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Full Length Research Paper

Diversity of NosZ gene in three municipal wastewater treatment plants located in different geographic regions

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Diversity of nitrous oxide reductase genes (NosZ) in activated sludge was compared in the biological treatment process (BTCs, anaerobic, anoxic and aerobic process) of three municipal wastewater treatment plants located in different geographic regions. A total of 18 specific bands generated after polymerase chain reaction- denaturing gradient gel electrophoresis (PCR-DGGE) analysis. The diversity of NosZ in MWTPs of Nanjing is higher than that of Xiamen and Foushan, and the similarity of DGGE bands from BTCs in the same MWTP is obviously higher than that of the same BTC in different MWTPs. 12 of 18 unique bands were selected, sequenced and identified by searching against Genbank using BLASTn, and the highest identity and score values of the isolated sequences were the NosZ gene of uncultured bacterium in non-redundant database of Genbank. However, the most probable affiliations of the bacteria harboring the NosZ gene were the denitrifying bacteria from α- or β-proteobacteria, including *Alicycliphilus denitricans*, *Herbaspirillum sp*., *Paracoccus denitrificans*, and *Rhodopseudomonas palustris*. Phylogenetic analysis of the twelve sequences indicated that all the sequences could be divided into two main clusters. Each cluster not only contains species from α-proteobacteria but also from β-proteobacteria.

Key words: Denaturing gradient gel electrophoresis (DGGE), municipal wastewater treatment plants, nitrous oxide reductase, diversity.

INTRODUCTION

N_2O is a kind of greenhouse gas having long estimated half-life (approximately 120 years) and has a global warming potential about 310 times that of carbon dioxide (Stres et al., 2004). 0–14.6% of the nitrogen can be converted into N_2O in full-scale wastewater treatment plants (MWTPs), and 90% of the N_2O emission happened in the activated sludge compartments (Czepiel et al., 1995; Tallec et al., 2008; Kampschreur et al., 2009). N_2O can be deoxidized into molecular nitrogen with the help of nitrous oxide reductase that encoded by NosZ gene, and this process is the last step in the complete denitrification pathway (Throbäck et al., 2004).

The denitrification process is an important way for N_2O removal. However, the denitrifying bacteria are phylogenetically diverse (You, 2005). About 130 denitrifying bacteria species have been identified from more than 50 genera (Zumft, 1992). NosZ gene has been used to target the denitrifying bacteria in various samples by using polymerase chain reaction- denaturing gradient gel electrophoresis (PCR-DGGE) technique (Kandeler et al., 2006; Dandie et al., 2007), because PCR-DGGE can simultaneously analyze a large number of community samples for structural comparison of microbial communities from different environments (Miura et al., 2007; Liu et al., 2002).The distribution of NosZ gene has been studied in MWTPs to reveal the diversity of NosZ gene in the MWTPs, although many of the bacteria that having the NosZ gene were not identified in MWTPs. Recently, it was shown to have the greatest level of congruence with 16S rRNA-based taxonomic classification in the molecular markers for denitrifiers (Jones et al., 2008).

Processes anoxic/anaerobic/aerobic (A/A/O) has been widely adopted to remove N and P in waste water in

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MWTPs and its relative strict anaerobic, anoxic and aerobic process provides convenient model to study the difference in the diversity of NosZ gene among these different biotechnical tanks. In this study diversity of NosZ gene in activated sludge collected from different biochemical treatment compartments in three MWTPs were compared by using PCR-DGGE and subsequent sequence analysis. The impact of the influent and the process of wastewater treatment on the diversities of NosZ gene were discussed.

MATERIALS AND METHODS

Wastewater treatment plants and sample collection

The three MWTPs located in different geographic areas of China were analyzed, that is, plant NJ in Nanjing (118°50'E 32°02'N); plant XM in Xiamen (118°04' E, 24°26' N); and plant FS in FoShan (113°06'E 23°02'N). Plant NJ and FS mainly received municipal wastewater while Plant XM received industrial wastewater about 70% of the influent. All the MWTPs used a similar process based on Anaerobic-anoxic-aerobic (A^2O). The operating data were listed (Table 1) and information of the processes was provided in Figure 1.

Activated sludge samples were collected from each biological treatment compartments (BTCs; anaerobic, anoxic and aerobic process) of the plant NJ at May and September, 2008; and for plant XM and FS at September 2008. Activated sludge samples were placed in sterile polypropylene tubes in a box with dry-ice, taken to the lab within 24 h and stored at -20°C.

Extraction and purification of DNA

Total DNA was extracted as described by Zhou et al. (1996). Further purification of the activated sludge DNA extracts were done with EZ Spin Column DNA Gel Extraction Kit UNlQ-10 (Sangon, ShangHai, China). DNA quality and quantity was verified by agarose gel electrophoresis and Ultraviolet spectrophotometer.

NOSZ gene fragment amplification

The primers NosZF-GC (5'- GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG CCG YTG TTC MTC GAC AGC CAG-3') and NosZ1622R (5'-CGS ACC TTS TTG CCS TYG CG-3') (Kloos et al., 2001; Throbäck et al., 2004) synthesized by Jinsite Biotechnology (Nanjing, JiangSu, China) were used to amplify fragments of the NosZ genes. The universal 40 bp GC-clamp added to PCR fragment was used for mutation analysis by DGGE. Amplification was performed in a total volume of 25 µL with 2.5 µL 10×PCR buffer, 1.5 µl Mg^{2+} (25 mM), 0.5 µL dNTP (10 mmol/L), 0.2 U Taq polymerase (TianGen, Beijingi, China), 1 µl primers (10 µM each), 1 µL DNA extract and 18.3µL sterile deionized water. Touchdown PCR was performed according to the method described by Enwall et al. (2005). The PCR products were analyzed on 1% agarose gel and quantified using a 100 bp DNA ladder-H1 (Jinsite Biotech, NanJing, China).

DGGE profiling

The DGGE was performed according to the method of Throbäck et al. (2004) by using the Dcode TM Universal Mutation Detection System (Bio-Rad Co.,USA). After electrophoresis for 17 hours at 130 V and 60°C, the gel was stained with rapid silver staining (Sugano et al., 1993). Gel images were photographed with a GS-800 Grayscale scanner and analyzed with Quantity One software (Bio-Rad Laboratories, Inc, USA). The experiments were carried out in triplicate.

The processing of the DGGE gel patterns was done by the Bio-rad software Quantity one 4.3.0. DGGE sketch map was generated automatically to provide an access to clear insight into the DGGE patterns. DGGE fingerprints were automatically scored by the presence or absence of co-migrating bands. Dendrogram was constructed with unweighted-pair group method using average linkages (UPGMA) algorithms to compare the diversity of denitrifier community encoded by NosZ gene in MWTPs.

Dice coefficients on the DGGE patterns were calculated for representing the similarity between different samples. Dice index (Cs) were calculated by the formula (Dice, 1945): Cs=2j/ (a+b), where j is the number of bands common to samples A and B, and a and b are the number of bands in samples A and B, respectively.

Sequencing

To confirm whether the bands were from NosZ gene, 12 unique discernable DGGE bands from each lane were excised and re-amplified for nucleotide sequence analysis. The target bands were carefully excised from the DGGE gel with a sterile scalpel. Each band was washed with 60 µL sterile ddH_2O faster, crumbled and then stored in 40 µL ddH_2O. After incubation at 4°C for 12 h, 2 µL of elute was used as template to amplify the sequence by using primers NosZF-GC and NosZ1622R. PCR was performed according the methods described above. The PCR products were sequenced by Nanjing Jinsite Biotechnology Company.

BLAST and phylogenetic analysis

The homologue was analyzed by the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) Alignment of DNA sequences was done with the ClustalX 1.83 program, and phylogenetic tree was edited with Mega 4.1. Phylogenetic tree was constructed by the neighbor-joining method with 1,000 replicate trees.

Nucleotide sequence accession numbers

The twelve partial sequences of NosZ gene were submitted to the GenBank under accession numbers: HQ842598 to HQ842609.

RESULTS AND DISCUSSION

The behavior of the treatment process related to the microbial community structure closely, and the DGGE profiles of NOSz gene could express the nitrification stability. As shown in Figures 2A and B, each sample exhibits district DGGE patterns. 13, 8 and 8 bands were, respectively, detected in anaerobic, anoxic and aerobic process of plant NJ at May, 2008 and 7, 9 and 8 at September 2008, respectively; for plant FS, 3, 3 and 3, respectively; and for plant XM, 6, 6 and 5, respectively. Hierarchical cluster analysis was used to demonstrate similarities in the banding profiles of samples, and the results were presented in the form of UPGMA dendrograms. As shown in Figure 3, all the samples were

Table 1. Summary of operating data of the three MWTPs at full scale.

MWTPS	Month	C (t/d)	T (°C)	pH	The characteristics of influent (mg/L)						The removal rate of WMTPs treatment (%)					
					BOD_5	COD	SS	NH_4^+-N	TN	TP	BOD_5	COD	SS	NH_4^+-N	TN	TP
Plant NJ	May	40000	24.1	6.91	92.9	206.8	276.0	15.2	-	3.1	91.3	80.6	97.3	80.2	-	91.9
	Sept	40000	26.1	6.96	125.9	298.8	467.5	23.9	-	4.5	93.4	84.5	97.5	82.4	-	84
Plant FS	Sept	44000	27	7.21	282	1130	504	-	66.3	10.1	-	77.2	94.1	81.2	68.6	71.2
Plant XM	May	200000	25.6	7.03	113.8	260	266.5	21.6	27.7	4.3	95.9	93.0	95.6	88.6	50.9	84.1

C, the treatment capacity of MWTPs; T, average temperature of the month; BOD_5, biochemical oxygen demand for 5 days; COD, chemical oxygen demand; SS, suspended solids; NH_4^+-N, ammonia nitrogen; TN, total nitrogen; TP, total phosphorus.

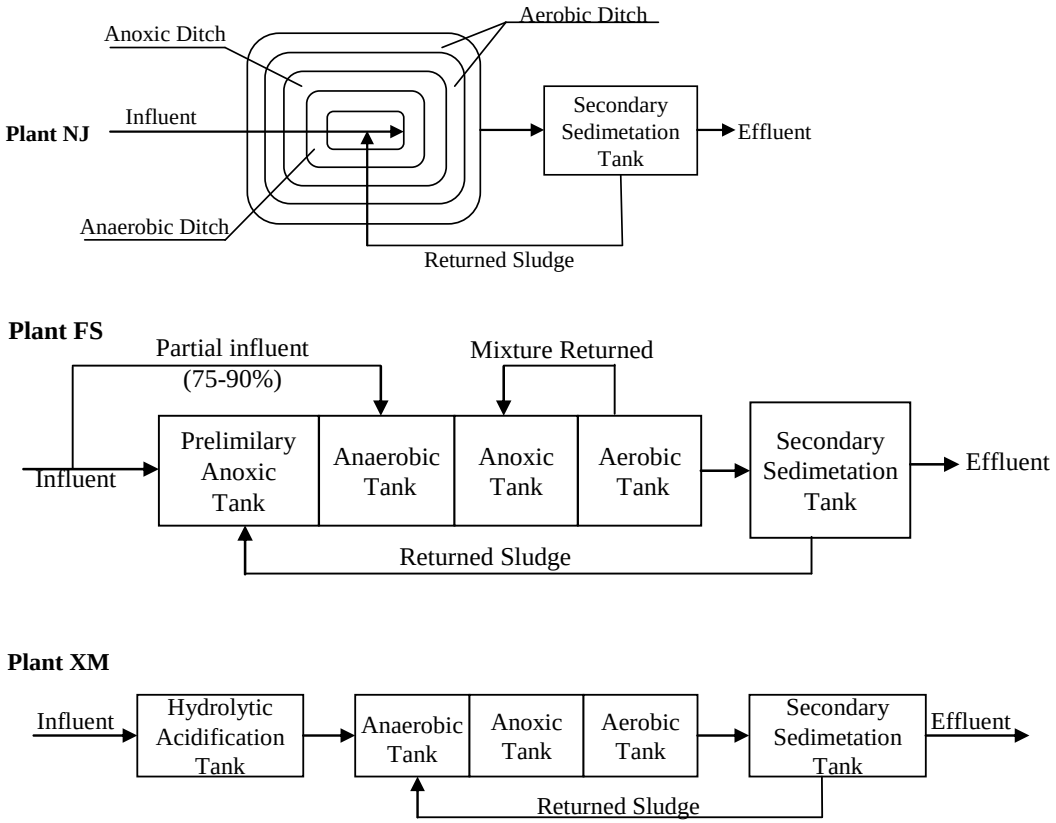

Figure 1. The schematic diagram of flow chart of the three MWTPs.

Table 2. The Dice coefficient of activated sludge in different BTCs.

Lane	FS1	FS2	FS3	XM1	XM2	XM3	NJ1	NJ2	NJ3	NJ4	NJ5	NJ6
FS1	100.0											
FS2	83.3	100.0										
FS3	83.9	97.9	100.0									
XM1	73.0	74.8	76.5	100.0								
XM2	70.0	75.2	76.9	91.8	100.0							
XM3	71.3	82.0	83.7	85.5	90.9	100.0						
NJ1	63.6	61.8	63.3	62.3	62.3	61.2	100.0					
NJ2	64.4	62.5	64.1	63.0	62.9	61.8	92.4	100.0				
NJ3	60.1	58.8	60.2	59.5	59.6	58.5	88.3	89.0	100.0			
NJ4	67.6	68.4	70.0	68.3	68.1	67.1	88.3	89.4	84.7	100.0		
NJ5	63.5	62.6	64.1	63.0	63.0	61.9	72.6	79.3	78.9	81.7	100.0	
NJ6	59.0	60.5	61.9	61.1	61.1	60.1	80.5	85.0	78.9	88.9	85.7	100.0

Lane 1, 2, 3 are aerobic tank, anoxic tank, anaerobic tank of Plant C in May 2008 respectively; Lane 4, 5, 6 are aerobic tank, anoxic tank, anaerobic tank of Plant B in September 2008, respectively; Lane 7, 8, 9 are aerobic tank, anoxic tank, anaerobic tank of Plant A in September 2008, respectively; Lane 10, 11, 12 are aerobic tank, anoxic tank, anaerobic tank of Plant A in May 2008, respectively.

divided into 3 major groups and samples from the same MWTPs were clustered into the same group. Table 2 shows the Dice coefficients of activated sludge in the BTCs. The Dice coefficients between BTCs in the same plant were 81.7-92.4% in Plant A, 88.3-92.4% in Plant B, and 83.3-97.9% in Plant C. The Dice coefficients of the same BTC in different plants were 63.6-73, 62.5-75.5 and 60.2-83.7% for the anaerobic tank, anoxic tank and aerobic tank, respectively. Dice coefficients of different BTCs in the same plants are higher than that of the same BTC in different plants.

The changes in diversity of bacteria community may be associated with the influent, the operational parameter of MWTPs and the operation for treating activated sludge (Forney et al., 2001). In this study, 7-13 bands in Plant NJ, 3 in FS and 5-6 in XM were detected, indicating the diversity of NosZ gene in NJ is higher that that in FS and XM and that bacterial harboring NosZ gene might contribute to the removal of N_2O. The diversity of denitrifying bacteria in plant NJ is higher than that in plant XM and FS, since NosZ gene can be used to target the denitrifying bacteria instead of 16S rRNA gene sequences (Ishii et al., 2011). In this study, Fig.3 and Tab 2 show that diversity of NosZ gene among there BTCs in the same plant is higher than that in the same BTCs among three plants, indicating influents may affect the denitrifying community significantly than the operating process. Similarly, Miura et al. (2007) found that influent wastewater composition had a larger impact on bacterial community structures. However, the differences in NosZ gene diversity were also detected between May and September in Plant NJ, supporting the viewpoints that the season might also affect the diversity (Mergel et al., 2001). Differences of microbial community structure between sampling sites was greater than seasonal variability within each site (Bossio et al., 2006), while Smith et al. (2010) showed that seasonal variation had the largest influence on the diversity of denitrifier populations. The higher similarities among BTCs in the same plant may be ascribed to the return of activated sludge from the sedimentation tank, which brings the mixing of the activated sludge and makes the bacterium community consistent in different BTCs. Further, some bacteria can survive and even maintain their initial capacity in different environment (Jensen et al., 1993; Bodelier et al., 1996), which can also increase the similarity. Liu et al. (2007) analyzed bacterial community structures in two sewage treatment plants and found that there existed common bands between raw sewage and activated sludge of the same MWTPs. Twelve bands indicated in the DGGE pattern (Figure 2B) were selected, cloned and sequenced for further analysis. The identities of the NosZ gene sequences were confirmed by searching against the international sequence database Genbank using the BLAST programs. As shown in Table 3, the highest identity and score values of the isolated sequences was the NosZ gene from uncultured bacterium.

Coexistence of different bacteria that perform similar function implies functional redundancy that may allow communities to maintain physiological capabilities when conditions change (Siripong and Rittmann, 2007), and a genetically diverse microbial community stabilized the denitrifying performance (Yoshie et al., 2001). It has been indicated that Proteobacteria was the dominant bacteria in wastewater treatment system (Snaidr et al., 1997). In this study, the most probable affiliations of the identified bacteria harboring the NosZ gene were from α-, β-Proteobacteria including *Alicycliphilus denitricans*, *Herbaspirillum* sp., *Paracoccus denitrificans*, and *Rhodopseudomonas palustris* while the non-identified organism were search for S7 in Genbank. Similarly, the isolated NosZ genes of denitrifying bacteria from

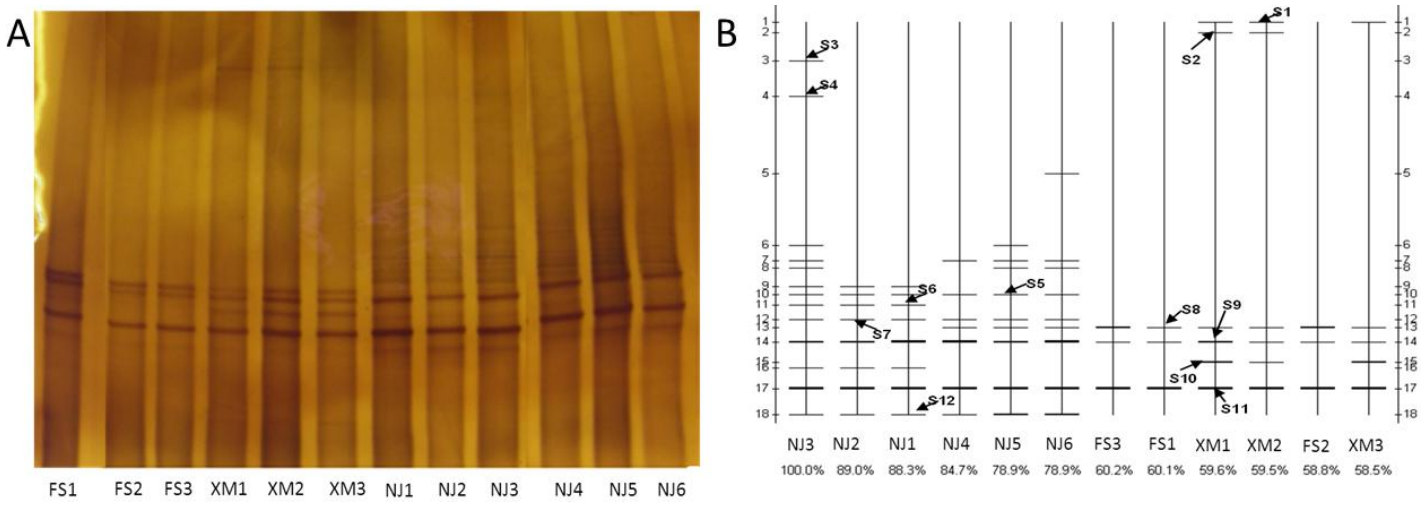

Figure 2. Photograph (A) and Sketch map (B) of NosZ gene bands of activated sludge in anaerobic tank (NJ1, NJ4, XM1 and FS1), anoxic tank (NJ2, NJ5, XM2 and FS2) and aerobic tank (NJ3, NJ6, XM3 and FS3) of MWTPs, NJ (NJ1-6), XM (XM1-3) and FS (FS 1-3). Bands S1-S12 were reamplified and sequenced.

Table 3. Sequence analysis of bands encoded by NosZ gene.

Band NO.	Accession NO. in Genbank	Most similar strain (accession NO.), score values, identity	Most probable affiliation
S1	HQ842601	Uncultured bacterium (HQ148889.1), 462, 88%	Alicycliphilus denitrificans[β]
S2	HQ842600	Uncultured bacterium (HQ148889.1), 504, 89%	Alicycliphilus denitrificans[β]
S3	HQ842609	Alicycliphilus denitrificans (CP002449.1), 280, 77%	Alicycliphilus denitrificans[β]
S4	HQ842606	Uncultured bacterium (HQ148889.1), 298, 81%	Alicycliphilus denitrificans[β]
S5	HQ842605	Uncultured bacterium (EU271750.1), 271, 79%	Alicycliphilus denitrificans[β]
S6	HQ842604	Uncultured bacterium (FJ209398.1), 223, 76%	Alicycliphilus denitrificans[β]
S7	HQ842603	Uncultured bacterium (GU362730.1), 145, 69%	Uncultured bacterium
S8-a	HQ842599	Paracoccus denitrificans (CP000490.1), 455, 83%	Paracoccus denitrificans[α]
S9-a	HQ842598	Uncultured bacterium (HQ148899.1), 540, 88%	Rhodopseudomonas palustris[α]
S10-a	HQ842608	Uncultured bacterium (AY577575.1), 493, 88%	Paracoccus denitrificans[α]
S11	HQ842607	Uncultured bacterium (HQ625235.1), 511, 89%	Alicycliphilus denitrificans[β]
S12	HQ842602c	Uncultured bacterium (EU271750.1), 439, 85%	Herbaspirillum sp.[β]

[α]α- *Proteobacteria*; [β] β- *Proteobacteria*.

sediments, soil and MWTPs were closely related to the genes of the uncultured bacteria and can be to α, β and γ subclasses of the *Proteobacteria* (Zhang et al., 2006; Magalhães et al., 2008; Pei et al., 2010).

Paracoccus sp, *R. palustris* and *A. denitrificans* (Table 3) were, respectively, the most probable affiliations of S8, S9 and S11 gene (Figure 2), indicating these bacteria may be the dominant bacteria. *A. denitrificans* is a Gram-negative denitrifying bacterium and can use nitrate, nitrite and oxygen as the electron acceptors (Mechichi et al., 2003; Weelink et al., 2008), and *Paracoccus sp*. was first isolated by Robertson et al. (1983) from desulphurization and denitrification systems. Recent report showed that many species of *Herbaspirillum* are denitrifiers and are most likely responsible for denitrification under the specified conditions (Ishii et al., 2011). Of course, the common bands may be attributed to their wide adaptability. For example, *Paracoccus sp* is able to be found in soil, sludge and environments, where the oxygen availability can change drastically (Robertson and Kuenen, 1983) while *R. palustris* is commonly found in soil and water and can survive in both aerobic and anaerobic conditions.

A phylogenetic tree was constructed for all twelve sequences from DGGE bands along with some similar sequences in Genbank with neighbor-joining method (Figure 4). Phylogenetic analysis demonstrated that all these sequences were grouped into two main cluster, that

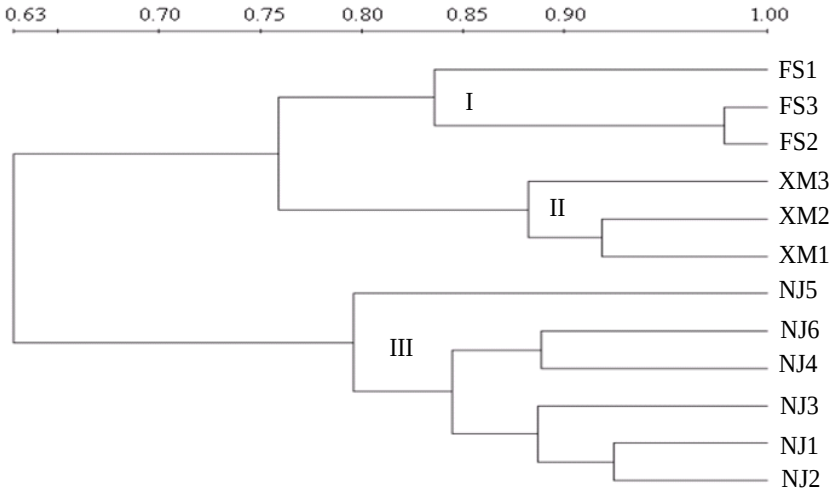

Figure 3. Dendrograph generated by UPGAM cluster analysis comparison of DGGE patterns. NosZ gene bands of activated sludge in anaerobic tank (NJ1, NJ4, XM1 and FS1), anoxic tank (NJ2, NJ5, XM2 and FS2) and aerobic tank (NJ3, NJ6, XM3 and FS3) of MWTPs, NJ (NJ1-6), XM (XM1-3) and FS (FS 1-3).

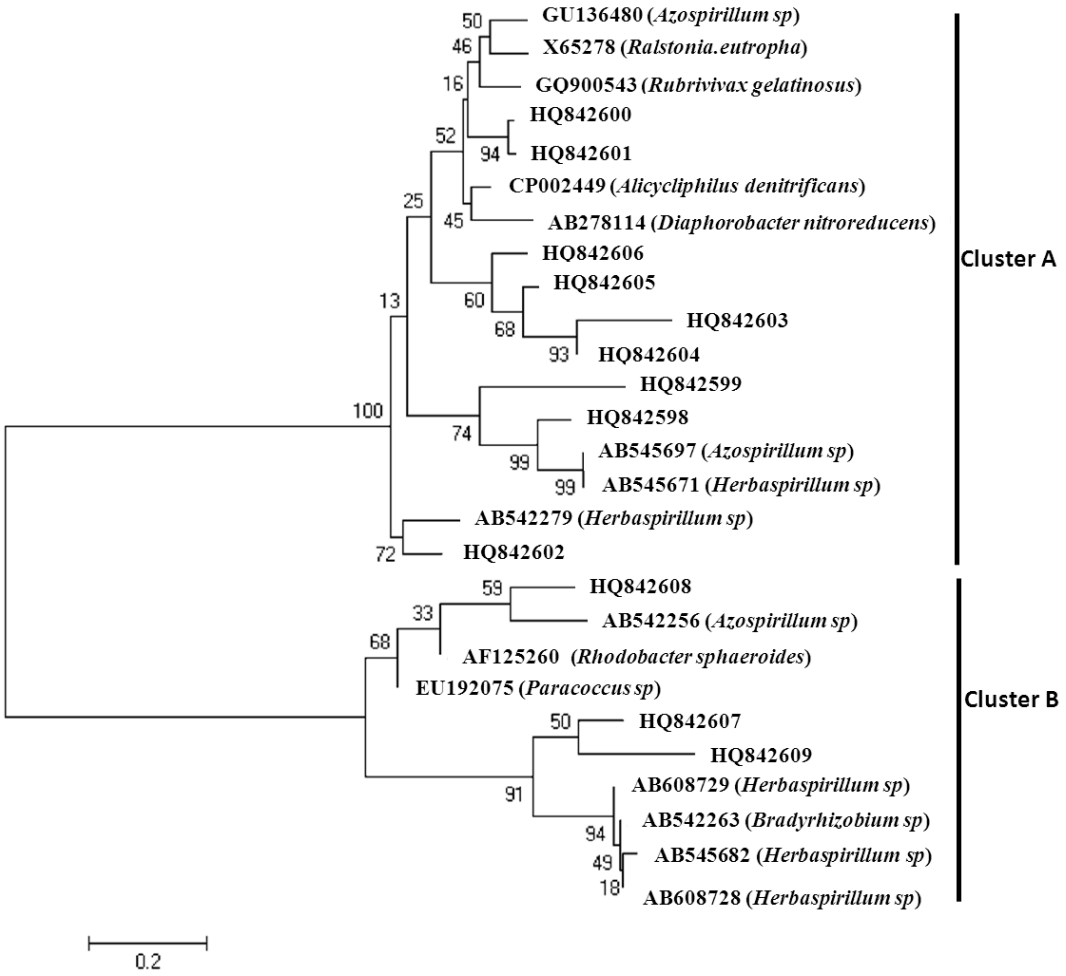

Figure 4. Phylogenetic tree of the NosZ gene based on nucleic acid sequence. , sequence from Genbank; HQ842598-HQ842609 were obtained by this study.

is, clusters A and B (Figure 4). Among the twelve sequenced bands, sequences of band S3, S10 and S11 fell into Cluster B, while the other nine sequences belong to cluster A. However, each cluster not only contains species from α- subclasses of the *Proteobacteria* but also from β- subclasses of the *Proteobacteria* (Figure 4 and Table 3). These results demonstrate that phylogenetically distantly related bacteria may carry highly similar functional gene sequences (Ishii et al., 2011). Thus, there are limitations on the identification of denitrifiers by solely using functional gene sequence information. Since most of the NosZ sequence data are isolated from uncultured bacteria in this study and in Genbank (Dell et al., 2010), further studies are needed to link the functional genes to the specific bacteria.

Conclusions

The diversity of NosZ gene was detected in different MWTPs by PCR-DGGE. The similarities of different BTCs in the same MWTP were higher than that of the same BTC in different MWTPs, indicating the influents might affect the redundant of NosZ gene than the oxygen level. The diversity of NosZ gene is higher in NJ than that in XM and FS. The nucleic acid sequences of 12 DGGE bands were sequenced and identified by using BLAST analysis. Phylogenetic analysis showed these nucleic acid sequences were grouped in to two main clusters. The highest similar nucleic acid sequences of these bands were from NosZ gene of uncultured bacteria, while the most probable affiliation of the bacterial harboring these sequences was related to *Proteobacteria*.

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