

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

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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, *nxrA* 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; *Nitrosomonas* 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

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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 (*nrx*) 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, *nrxA* 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 *nrxA*) 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 *nrxA* 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 *amoA* and *nrxA* 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 (*nrxA*) 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 Topsis (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

Winogradsky 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 (Eppendorf) 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 amplification 16S rDNA, *amoA* and *nrxA* 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 MgCl₂, 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 MgCl₂, 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, *nrxA* 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 mM of each dNTP, 1.5 mM MgCl₂, 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 Genfisher2 [BiBiServ (Bielefeld Bioinformatics Service, a unit of the Institute for Bioinformatics (bibiserv.techfak.uni-bielefeld.de/genfisher2/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.

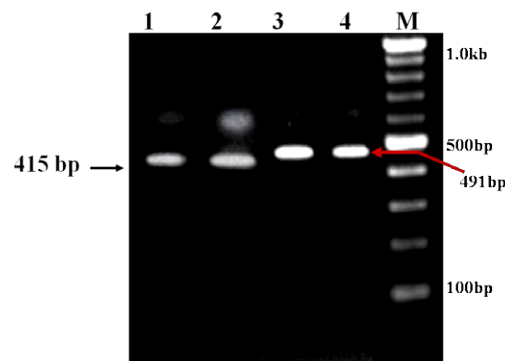


Figure 1: PCR detection of *amoA* gene of the genus *Nitrosomonas*. 2.0% Agarose gel showing the typical *amoA* PCR amplicons of about 415 bp fragments in Lane 1 and 2 for *Nitrosomonas europaea* (KF618624 and KF618625); and Lane 3 and 4 showing of about 491 bp fragments of *Nitrosomonas eutropha* (KF618623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

[59] expanded this database with partial *nrxA* 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 *nrxA* 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 *nrxA* 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 *nrxA* 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

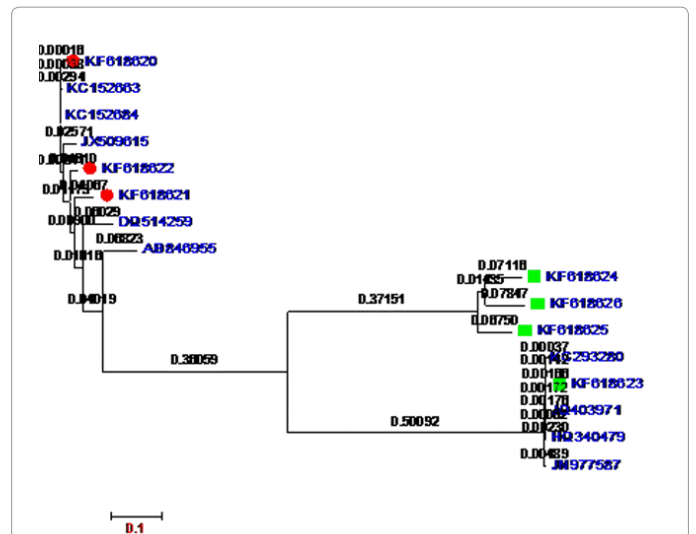


Figure 4: Phylogenetic distance tree showing the interrelationship between the query sequence and reference sequences (microbes were isolated from wastewater sources of the East Kolkata Wetland) of different species of *Nitrosomonas* specific *amoA* gene and *Nitrobacter* specific *nrxA* gene with their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

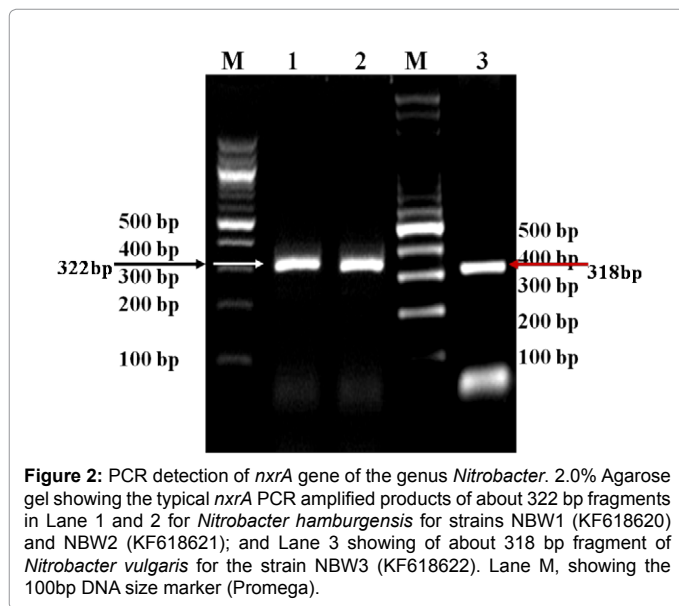


Figure 2: PCR detection of *nrxA* gene of the genus *Nitrobacter*. 2.0% Agarose gel showing the typical *nrxA* PCR amplified products of about 322 bp fragments in Lane 1 and 2 for *Nitrobacter hamburgensis* for strains NBW1 (KF618620) and NBW2 (KF618621); and Lane 3 showing of about 318 bp fragment of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

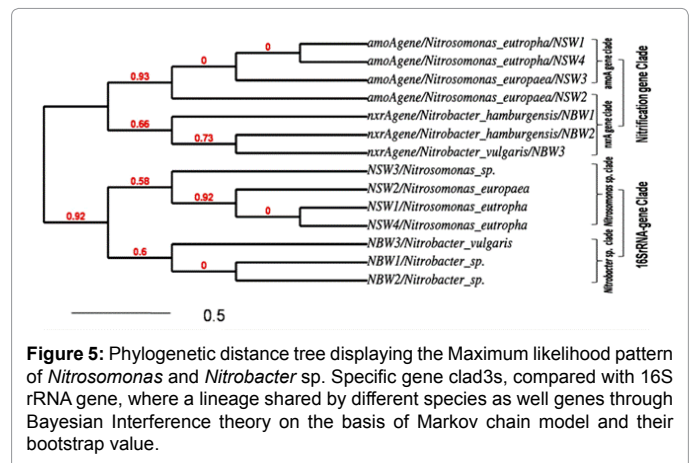


Figure 5: Phylogenetic distance tree displaying the Maximum likelihood pattern of *Nitrosomonas* and *Nitrobacter* sp. Specific gene clad3s, compared with 16S rRNA gene, where a lineage shared by different species as well genes through Bayesian Interference theory on the basis of Markov chain model and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

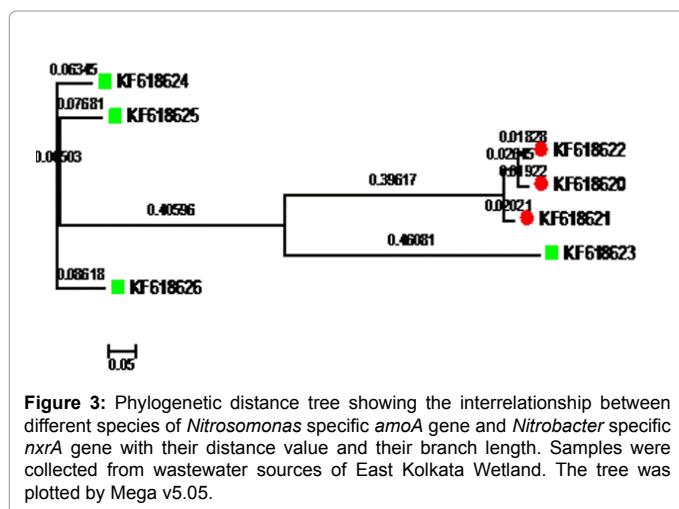


Figure 3: Phylogenetic distance tree showing the interrelationship between different species of *Nitrosomonas* specific *amoA* gene and *Nitrobacter* specific *nrxA* gene with their distance value and their branch length. Samples were collected from wastewater sources of East Kolkata Wetland. The tree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

sequences and *nrxA* gene sequences to study phylogenetic distance within the species, It has been dramatically found that, 16S rDNA sequences, *amoA* sequences and *nrxA* sequences of *Nitrosomonas* and *Nitrobacter* belong in different clades, but, *amoA* clusters and *nrxA* clusters are more closed in the tree position as because they are more conserved than 16S rDNA 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 *nrxA* 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 *nrxA* 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 *nrx*) are potential target gene candidates for studies of *Nitrosomonas* and *Nitrobacter* in East Kolkata Wetlands, West Bengal (Figures 4 and 5). Thus, *amoA* and

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.

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