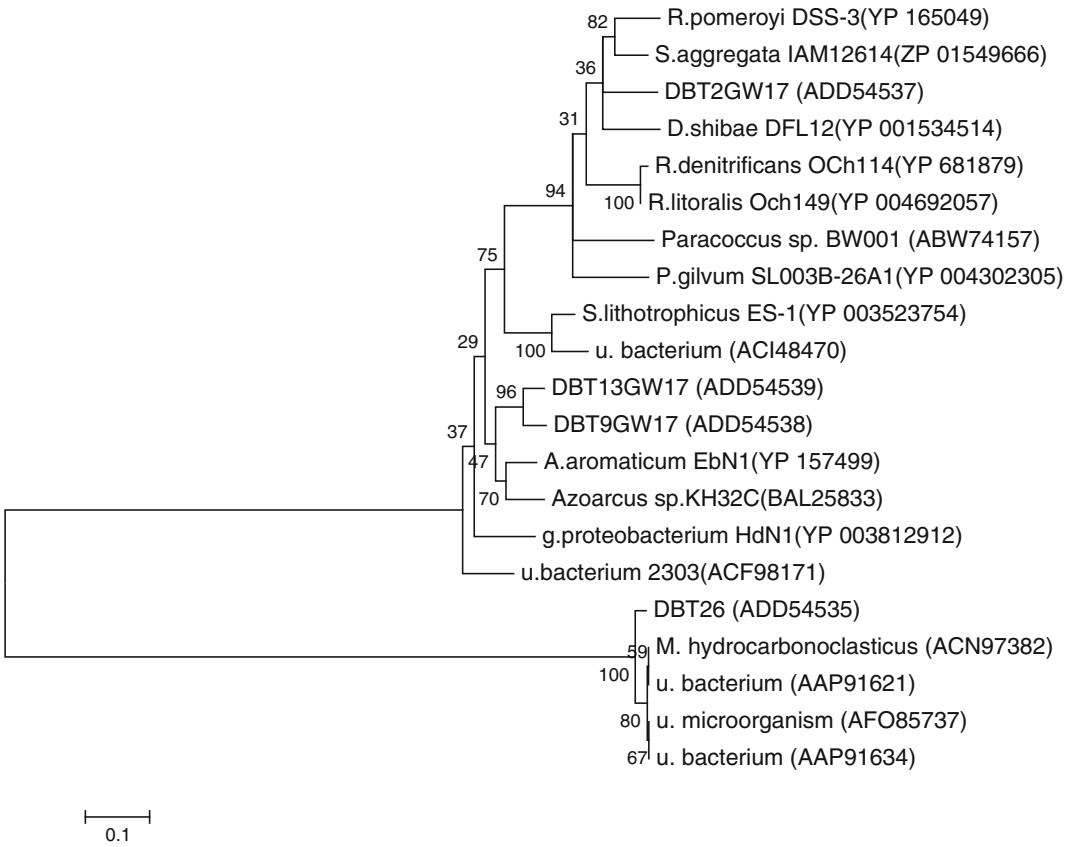

Fig. 4 Phylogenetic tree based on predicted amino acids encoded by nirS as determined by maximum likelihood method

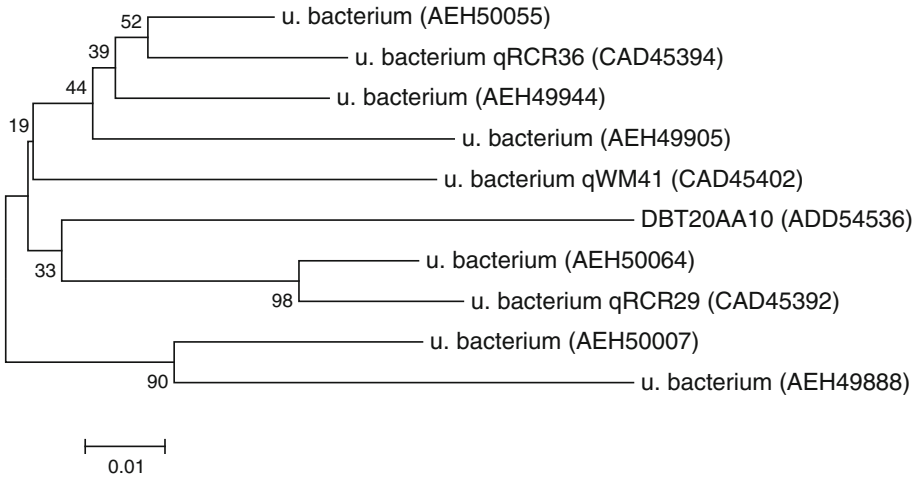

Fig. 5 Phylogenetic tree based on predicted amino acids encoded by qnorB as determined by maximum likelihood method

similarity with *nitrous oxide reductase* genes of Alpha proteobacterium (GenBank: AFC41135). The Clone DBTNZ51 has 93–98 % similarity with *nosZ* sequences from *Rhodobacterales* bacterium Y4I (GenBank: ZP_05078392), *Ruegeria pomeroyi* DSS-3 (GenBank: YP_164881) and *Roseobacter* sp. SK209-2-6 (GenBank: ZP_01754658). Krishnani (2010) has reported three *nosZ* clones having 82 % similarity with *Marinobacter* sp.

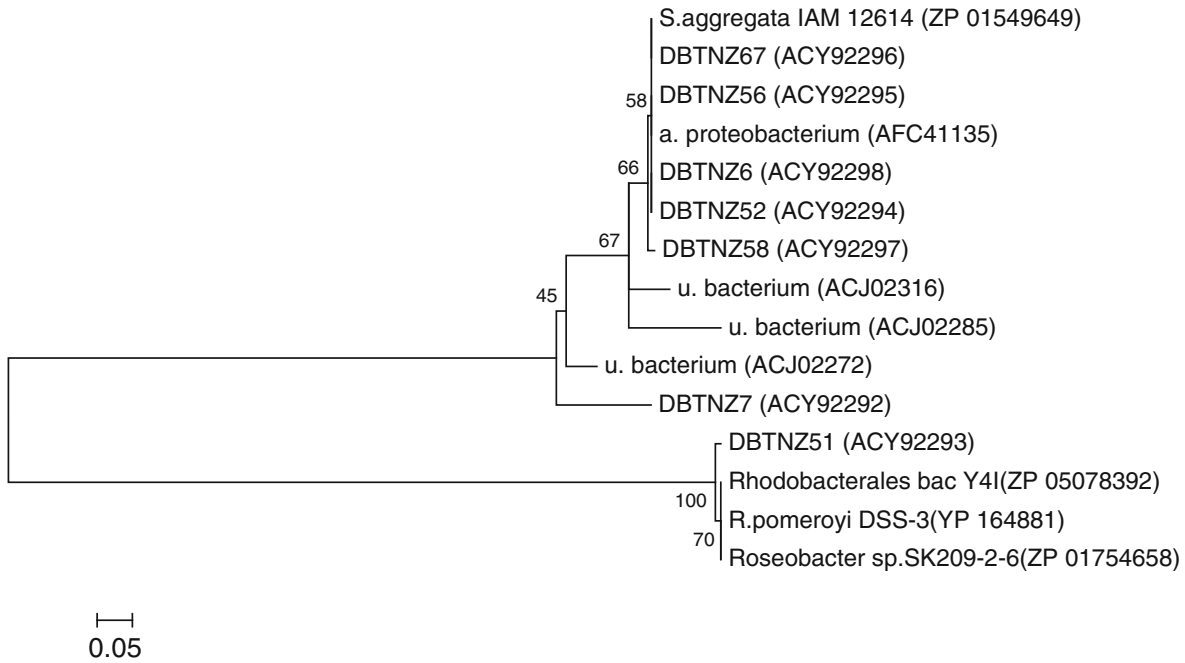

Fig. 6 Phylogenetic tree based on predicted amino acids encoded by nosZ as determined by maximum likelihood method

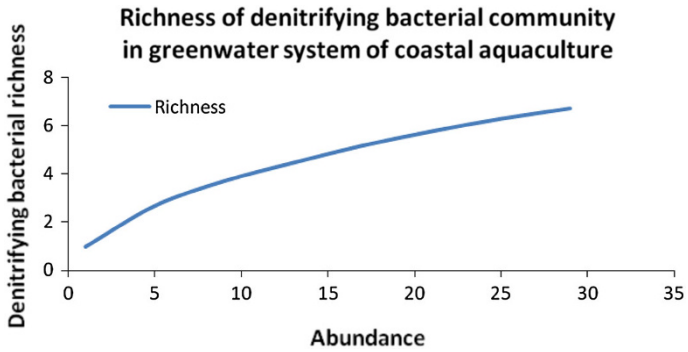

Fig. 7 Richness of denitrifying bacterial community in greenwater system of coastal aquaculture

The richness of denitrifying bacteria is presented in Fig. 7. The results revealed higher denitrifying bacterial diversity in the greenwater system of coastal aquaculture as compared to earlier studies on denitrifying functional genes in Indian coastal aquaculture, which retrieved less denitrifying bacterial diversity with the same primers (Krishnani et al. 2009a, b; Krishnani 2010).

Conclusions

In the present study, the diversity of genes (*narG, napA, nirS, qnorB,* and *nosZ*) encoding complete denitrification in Indian coastal aquaculture has been investigated. To our knowledge, this is the first report for identification of complete denitrification genes especially *qnorB* gene in Indian coastal aquaculture. Maximum clones have similarity with uncultured bacterial clones which revealed that many bacteria yet to be cultured in future to utilize their metabolites. To achieve this objective, studies must be performed in a way that successfully relates microbial diversity and activity. The results confirmed that metagenomic library creation is specific, sensitive and can be used for analysis of uncultivable denitrifying bacterial community from aquatic environment. In conclusion, the denitrifying communities from the greenwater system of coastal aquaculture

are phylogenetically diverse from other environments. This work could be useful in planning the future strategy for understanding of denitrifying communities, nitrogen fluxes, greenhouse gases and their mitigation in coastal aquaculture systems.

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Conflict of interest The authors declare that they have no competing interests.

Authors' contributions VK and KKK defined the research theme, designed methods and experiments. VK carried out the laboratory experiments. VK and KKK analyzed the data and interpreted the results. VK and KKK wrote the manuscript. Both authors read and approved the final manuscript.

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