Journal of Aquaculture Research & Development

Saha et al., J Aquac Res Development 2014, 5:6
DOI: 10.4172/2155-9546.1000270

Research Article Open Access

Introduction to Establish the Comparative Analysis of 16S rRNA Gene Sequences with *amoA* and *nxrA* for Nitrifying Bacteria Isolated from East Kolkata Wetland: an International Ramsar Site

Saha M[1]*, Sarkar A[2] and Bandhophadhyay B[1]

[1]*Department of Biotechnology, Vidyasagar University, West Bengal-721 102, India*
[2]*Department of Aquatic Animal Health, West Bengal University of Animal and Fishery Sciences, Kolkata-700 094, India*

Abstract

The nitrogen cycle is most complex and very important for the life on earth. Nitrifying bacteria which carried out nitrogen cycling process play a vital role in water quality control, thus creating a genomic fingerprint database for surveillance and monitoring of genetic variability of nitrifying bacteria is very important and also to establish a Biosecurity protocol for the Bheries located in different areas of West Bengal. The currently evolutionary relationships and the natural diversity of Ammonia Oxidizing Bacteria (AOB) and Nitrite Oxidizing Bacteria (NOB) is mainly based on comparative sequence analyses of their genes encoding the 16S rRNA and the active site polypeptide of the ammonia monooxygenase (*AmoA*) and Nitrite oxidoreductase (*NxrA*) in the East Kolkata Wetland. This study extended significantly the 16S rRNA and *amoA* databases for AOB, n*xrA* databases for NOB. Therefore, either of the functional markers (*amoA* or *nxrA*) can be used to trace ammonia oxidizers or structural marker (16S rDNA) can be used to trace specific species in environmental studies. These techniques included the use of 16S rDNA genes to characterize natural AOB and NOB populations and to analyze their taxonomic and phylogenetic features. The current perception of AOB and NOB phylogeny established by comparative 16S rRNA sequence analysis could be confirmed independently by exploiting the gene *amoA* and *nxrA* genes and proved as an alternative phylogenetic marker. The aim of our work is to investigate the potential of the ammonia monooxygenase subunit A gene (*amoA*) and Nitrite oxidoreductase (*nxrA*) gene as functional markers for AOBs and NOBs along with 16S rDNA sequence analysis in EKW. For ecological surveillance, genes like *amoA* and *nxrA* specific for the nitrifying bacteria under present research work could be a more reliable tool.

Keywords: Ammonia oxidizing bacteria; Nitrite oxidizing bacteria; 16S rDNA-PCR; *Nitrosomnas* sp; *Nitrobacter* sp; East Kolkata Wetland

Introduction

Environmental and bioremediation interest in Ammonia Oxidizing Bacteria (AOB) and Nitrite-Oxidizing Bacteria (NOB) has increased tremendously in recent years as both of these bacteria plays a major role in the nitrogen cycle, i.e., Nitrification, which occurs in soils [1], ocean water [2], freshwater lakes [3], wastewaters [4], and aquaria [5]. Waste water fed fish ponds in West Bengal, India, is called Bheris having a distinctly different architecture resulting in extensive purification of waste along with integrated resource recovery [6]. East Kolkata Wetlands constitute one huge system of marshes located within the peri-urban interface of Kolkata City of West Bengal. It acts as a natural sewage treatment plant in the city and side by side generates products like paddy, vegetables and fish utilizing the sewage [7-10]. East Kolkata Wetland was declared as a Ramsar site in November 2002 as per "Ramsar Guidelines". The purification of the waste products is mainly based on microbial activity. The hot and humid climate all throughout the year favours this site to act as an incubator for a diverse group of microbes [11]. Thus the site was selected to create a composite dataset of various nitrifying bacteria which can be applicable in biotechnology and bioremediation purpose in different parts of the world.

To establish AOB and NOB phylogeny with compare to 16S rRNA sequence analysis, *amoA* gene for AOB and *nxrA* NOB gene could be the potential target gene candidates for the confirming ecological phylogeny of nitrifying bacteria. Genus Nitrosomonas and Nitrobacter, both are chemolithoautotrophic and members of the Proteobacteria class [12]. Genus *Nitrosomonas* form a distinct group within the β subdivision were as nitrite-oxidizing bacteria like Nitrobacter occurring at α, δ, and γ subdivisions. Study of AOB and NOB of conventional cultivation techniques is also extremely difficult because of their long generation times and low growth rates [13]. Therefore, a rapid, culture-independent detection technique for nitrifiers would be useful to provide a record of the natural availability and role of these microbes in bioremediation of various Bheries located in the East Kolkata Wetland.

Especially the uses of cultivation-independent techniques have enhanced the knowledge on the biogeography of this considerable specialized group of bacteria [14-16]. AOB is among the rare cases in microbial ecology, where pure cultures of nearly all recognized lineages are available. The latter is provided to connect ecophysiology with the diversity and distribution of these bacteria [14,17]. Based on structural (16S rRNA) as well as functional (*amoA*) gene relationships, seven major lineages of AOB are recognized [13,16,18]: *Nitrosospira, Nitrosomonas europaea/Nitrosococcus mobilis, Nitrosomonas communis, Nitrosomonas marina, Nitrosomonas oligotropha, Nitrosomonas cryotolerans* and Nitrosomonas species. Despite the fact that all these lineages carry out the same basic energy-generating biochemical reaction, AOB seems to occupy clearly different niches

***Corresponding author:** Mousumi Saha, Department of Biotechnology, Vidyasagar University, West Bengal-721102, India, Tel: +91 9830736189; E-mail: mou.cute@gmail.com

Received April 14, 2014; **Accepted** September 23, 2014; **Published** October 03, 2014

Citation: Saha M, Sarkar A, Bandhophadhyay B (2014) Introduction to Establish the Comparative Analysis of 16S rRNA Gene Sequences with *amoA* and *nxrA* for Nitrifying Bacteria Isolated from East Kolkata Wetland: an International Ramsar Site. J Aquac Res Development 5: 270. doi:10.4172/2155-9546.1000270

and can therefore be classified into ecophysiological groups [19]. Niche differentiation within AOB communities has also been noted with respect to salinity [20-23].

Gene *amoA* codes for the active site of ammonia monooxygenase [24], and it has been widely used for the detection of ammonia oxidizers, particularly in natural environments [25-27]. The oxidation of ammonia to nitrite by chemoautotrophic ammonia-oxidizing bacteria (AOB) are performed in two steps following ammonia monooxygenase (AMO) catalyses the oxidation of ammonia to hydroxylamine in an endergonic reaction and hydroxylamine is oxidized to nitrite by a hydroxylamine oxidoreductase (HAO) in an exergonic reaction. The latter reaction is the sole reaction by which the AOB produces energy for cell growth [28]. Nitrite oxidoreductase (nxr) is the key enzyme responsible for the oxidation of NO_2^- to NO_3^- in nitrite-oxidizing bacteria.

The 16S rDNA, *amoA* genes for AOB and 16S rDNA, *nxrA* genes for NOB were evaluated individually and then compared in order to determine the best method. The above three markers for taxonomic and phylogenetic information were also valid separately. Therefore, either of the functional markers (*amoA* or *nxrA*) can be used to trace ammonia oxidizers or structural marker (16S rDNA) can be used to trace specific species in environmental studies. These techniques understand the use of 16S rDNA genes to characterize natural AOB and NOB populations [29-33] and to analyze their taxonomic and phylogenetic features [13,18,25, 34,35]. Although, 16S rDNA sequences are appropriate for providing a comprehensive long-term evolutionary view of AOBs and NOBs [36]. The composite dataset, consisting of 16S rDNA, *amoA*, and *nxrA* sequences, provided more information and therefore resulted in the most accurate classification. The current perception of AOB and NOB phylogeny established by comparative 16S rRNA sequence analysis could be confirmed independently by exploiting the gene a*moA* and *nxrA* genes and proved as an alternative phylogenetic marker. The aim of our work is to investigate the potential of the ammonia monooxygenase subunit A gene (*amoA*) and Nitrite oxidoreductase (*nxrA)* genes as functional markers for AOBs and NOBs along with 16S rDNA sequence analysis of EKW.

Materials and Methods

Samples collection and processing

East Kolkata Wetland in Kolkata was chosen for the present study as it is a wastewater fishery. Among which research work was initiated under the East Kolkata Wetland at Nalban Fisheries (owned by the Government of West Bengal and Runed by State Fisheries Development Co-operation) and Jhagrasisa bhery (Runed by private organization).

A total 6 fish ponds were selected from the above two bheries and marked as N1, N2, N3 for Nalban Fishery and J1, J2, J3 for Jhagrasisa bhery in the East Kolkata Wetland. 25 ml each of water samples were collected from the above ponds in two aliquots. Samples were also collected from Topsia (Sewage Pumping Station) and from different channels like Ambedkar Bridge, Bantala, Bamunghata, Ghosher Khal which is connected to the fish pond and Ghusighata (outlet). Samples from each site was collected in winter, summer, post monsoon and monsoon at around 6.30 -7.00 in the morning in a sterile plastic bottle (Tarson) from a depth of 1-2.0 cm below the surface of the water and kept in the ice box (Milton, India) for immediate culture in the laboratory. A total of 96 samples were collected and studied, out of which 23 isolates are found as *Nitrosomonas* sp. and 18 are found as *Nitrobacter* sp. Genus are initially identified by various biochemical tests.

For the isolation of nitrifying bacteria samples were processed on Winogradskyi medium. Winogradsky phase 1 medium was used for the isolation of *Nitrosomonas* sp. and Winogradsky phase 2 medium was used for the isolation of *Nitrobacter* sp. [37-39]. All tubes were placed in the incubator at 28°C for 90 hrs aerobically [38,40,41]. Gram staining initially confirmed the bacteria [42] and suspected cultures were evaluated for the presence of ammonia and nitrite and vice versa for production of nitrite and nitrate. These tests were performed and confirmed by a series of reaction. Selected cultures were preserved for further studies at -80°C with 30-40% glycerol stock.

Genomic DNA isolation

Bacterial biomass from 10 ml of culture was collected by centrifugation for 5 mins at 10000×g and resuspended in a 2 ml polypropylene tube (Eppendrof) containing glass beads with 500 ml buffer containing 20 mM sodium acetate, 1 mM EDTA (pH 5.5), 50 ml 25% SDS and 600 ml phenol: chloroform: isoamyl alcohol (25: 24: 1). Cells were lysed by the addition of 200 µl of 10 mg ml^{-1} lysozyme and incubation at 37°C for 60 min. 20% Sodium dodecyl sulfate was added to a final concentration of 1% and incubated at 37°C for 60 min. The Proteinase K solution was added (10 mg/ml) to a final concentration of 2 mg/ml and incubated at 50°C for 35 min and the mixture was then centrifuged at 10 000 g×10 min at 4°C. The aqueous phase was transferred carefully to a fresh tube, mixed with 600 ml chloroform: isoamyl alcohol (24: 1) and centrifuged 10000 g x 10 min. The aqueous phase was transferred to a fresh tube and, after the addition of 0.1 vol. 3 M sodium acetate. DNA was precipitated by incubation with 0.6 volumes of isopropanol and 5 ml glycogen (5 mg/ml) for 1 h and subsequently pelleted by centrifugation at 10 000 g×0 min at 4°C. Pellets were washed with 1 ml ice-cold 70% alcohol and dried it in Maxi Vacuum Dryer and resuspended in 500 µl TE buffer (pH 8.0). The DNA concentration was estimated by visual comparison with the standard DNA size markers after electrophoresis through 0.8% agarose gel, stained with 0.5 mg ml^{-1} ethidium bromide (Sigma Chemicals Co.).

PCR amplification16S rDNA, amoA and nxrA gene

The 16S rRNA genes were amplified by PCR using the universal primers: forward primer 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 1492R 5'-GGT TAC CTT GTT ACG ACT T-3' (Chromous Biotech Ltd., Bengaluru, India) [43]. PCR reactions have been carried out by following the series of standardizing experimental protocol as annealing temperature, concentration of $MgCl_2$, template DNA, Taq DNA polymerase, dNTP's and primers. The PCR reaction components consist of 200 mm dNTP, 20 pico moles of primer, 2 units of Taq DNA polymerase enzyme, assay buffer with working concentration of 1.5 mM $MgCl_2$, 20-30 ng template DNA in an assay volume of 25 µl. These concentrations were determined by a series of preliminary standardizing experiments.

The amoA fragment was amplified from AOBs by using the primer *amoA*-3F 5'-GGT GAG TGG GYT AAC MG-3' and *amoB*-4R 5'-GCT AGC CAC TTT CTG G-3' [4]. For NOBs, *nxrA* sequences were identified by using the primer sequence as F1norA 5'- CAG ACC GAC GTG TGC GAA AG-3' for forward reaction and the R1norA 5'- TCY ACA AGG AAC GGA AGG TC-3' (Y may be C or T) for the reverse reaction. The reaction mixture contained 20 ng of template DNA, 0.5 mM of each primer, 1XPCR buffer (Genei, Bangalore, India), 200 mMof each dNTP, 1.5 mM $MgCl_2$, 2.5U Taq DNA polymerase (Sigma, USA).

PCR programme of 16S rRNA gene, amoA and nxrA gene amplification

A cycling program was performed using a Thermal Cycler (Thermo Scientific) with an initial denaturation step at 95°C×3 min, 35 cycles of denaturation at 94°C×1 min, annealing at 55°C×1 min, and extension at 72°C×2 min, and final extension at 72°C×3 min.

A cycling program was performed using a Thermal Cycler (Thermo Scientific) with an initial denaturation step at 94°C×3 min, 35 cycles of denaturation at 94°C×1 min, annealing at 57°C×1 min, and extension at 72°C×1 min, and final extension at 72°C×5 min. For nxrA gene, the PCR thermocycling program was 94°C×3 min, followed by 30 cycles of 94°C×30 s, 55°C×45 s and 72°C×45 s, and a final extension step at 72°C×5 min.

The presence of the *amoA* and *nxrA* gene products were examined and visualized by electrophoresis on 1.5% agarose gel for 16S rRNA amplicons and 2.0% agarose gel for *amoA* and *nxrA* gene products in TBE buffer. The gels were stained with EtBr (Sigma) and viewed in the Gel Doc System (Biorad Gel Doc. 2000 system).

Sequencing of PCR amplified gene products

The PCR products were purified by the Exosap treatment in the Departmental Molecular Biology Laboratory. For the validation and identification, chosen strains were observed on agarose gel and selected for sequencing. In total, the PCR amplified products were sequenced by Ion torrent Sequencer from the Xcelris Lab Ltd, Ahmedabad, India.

Computer assisted pattern analysis

Chromatogram of sequence data confirmed the peak, reproducibility, quality and gene sequences of amplicons. 16S rDNA sequence data are arranged as complementary and consensus through online Reverse-Complement tools: (www.bioinformatics.org/sms/rev_comp.html) and Genefisher2 [BiBiServ (Bielefeld Bioinformatics Service, a unit of the Institute for Bioinformatics (bibiserv.techfak.uni-bielefeld.de/genefisher2/submission.html)]. Identification of species and similarity calculations were performed, comparing sequences of approximately 1500 bases with sequences available in GenBank using BLAST network services (blast.ncbi.nlm.nih.gov/). Multiple sequence alignments (MSA) are carried out by ClustalW following all the sequence pairs were aligned separately for the calculation of distance matrix and constructed a guide tree and sequences are aligned according to the hierarchy.

Phylogenetic analysis

Phylogenetic relationship, similarity index, distance between all isolates and maximum likelihood pattern analysis outlined. Gene sequences of *amoA* and *nxrA* fragments were also compared with 16S rDNA sequence by multiple sequence alignment and phylogenetic tree generated by Mega v5.05. The 16S rDNA sequences in this study were submitted to the GenBank database by using Sequin software (www.ncbi.nlm.nih.gov/projects/Sequin/) under nucleotide accession number. Analysis of the 16S rRNA gene proved as useful taxonomic tools with discriminatory properties for identification up to the species level.

Results and Discussion

For nitrification, the *amoA* gene encoding ammonia monooxygenase for NOBs and *nxrA* gene encoding nitrate reductase for AOBs have been used successfully to characterize the diversity and phylogenetic studies of wastewater nitrifying microbial communities [13]. The present study developed a molecular approach for targeting the *amoA* and *nxrA* gene through a PCR based analysis and sequencing. Identification tool was developed to explore the variability of multiple gene copies in different species of *Nitrosomonas* and *Nitrobacter* and the diversity of these genes in wastewater of EKW.

Amplification and sequencing of *amoA* gene fragment produced of about 491bp for *Nitrosomonas eutropha* (KF618623 and KF618626) (Figure 1); 415 bp for *Nitrosomonas europaea (*KF618624). 16S rRNA analysis could not determine the species of the strain NSW3 and designated as *Nitrosomonas* sp. (KF618625) through the BLAST network service of NCBI-database, whereas, PCR amplification of *amoA* gene designated the strain fragmented at 415bp position in agarose gel which confirmed the species *N. europaea*. Thus, the present investigation indicates the beauty of the study of *amoA* gene with 16S rRNA-PCR analysis for the genus *Nitrosomonas* (Figure 1). Purkhold et al. [13] claimed that the availability of AOB may be more representative of the natural AOB diversity than other molecular ecological studies, and almost all the *amoA* sequences obtained from a wastewater treatment plant could be assigned to recognize species of AOB, *Nitrosomonas* in particular [13]. However, only partial 16S rRNA sequences are available for many AOB species and most AOB have not yet been analyzed on the *amoA* level. Recently, the battery of molecular tools to infer the presence of AOB in the environment has been supplemented by PCR primers for specific amplification of the ammonia monooxygenase structural gene *amoA* [3,44-46]). While environmental 16S rDNA and *amoA* libraries significantly extended our knowledge on the natural diversity of AOB, biases introduced by DNA extraction, PCR amplification, and cloning methods [47-53] blur quantitative information on the community composition.

No equivalent tool targeting a functional gene was available for the NOB communities [54,55]. Until now, however, no information on the diversity and the distribution of nxrA sequences in environmental samples were available. Recently, molecular tools based on 16S rRNA gene [56] suggested a higher diversity of NOB in soils. Here, the Nitrobacter diversity in soils was investigated by targeting the functional gene *nxrA* encoding an essential enzyme for nitrite oxidation, the NXR. In Nitrobacter, the oxidation of nitrite to nitrate is performed by the nitrite oxidoreductase (NXR), encoded by the nxr operon [57] formerly named "nor" [58] More recently, Vanparys et al.

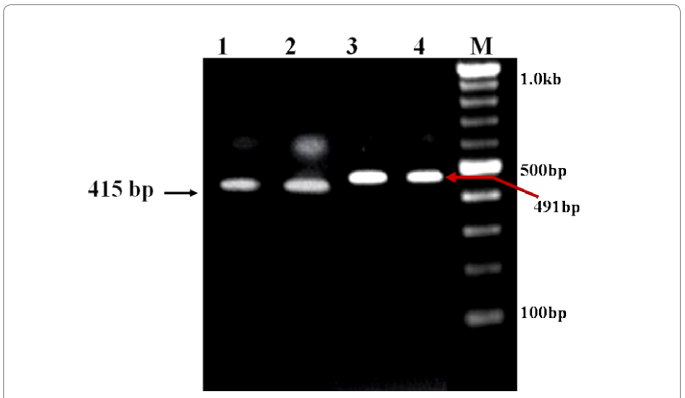

Figure 1: PCR detection of *amoA* gene of the genus *Nitrosomonas*. 2.0% Agarose gel showing the typical *amoA* PCR amplicons of about 415 bp fragments in Lane 1 and 2 for *Nitrosomonas europaea (*KF618624 and KF618625); and Lane 3 and 4 showing of about 491 bp fragments of *Nitrosomonas eutropha* (KF618623 and KF618626). Lane M, showing the 100bp DNA size marker (Promega).

[59] expanded this database with partial nxrA sequences (318 bp) from five pure cultures and 34 environmentally derived sequences.

In our study, it has been found through 16S rRNA gene sequence that the strain NBW3 belongs to the *Nitrobacter vulgaris* (KF618622) hierarchy with the size of about 318bp. However, strain NBW1 (KF618620) and NBW2 (KF618621) (Figure 2) clustered together with genus Nitrobacter on the basis of 16S rRNA phylogeny, but the species were not identified. Amplification of *nxrA* gene fragment produced of about 322 bp. A similar result was described by Poly et al., Wertz et al., and Willie et al., [60-62]. They also derived 322bp gene fragment of *nxrA* from *Nitrobacter hamburgensis*. Therefore, the both NBW1 and NBW2 strains, which also produced the same as above, may be under the species *Nitrobacter hamburgensis* (Figure 3).

Phylogenetic studies of *amoA* and *nxrA* gene products, showing an interrelationship between the same genus as produced *Nitrosomonas* cluster and *Nitrobacter* cluster, but they do not mix up together. By the same way, reference sequences from the NCBI-database compared with our query sequences and results obtained the same as above. When comparison occurred between 16S rRNA gene sequences, *amoA* gene sequences and *nxrA* gene sequences to study phylogenetic distance within the species, It has been dramatically found that, 16S rDNA sequences, *amoA* sequences and *nxrA* sequences of *Nitrosomonas* and *Nitrobacter* belong in different clades, but, *amoA* clusters and *nxrA* clusters are more closed in the tree position as because they are more conserved than 16S rRNA genes and likely to be species specific.

Although it was established previously that 16S rDNA is a good phylogenetic marker, especially concerning the avoidance of ambiguous classifications. But studies of environmental samples based on the analysis of *amoA* or *nxrA* provide some significant advantages: the genes are specific and provide a phylogeny consistent with the current taxonomic outlines. For ecological surveillance, genes like *amoA* and *nxrA* specific for the nitrifying bacteria under present research work could be a more reliable tool along with the use of the 16S rDNA only, since this reduces the number of non-target organisms detected. The genes encoding nitrification (amo and nxr) are potential target gene candidates for studies of *Nitrosomonas* and *Nitrobacter* in East Kolkata Wetlands, West Bengal (Figures 4 and 5). Thus, *amoA* and

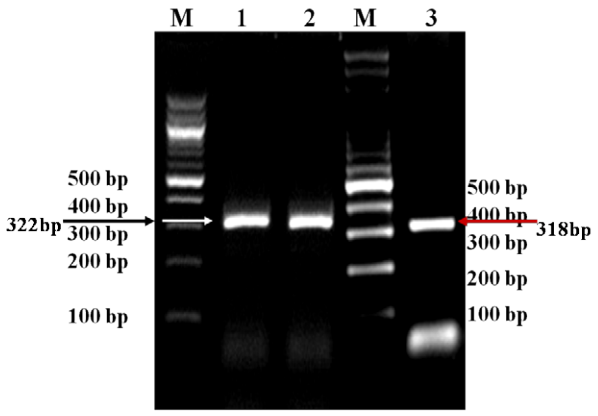

Figure 2: PCR detection of *nxrA* gene of the genus *Nitrobacter*. 2.0% Agarose gel showing the typical *nxrA* PCR amplified products of about 322 bp fragments in Lane 1 and 2 for *Nitrobacter hamburgensis* for strains NBW1 (KF618620) and NBW2 (KF618621); and Lane 3 showing of about 318 bp fragment of *Nitrobacter vulgaris* for the strain NBW3 (KF618622). Lane M, showing the 100bp DNA size marker (Promega).

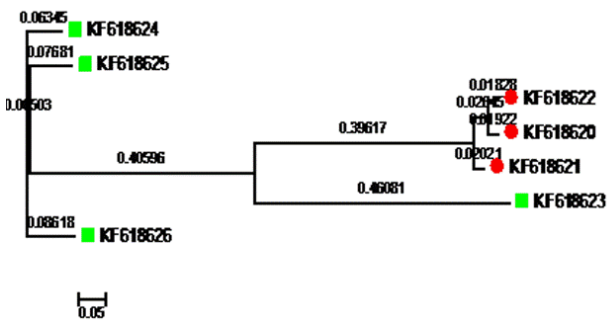

Figure 3: Phylogenetic distance tree showing the interrelationship between different species of *Nitrosomonas* specific *amoA* gene and *Nitrobacter* specific *nxrA* gene with their distance value and their branch length. Samples were collected from wastewater sources of East Kolkata Wetland. The tree was plotted by Mega v5.05.

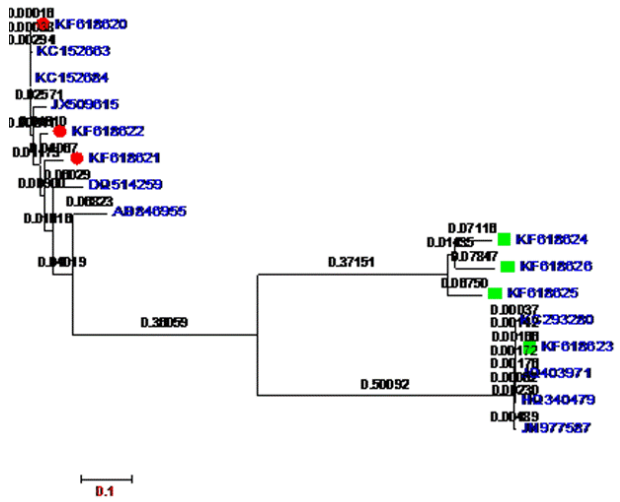

Figure 4: Phylogenetic distance tree showing the interrelationship between the query sequence and reference sequences (microbes were isolated from wastewater sources of the East Kolkata Wetland) of different species of *Nitrosomonas* specific *amoA* gene and *Nitrobacter* specific *nxrA* gene with their distance value and their branch length.

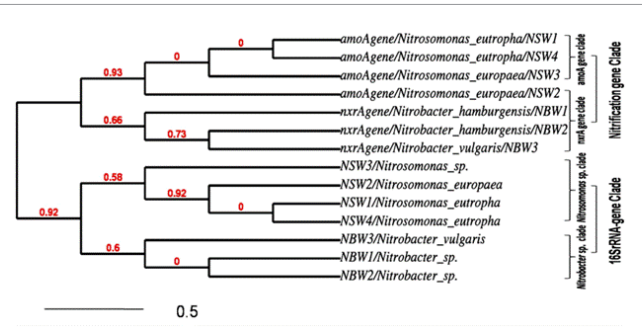

Figure 5: Phylogenetic distance tree displaying the Maximum likelihood pattern of *Nitrosomonas* and *Nitrobacter* sp. Specific gene clad3s, compared with 16S rRNA gene, where a lineage shared by different species as well genes through Bayesian Interference theory on the basis of Markov chain model and their bootstrap value.

nxrA gene can be considered as potential marker leading to taxonomic and diversity studies of AOBs and NOBs.

Conclusion

In conclusion, a molecular tool was developed based on functional gene *amoA* and *nxrA* to explore the diversity of *Nitrosomonas* and *Nitrobacter* in wastewater of EKW. The application of these tools together showed that the diversity and phylogenetic may differ according to strains. From our current study, it can also be concluded that genes encoding the 16S rRNA and the active site polypeptide of the *amoA* and *nxrB* can be the best molecular tool for phylogenetic relationships and diversity of AOB and NOB in EKW.

References

1. Killham K(1986) Heterotrophic nitrification(J. I. Prosser ed.), Nitrification. IRL Press, Oxford, 117-126.
2. Ward BB(1986) Nitrification in marine environments, *In* J. I. Prosser (ed.), Nitrification. IRL Press, Oxford, 157-184.
3. Hall G(1986) Nitrification in lakes(J. I. Prosser ed.), Nitrification. IRL Press, Oxford,127-156.
4. Painter HA (1986) Nitrification in the treatment of sewage and waste-water, *In* J. I. Prosser (ed.), Nitrification. IRL Press, Oxford, 185-211.
5. Kawai A, Sugiyama M, Shiozaki R, Sugahara I(1971) Microbiological studies on the nitrogen cycle in aquatic environments. Mem. Res. Inst. Food Sci. Kyoto Univ 32: 7-15.
6. Ray Chaudhuri S, Salodkar S, Sudarshan M, Mukherjee I, Thakur AR (2008) Role of water hyacinth-mediated phytoremediation in waste water purification at east Calcutta wetland. J Integ Environ Sci 5: 53-62.
7. Ray Chaudhuri S, Mishra M, Nandy P, Thakur AR(2008) Waste management: A case study of ongoing traditional practices at East Calcutta Wetland. American Journal of Agricultural and Biological Sciences 3: 315-320.
8. Ray Chaudhuri S, Mishra M, Salodkar S, Sudarshan M, Thakur AR(2008) Traditional Aquaculture Practice at East Calcutta Wetland: The Safety Assessment. American Journal of Environmental Sciences 4:140-144.
9. Ray Chaudhuri S, Salodkar S, Sudarshan M, Thakur AR (2007) Integrated Resource Recovery at East Calcutta Wetland how safe is these? International Journal of Agricultural and Biological Scienes 2: 75-80
10. Pradhan A, Bhaumik P, Das S, Mishra M, Khanam S, et al. (2008) Phytoplankton diversity as indicator of water quality for fish cultivation. American Journal of Environmental Sciences 4: 140-144.
11. Raychaudhuri S, Thakur AR(2006) Microbial genetic resource mapping of East Calcutta Wetland. Current Science 91: 212-217.
12. Wheaton FW, Hochheimer JN, Kaiser GE, Kronos MJ, Libey GS, et al. (1994) Nitrification filter principles. Timmons MB and Losordo TM (ed.), Aquacult water reuse sys. Engineering design and management. Elsevier, Amsterdam. 101-126.
13. Purkhold U, Pömmerening-Röser A, Juretschko S, Schmid MC, Koops HK, et al. (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA*sequence analysis: implications for molecular diversity surveys. Appl Environ Microbiol 66: 5368-5382.
14. Koops HP, Purkhold U, Pommering-Röser A, Timmermann G, Wagner M (2003) The Lithoautotrophic Ammonia-oxidizing Bacteria (3rdedn). The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community.
15. Stephen JR, McCaig AE, Smith Z, Prosser JI, Embley M (1996)Molecular diversity of soil and marine 16S rRNA gene sequences relatedto Z-subgroup ammonia-oxidizing bacteria. Appl Environ Microbiol 62: 4147-4154.
16. Kowalchuk G, Stephen JR (2001) Ammonia-oxidising bacteria: amodel for molecular microbial ecology. Annu Rev Microbiol 55: 485-529.
17. Koops HP, Pommering-Röser A (2001) Distribution andecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol Ecol 37: 1-9.
18. Purkhold U, Wagner M, Timmermann G, Pommering-Röser A,Koops HP (2003) 16S rRNA and amoA-based phylogeny of 12 novelbetaproteobacterial ammonia-oxidizing isolates: extension of the datasetand proposal of a new lineage within the nitrosomonads. IntJ SystEvol Microbio 53: 1485-1494.
19. Suwa Y, Sumino T, Noto K (1997) Phylogenetic relationship ofactivated sludges isolates of ammonia oxidizers with different sensitivitiesto ammonium sulfate. J Gen Appl Microbiol 43: 373-379.
20. Bie de M, Speksnijder AGCL, Kowalchuk GA, Schuurman T, Zwart G, et al. (2001) Shifts inthe dominant populations of ammonia-oxidizing Z-subclass Proteobacteria along the eutrophic Schelde estuary. Aquat Microb Ecol 23: 225-236.
21. Bollmann A, Laanbroek HJ (2002) Influence of oxygen partialpressure and salinity on the community composition of ammoniaoxidizingbacteria in the Schelde estuary. Aquat Microb Ecol 28: 239-247.
22. Stehr G, Böttcher B, Dittberner P, Rath G, Koops HP (1995) Theammonia oxidizing nitrifying population of the river Elbe estuary. FEMSMicrobiolEcol 17: 177-186.
23. Cebron A, Berthe T, Garnier J (2003) Nitrification and nitrifyingbacteria in the lower Seine river and estuary (France). Appl Environ Microbiol 69: 7091-7100.
24. McTavish H, Fuchs JA, Hooper AB (1993) Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonaseuropaea*. J Bacteriol 175: 2436-2444.
25. Aakra A, Utåker JB, Nes IF (2001) Comparative phylogeny of the ammonia monooxygenase subunit A and 16S rRNA genes of ammoniaoxidizing bacteria. FEMS Microbiol Lett 205: 237-242.
26. Gieseke A, Purkhold U, Wagner M, Amann R, Schramm A (2001) Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Appl Environ Microbiol 67: 1351-1362.
27. Juretschko S, Timmermann G, Schmid M, Schleifer KH, Pömmerening- Röser A, et al. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcusmobilis*and *Nitrospira*-like bacteria as dominant populations. Appl Environ Microbiol 64: 3042-3051.
28. Wood PM (1986) In: Nitrification, (Prosser, J.I., Ed.), IRL Press, Oxford. 20: 39-62.
29. Bothe H, Jost G, Schloter M, Ward BB, Witzel K (2000) Molecular analysis of ammonia oxidation and denitrification in natural environments. FEMS Microbiol Rev 24: 673-690.
30. Calvó L, Vila X, Abella CA, Garcia-Gil LJ (2004) Use of the ammoniaoxidizing bacterial-specific phylogenetic probe Nso1225 as a primer for fingerprint analysis of ammonia-oxidizer communities. Appl Microbiol Biotechnol 63: 715-721.
31. Kowalchuk GA, Stienstra AW, Heilig GH, Stephen JR, Woldendorp JW (2000) Molecular analysis of ammonia-oxidizing bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). FEMS Microbiol Ecol 31: 207-215.
32. Rowan AK, Snape JR, Fearnside D, Barer MR, Curtis TP, et al. (2003) Composition and diversity of ammonia-oxidizing bacterial communities in wastewater treatment reactors of different design treating identical wastewater. FEMS Microbiol Ecol 43: 195-206.
33. Sinigalliano CD, Kuhn DN, Jones RD (1995) Amplification of the *amoA*gene from diverse species of ammonium-oxidizing bacteria and from an indigenous bacterial population from seawater. Appl Environ Microbiol 61:2702-2706.
34. Aakra A, Utåker JB, Pömmerening-Röser A, Koops HP, Nes IF (2001) Detailed phylogeny of ammonia-oxidizing bacteria determined by rDNA sequences and DNA homology values. Int J Syst Evol Microbiol 51: 2021-2030.
35. Calvó L, Garcia-Gil LJ (2004) Use of *amoB*as a new molecular marker for ammonia-oxidizing bacteria. J Microbiol Methods 57: 69-78.
36. Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25: 39-67.
37. Odokuma LO, Akponah E(2004) Inhibition of nitrification and carbon dioxide evolution as rapid tools for ecotoxicogical assessment of drilling fluids. African J Appl Zool Environ Biol 6: 16-24.
38. Odokuma LO, Akponah E (2008) Response of *Nitrosomonas*, *Nitrobacter*and *Escherichia coli* to drilling fluids. J Cell and Animal Biol 2: 43-54.
39. Ogugbue CJ Oranusi NA (2005) Inhibitory effect of Azo dyes on ammonia –N oxidation of *Nitrosomonas*. African J Appl Zool Environ Biol 7: 61-67.

40. Holt JG, Hendricks Bergey D (1993) R.S. Breed, ed. Bergey's Manual of Determinative Bacteriology (9thedn). USA.

41. Grundmann GL, Neyra M, Normand P (2000) High-resolution phylogenetic analysis of NO_2--oxidizing Nitrobacter species using the rrs-rrl IGS sequence and rrl genes. Int J Syst Evol Microbiol 5: 1893-1898.

42. Beaumont HJE, Hommes NG, Sayavedra-Soto LA, Arp DJ, Arciero DM, et al. (2002) Nitrite Reductase of *Nitrosomonaseuropaea*is Not Essential for Production of Gaseous Nitrogen Oxides and Confers Tolerance to Nitrite. J Bacteriol 184: 2557-2560.

43. Stackebrandt E, Murray RGE, Truper HG (1988) *Proteobacteria*classis nov., a name for the phylogenetic taxon includes the 'purple bacteria and their relatives'. Int J Syst Bacterio 38: 321-325.

44. Holmes AJ, Costello A, Lidstrom ME, Murrell JC (1995) Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol. Lett. 132:203-208.

45. Mendum TA, Sockett RE, Hirsch PR (1999) Use of molecular and isotopic techniques to monitor the response of autotrophic ammonia-oxidizing populations of the beta subdivision of the class *Proteobacteria*in arablesoils to nitrogen fertilizer. Appl Environ Microbiol 65: 4155-4162.

46. Rotthauwe JH, Witzel KP, Liesack W (1997)The ammonia monooxygenase structural gene *amoA*as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63: 4704-4712

47. Chandler DP, Fredrickson JK, Brockman FJ(1997) Effect of PCR template concentration on the composition and distribution of total community16S rDNA clone libraries. Mol Ecol 6: 475-482.

48. Farrelly V, Rainey FA, Stackebrandt E(1995) Effect of genome size and *rrn*gene copy number on PCR amplification of 16S rRNA genes from amixture of bacterial species. Appl Environ Microbiol 61: 2798-2801.

49. Polz MF, Cavanaugh CM(1998) Bias in template-to-product ratiosin multitemplate PCR. Appl. Environ Microbiol 64: 3724-3730.

50. Reysenbach AL, Giver LJ, Wickham GS, Pace NR(1992) Differential amplification of rRNA genes by polymerase chain reaction.Appl. Environ. Microbiol 58: 3417-3418.

51. Suzuki M, Rappe MS, Giovannoni SJ(1998) Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Appl Environ Microbio 64: 4522-4529.

52. Suzuki MT, Giovannoni SJ(1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62: 625-630.

53. Wintzigerode F, Goebel UB(1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21: 213-229.

54. Treusch H, Leininger S, Kletzin A, Schuster SC, Klenk HP, et al. (2005) Novel genes for nitrite reductase and Amorelated proteins indicate a role of uncultivated mesophiliccrenarchaeota in nitrogen cycling. Environ Microbiol 7: 1985-1995.

55. Francis CA, Roberts KJJ, Beman M, Santoro AE, Oakley BB (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc Natl Acad Sci USA 102: 14683-14688

56. Freitag T, Chang L, Clegg CD, Prosser JI (2005) Influence of inorganic nitrogen regime on the diversity of nitrite-oxidizing bacteria in agricultural grassland soils. Appl Environ Microbiol 71: 8323-8334.

57. Starkenburg SR, Chain PSG, Sayavedra-Soto LA (2006) Genome sequence of the chemolithoautotrophicnitriteoxidizing bacterium Nitrobacterwinogradskyi Nb-255.Appl Environ Microbiol 72: 2050-2063.

58. Kirstein K, Bock E (1993) Close genetic relationship between Nitrobacterhamburgensis nitrite oxidoreductase andEscherichia coli nitrate reductase. Arch Microbiol 160: 447-453.

59. Vanparys B, Spieck E, Heylen K, Wittebolle L, Geets J, et al. (2007) The phylogeny of the genus Nitrobacter based on comparative rep-PCR, 16S rRNA and nitrite oxidoreductase gene sequence analysis. Syst Appl Microbiol 30: 297-308.

60. Poly F, Wertz S, Brothier E, Degrange V (2008) First exploration of Nitrobacter diversity in soils by a PCR cloning-sequencing approach targeting functional gene nxrA. FEMS Microbiol Ecol 63: 132-140.

61. Wertz S, Poly F, Le Roux X(2008) Development and application of a PCRdenaturing gradient gel electrophoresis tool to study the diversity of Nitrobacter-like nxrA sequences in soil. Fems Microbiol Ecol 63: 261-271.

62. Willie FH, Akihiko T, Poly F, Xavier Le Roux, Ken Kristensen, et al. (2009) The Effect of Hydroxylamine on the Activity and Aggregate Structure of Autotrophic Nitrifying Bioreactor Cultures. Biotechnology and Bioengineering, 102.