

Heterotrophic nitrogen removal bacteria in sedimentary and water of striped catfish ponds in the Mekong Delta, Vietnam

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Abstract: A total of 1682 heterotrophic nitrogen removal (HNR) bacteria isolated from sedimentary and water of striped catfish ponds were classified in four kinds of heterotrophic ammonia-oxidizing bacteria (402 isolates), nitrite-oxidizing bacteria (438 isolates), nitrate-oxidizing bacteria (444 isolates) and heterotrophic nitrifying and denitrifying bacteria (398 isolates). The virtually complete 16S rRNA gene was PCR amplified and sequenced. The sequences from the selected HNR bacteria showed high degrees of similarity to those of the GenBank references strains (between 97% and 99.8%). Phylogenetic trees based on the 16S rDNA sequences displayed high consistency, with nodes supported by high bootstrap (500) values. These presumptive HNR isolates were divided four groups that included members of genera *Arthrobacter*, *Corynebacterium*, *Rhodococcus* (high G+C content gram-positive bacteria), *Bacillus* (low G+C content gram-positive bacteria) and *Pseudomonas* (gram-negative bacteria). Based on Pi value (nucleotide diversity), heterotrophic ammonium-oxidizing bacteria group had highest values and heterotrophic nitrifying-denitrifying bacteria group had the lowest values and Theta values (per sequence) from S of SNP for DNA polymorphism showed that heterotrophic nitrate-oxidizing bacteria group had the highest theta values in comparison of three groups. The present study, the HNR bacteria from sedimentary and water of striped catfish ponds, showed a very diverse community of HNR bacteria with a relatively high number of species involved in sedimentary and water samples and many isolates have nitrogen utilization ability at high concentration (800 – 1200 mM) and high G+C gram-positive bacteria strain occupied higher than low G+C gram-positive bacteria strain.

Keywords: Heterotrophic Nitrogen Removal, 16S Rna Gene Sequence, Biologic Nitrogen Removal, Sedimentary And Water Of Striped Catfish Ponds, Gram-Positive Bacteria

1. Introduction

The Mekong delta has a total freshwater area of 641,350 ha or 67.2% total water surface [1]; This delta has the most diversified farming activities and great potential for increased aquaculture production and Catfish farming started at the beginning of the 1960s that included catfish (*Pangasius bacourti*)(Vietnamese name: basa) cultured in small cages and striped catfish (*Pangasianodon hypophthalmus*)(Vietnamese name: tra) cultured mostly in latrine ponds [1]. Striped catfish culture in the Mekong delta is considered a success story of aquaculture in Vietnam with the production and export turnover reached 1,200,000 t worth USD\$ 1 billion in 2007; this result in new challenges for striped catfish

production to change towards more sustainable production with respect to its environmental impact [2].

Water quality in catfish production systems in the Mekong Delta was investigated to assess the potential impacts of this activity on the environment. The feed has been used to catfish with low quality as low protein, high carbohydrate content extensively and the residue of feed and catfish fall down in the bottom of ponds and they have used the anaerobic bacteria and the toxicities have released in the water as ammonia, hydrogen sulfur.....[3]. High N (especially ammonia) in fish-pond water may be toxic to fish and it may be cause eutrophication in canals, streams, small rivers, it threatens the quality of water used for household purposes by many farm-families. Nitrification is the process of converting ammonia to nitrate via nitrite and is mediated by two

groups of chemolithoautotrophic bacteria, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria [4]. Denitrification is part of the bioenergetic apparatus of the bacteria cell, where the N oxidations nitrate and nitrite and the gaseous N oxides NO and N₂O serve in lieu of O₂ as terminal acceptors for electron transport phosphorylation. Nitrogen removal (i.e., the conversion of ammonium and organic nitrogen to nitrogen gas forms) by heterotrophic microorganisms has attracted increasing interest recently in wastewater treatment [5][6] and they have usually been reported as the result of simultaneous heterotrophic nitrification and aerobic denitrification [7]. Specifically, the pathway has been widely accepted as the removal of NH₄⁺ to NO₂⁻ or NO₃⁻ (heterotrophic nitrification) and simultaneously aerobic conversion of the NO₃⁻ or NO₂⁻ to N₂O and/or N₂ (aerobic denitrification) as *Alcaligenes faecalis* [8], *Bacillus* sp. [9], *Acinetobacter calcoaceticus* [10] and they were isolated from other different sludges.

At present, molecular methods based on 16S rRNA have been used widely to study the population structure of bacteria domain. In this study, molecular methods based on 16S rRNA was used to identify the population composition of heterotrophic nitrogen removal (HNR) bacteria and drop plate count method [11] (Hoben and Somasegaran, 1982) to enumerate HNR bacteria in sedimentary and water of striped catfish ponds in the Mekong Delta, Vietnam. The aims of this study were to quantify HNR bacteria populations and identify their diversity in sedimentary and water of striped catfish ponds and isolates were identified gram staining, population and selected representative strains were identified at the molecular level using 16S rRNA sequence analysis.

2. Method

2.1. Isolation of Heterotrophic Nitrogen Removal Bacteria (HNRB)

The sources for isolating micro-organisms were solid waste (sedimentation) and wastewater of catfish-ponds (10 provinces/city) in the Mekong Delta (Figure 1), samples were stored at 15-20°C in plastic containers and they were moved to laboratory to stored in the refrigerator.

2.2. Media

Media were used in this study [10] with a standard medium was prepared for enrichment and isolation of bacteria by dissolving 10 g of peptone, 10 g of beef extract, and 5 g of NaCl in distilled water (per liter). This standard medium was autoclaved for 30 min at 121°C.

The ingredients of a basal medium in 100 ml distilled water (pH 8) were as follows: 0.4 g of NaCl, 2.15 g of Na₂HPO₄, 0.09 g of KH₂PO₄ and 3 ml of trace elements solution. The trace elements solution contained 0.3 g of MgSO₄·7H₂O, 0.1 g of MnSO₄, 0.112 g of H₃BO₃, 0.03 g of FeSO₄·7H₂O and 0.06 g of CaCl₂ (per liter). Different amounts of nitrogen and organic carbon sources were added

to basal medium for groups of nitrifiers or denitrifiers (Table 1). Each basal medium was autoclaved for 15 min at 110°C. The chemicals were purchased from Merck.

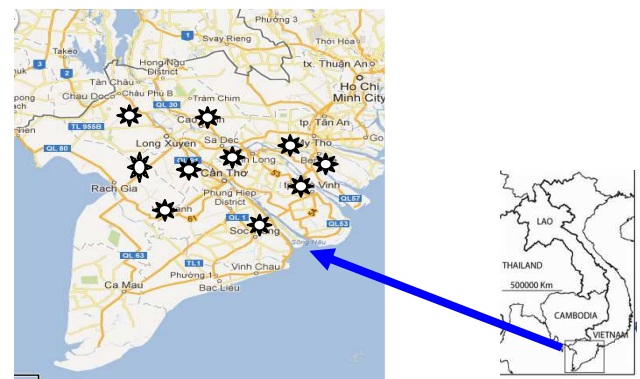


Figure 1. Samples were collected at sedimentary and water of striped catfish ponds of ten city/provinces (*) in the Mekong Delta, Vietnam (from Google map).

Table 1. List of nitrogen and carbon amount added in the basal medium (per 100 ml).

Component	Nitrogen and carbon amount			
	A	B	C	D
^a NH ₄ Cl solution (ml)	12			4
^b NaNO ₂ solution (ml)		4		4
^c NaNO ₃ solution (ml)			4	4
Glucose (g)	0.3	0.1	0.1	0.3

^a contained 1 mg/ml of NH₄⁺ -N

^b contained 1 mg/ml of NO₂⁻ -N

^c contained 1 mg/ml of NO₃⁻ -N

2.3. Count and isolation of Bacteria in the Material

The samples were agitated to obtain homogeneous suspensions between water and sedimentary in sterile distilled water. Suspended liquid (100 µl) was piped into a tube (10 ml) that contained the standard medium. After 48 h of aerobic incubation at 30°C and 120 rpm, 1 ml suspended liquid were suspended in 90 ml of sterile distilled water in flask-250 mL for 10 min a shaker [New Brunicks, USA]. The supernatant was appropriately diluted using sterile distilled water with 10⁻², 10⁻³...dilution. Five drops put on the media A (for ammonium), the media B (for nitrite), the media C (for nitrate) and the media D (combination of ammonium, nitrite, nitrate) with each dilution and they were incubated in 30°C. After 24 or 48 h, generated colonies were counted for calculating colony-forming units per 1 ml or 1 g of dry matter (CFU g⁻¹ DM). Simultaneously, each isolate was cultivated in each medium to detect the ability of ammonium, nitrite, nitrate or combination of three kinds of above nitrogen. Purified isolates were obtained by repeated streaking on fresh agar plates. A bacterium with high nitrogen removal efficiency was obtained and named and they were suspended in 20% glycerol solution at -80°C for



long-term storage.

2.4. DNA Extraction, PCR Amplification and 16S rRNA Gene Sequence Analysis

DNA was extracted from a bacterial suspension (1 ml from a TSB medium at 30°C and 120 rpm for 24h) to DNA protocol of Neumann *et al.* [12]. Primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTACGACTT-3')[13] were used to amplify 16S rRNA gene by a PCR protocol. Amplification was performed in a total volume of 50 µl in 0.2 ml Eppendorf tubes using a DNA thermocycle (BioRAD). The reaction mix was prepared using the following: 1 x PCR buffer (20 mM Tris-HCl-NH₄SO₄) with 5 µl, 4 µl dNTP (20 nmol of each deoxynucleoside triphosphate), 2 µl primer 8F; 2 µl primer 1492R (30 pmol of each primer), 0.5 µl BSA (100 µg of bovine albumin per ml), 2 µl of template DNA and 2.5 U of *Taq* DNA polymerase (Fermentas, Singapore) and 24 µl biH₂O. The standard thermal profile used for amplification of the 16S rRNA sequence was as follows: 5 min at 95°C; then 30 cycles consisting of 30 s at 94°C (denaturation), 30 s at 53°C (annealing), and 90 s at 72°C (elongation) and a final cycle of consisting of 10 min at 72°C. Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Partial 16S rRNA gene of selected isolates in each nitrogen group was sequenced by MACROGEN, Republic of Korea (dna.macrogen.com). Finally, 16S rRNA sequence of the isolate was compared with that of other microorganisms by way BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>); In each site (city or province) and the best isolate(s) (bacterial isolate can utilize the highest nitrogen concentration) in every group (bacteria utilize ammonia, nitrite, nitrate or combination of three nitrogen kinds) and 10 isolates of 10 sites were chosen to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequences to show relationships between heterotrophic nitrogen strains [14] and phylogenetic tree were constructed by the neighbor-joining method based on 500 bootstraps.

2.5. SNPs Discovery

The sequence data from 64 nitrogen removal bacterial isolates were analysed with SeqScape@ Software (Applied Biosystem, Foster City, CA, USA). SeqScape is a sequence comparison tool for variant identification, SNP discovery and validation. It considers alignment depth, the base calls in each of the sequences and the associated base quality values. Putative SNPs were accepted as true sequence variants if the quality value exceeded 20. It means a 1% chance basecall is incorrect.

2.6. Nucleotide Diversity (Θ)

Nucleotide diversity (Θ) was calculated by the method described by Halushka *et al.* [15].

$$n$$

$$\Theta = K/aL$$

$$a = \sum_{i=2}^n l(i-1)$$

$$i=2$$

where K is the number of SNPs identified in an alignment length, n is alleles and L is the total length of sequence (bp).

3. Results and Discussion

The suspended solid wastewaters have high concentration of ammonia and pH varied from 4.12 to 7.47 (Table 2) especially pH of sedimentary and water from striped catfish ponds related with nitrogen removal bacterial population closely (Figure 2).

Table 2. pH and nitrogen concentrations in piggery sewage of 13 city/provinces in the Mekong Delta, Vietnam.

Location	pH	NH ₄ ⁺ concentration in sewage (mg/wet litre)	
		Dry season	Wet season
AN GIANG	3.53 – 7.52	105 - 557	2110 – 2430
BẠC LIÊU	4.86 – 7.10	1226 - 1710	132 – 1596
BẾN TRE	4.63 – 7.08	1036 - 2018	122 – 458
CÀ MAU	3.73 – 7.33	521 - 584	127 – 386
CẦN THƠ	4.32 – 7.11	68 - 113	59 – 68
ĐỒNG THÁP	5.55 – 7.05	3000 - 3331	30 – 53
HẬU GIANG	4.03 – 7.58	185 - 4270	42 – 4056
LONG AN	4.95 – 7.49	1313 - 1877	963 – 1071
KIÊN GIANG	4.01 – 7.33	448 - 755	125 – 154
SÓC TRĂNG	6.51 – 8.02	18 - 23	370 – 401
TIỀN GIANG	5.71 – 7.41	616 - 837	854 – 903
TRÀ VINH	5.61 – 7.93	657 - 679	92 – 152
VĨNH LONG	5.56 – 7.44	1313 - 1877	42 - 4056

From 159 sedimentary+water samples of striped catfish pond, 1682 heterotrophic nitrogen removal bacterial isolates were isolated with 402 heterotrophic ammonia-oxidizing bacteria (HAOB) isolates, 438 heterotrophic nitrite-oxidizing bacteria (HNiOB) isolates, 444 heterotrophic nitrate-oxidizing bacteria (HNaOB) isolates and 398 heterotrophic nitrifying and denitrifying bacteria (HNDB) isolates (Table 3) especially many isolates can utilize at high concentration (800 – 1200 mM)(ammonium, nitrite or nitrate) in each group however the bacterial isolates having three kinds of nitrogen ability only grew on 300 mM (ammonium+nitrite+nitrate) media.

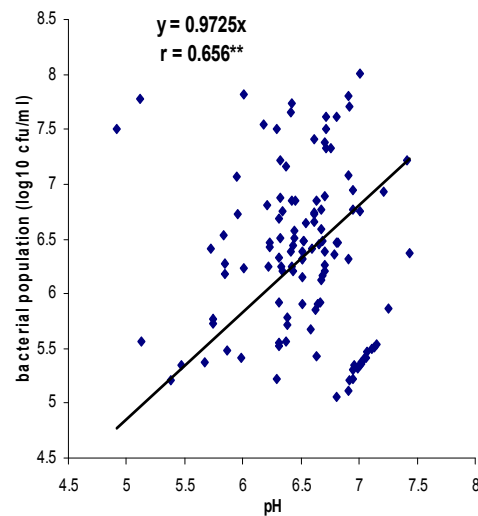


Figure 2. Correlation between pH and nitrogen removal bacterial population in sedimentary and water of striped catfish pond samples in 10 city/provinces on the Mekong Delta, Vietnam.

Table 3. Nitrogen removal bacterial isolates isolated from 159 sedimentary and water samples from striped catfish ponds in 10 city/provinces of the Mekong Delta, Vietnam.

No	City/ province	HA OB	HNi OB	HNa OB	Combination*	Total **
01	An Giang	55	65	52	75	247
02	Ben Tre	29	45	51	37	162
03	Can Tho	49	61	51	5	166
04	Dong Thap	58	55	55	51	219
05	Hau Giang	66	67	65	77	275
06	Kien Giang	22	29	31	32	114
07	Soc Trang	42	33	47	44	166
08	Tien Giang	13	30	32	10	85
09	Tra Vinh	40	27	18	27	112
10	Vinh Long	28	26	42	40	136
Total		402	438	444	398	1682

HAOB: Heterotrophic Ammonium Oxidation Bacteria

HNiOB: Heterotrophic Nitrite Oxidation Bacteria

HNaOB: Heterotrophic Nitrate Oxidation Bacteria

* bacterial isolates utilize three kinds of nitrogen (ammonium, nitrite, nitrate)

** total of four groups

Their colonies have round-shaped or not identified; milky; light brown, pink yellow; entire or lobate margin (Fig. 3) and all of them are Gram-positive by Gram stain. The cells were observed by SEM and appeared as short rods and most of them have motility (Figure 4).

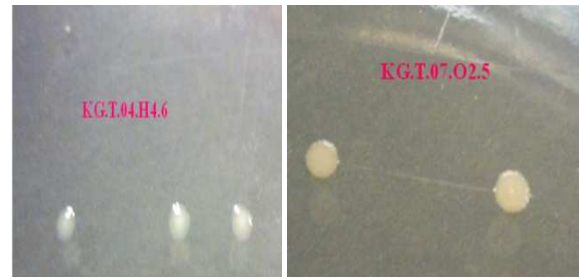


Figure 3. The colonies of several nitrogen removal bacterial isolates.

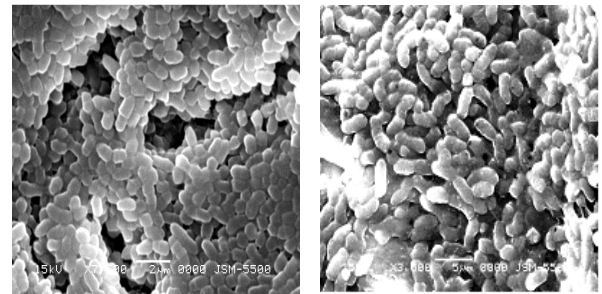
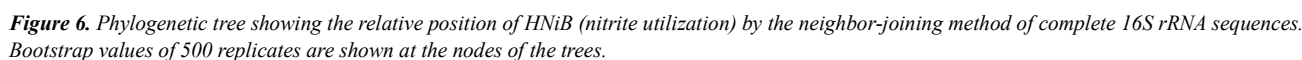
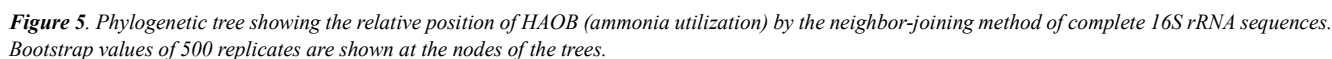


Figure 4. Scanning electron micrograph of two nitrogen removal bacterial isolates.

The fragments of 1485 bp 16S rRNA were obtained from PCR and sequencing. Homology searches of the 16S rRNA gene sequence of selected strain in GenBank by BLAST revealed that they had high similarity to sequences of Firmicutes phylum. A neighbor-joining phylogenetic tree in HAOB group showing the two clusters: big cluster with two clusters as cluster A1 *Bacillus* with VINHLONG, BENTRE, SOCTRANG, TRA VINH, KIEN GIANG isolates and cluster A2 with DONGTHAP, ANGIANG, CANTHO, HAU GIANG were classified as *Arthrobacter*. Cluster B with TIENGIANG was identified as *Pseudomonas* (Gram-negative bacteria)(Figure 5). The results showed that HAOB group had relationship closely even through they were isolated from various sites in the Mekong Delta.

In HNiOB group, phylogenetic tree showed that two clusters with genus *Arthrobacter* and *Rhodococcus* (high G+C gram-positive bacteria) in cluster A with HAUGIANG, VINHLONG, BENTRE, DONGTHAP, CANTHO, SOCTRANG and *Bacillus* and *Pseudomonas* (gram-negative bacteria) in cluster B with ANGIANG, TIENGIANG, KIENGIANG, TRAVINH (Figure 6).

In Figure 7 presented two clusters in HNaOB group with SOCTRANG, CANTHO, VINHLONG, KIENGIANG, HAUGIANG, TRAVINH, ANGIANG, BENTRE isolates were determined as genus *Bacillus* in cluster A while cluster B composed of TIENGIANG and DONGTHAP isolates were genus *Corynebacterium* and *Arthrobacter*. With HNDB group, phylogenetic tree showed that two clusters: cluster A composed of cluster A with ANGIANG, SOCTRANG, KIENGIANG, TRAVINH isolates in genera *Bacillus* and cluster B1 with HAUGIANG and TIENGIANG isolates were genus *Rhodococcus* and cluster B2 with BENTRE, CANTHO, DONGTHAP isolates were classified in genus *Arthrobacter* (Figure 8).



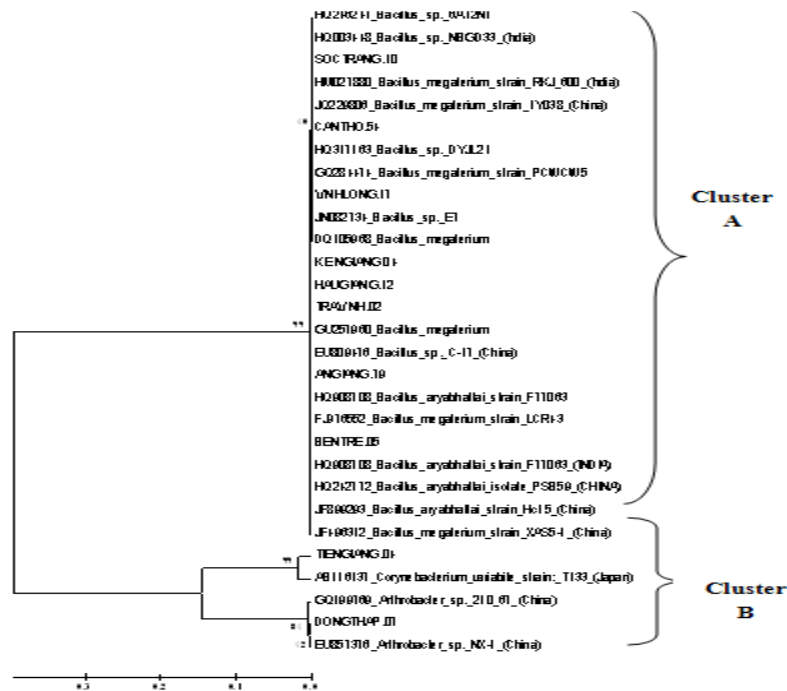


Figure 7. Phylogenetic tree showing the relative position of HNAB (nitrate utilization) by the neighbor-joining method of complete 16S rRNA sequences. Bootstrap values of 500 replicates are shown at the nodes of the trees.

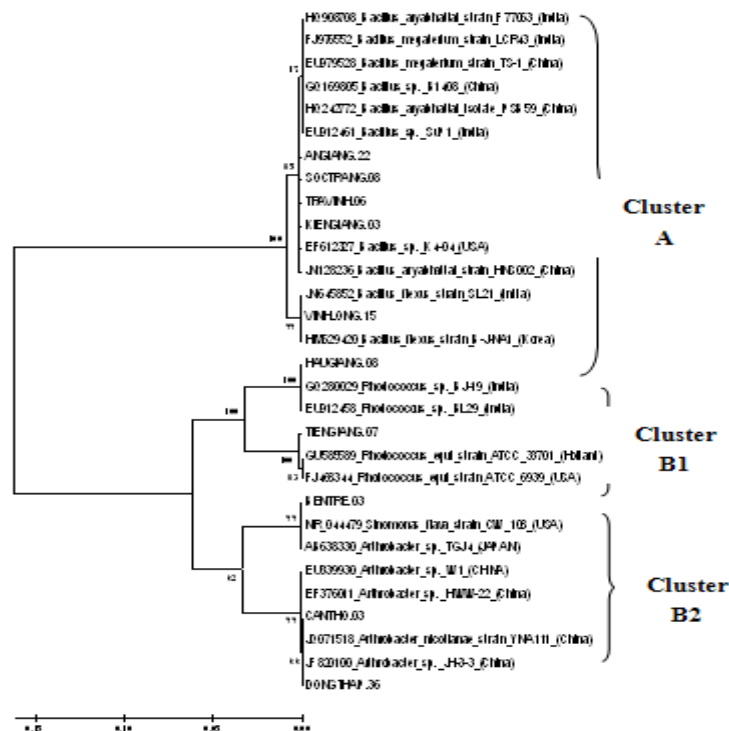


Fig.8. Phylogenetic tree showing the relative position of HNDB (ammonia, nitrite, nitrate utilization) by the neighbor-joining method of complete 16S rRNA sequences. Bootstrap values of 500 replicates are shown at the nodes of the trees.

Nucleotide polymorphism can be measured by many methods, for example, haplotype (gene) diversity, nucleotide diversity, (Pi), Theta (Θ) (per site) etc.

In this study, nucleotide diversity was estimated as Theta (Θ), the number of segregating sites [16], and its standard deviation (S Θ). These parameters were estimated by DNA

Sequence Polymorphism software version 4.0 [17].

Pi value explained nucleotide diversity of sequences for each gene. The higher values, the more diversity among HAOb group had highest values and HNDB group had the lowest values. Theta values (per sequence) from S of SNP for DNA polymorphism were calculated for each group.

HNaOB group had the highest theta values in comparison of three groups (Table 4).

Table 4. Nucleotide diversity (Θ) values of two ESTs using the programme DNAsp 4.0 [17].

Primer 8F 5'-AGAGTTTGATCCTGGCTCAG-3'

Primer 1492R 5'-TACGGTTACCTGTTACGACTT-3'

ESTs	HAOB	HNiOB	HNaOB	HNDB
Nucleotide diversity (Pi)	0.72447	0.71805	0.72206	0.70353
Theta (per sequence) from S	238.91 81.46	± 293.57 ± 110.13	318.70 ± 119.53	238.62 ± 81.37

Water pollution is one of the challenges, which threatens the striped catfish culture and environment and TAN concentration in water of catfish ponds varied from 0.033 – 4.602 mg/L and the catfishes died in ponds when TAN concentration was higher 2.0 mg/L [18] however high TAN concentration in water of catfish-ponds (in dry-season) has been diluted and removed by the rains and the flood from Mekong river in the wet-season therefore TAN concentration in water of catfish-ponds is lower than in wet-season (Table 2). Many strains of *Pseudomonas stutzeri* were isolated and identified in sedimentary and water of catfish-ponds in the Mekong Delta, Vietnam and many bacteria isolates can oxidize ammonia in the water and a few of isolates has high ammonia oxidation ability and they were applied to remove nitrogen in water of catfish-ponds [19]. *Pseudomonas stutzeri* strains have strong ability to remove nitrogen in wastewater in piggery wastewater [20] and they reported that *P. stutzeri* NS-2 strain had excellent denitrifying capability under aerobic and anaerobic conditions, as revealed by nitrate disappearance and nitrogen gas production. Nguyen *et al.* [21] reported that *Bacillus* is the most common group used for treating water pollution in aquaculture ponds. It comprises three common species including *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus megaterium*.

4. Conclusion

Bacillus is main genus in nitrogen remove bacteria groups which was found in sedimentary and water of striped catfish-ponds in the Mekong Delta, Vietnam and the best heterotrophic nitrogen removal strains will be applied to treat water pollution in catfish ponds.

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