Phylogenetic Characterization of Nitrifying Bacteria Isolated from East Kolkata Wetland

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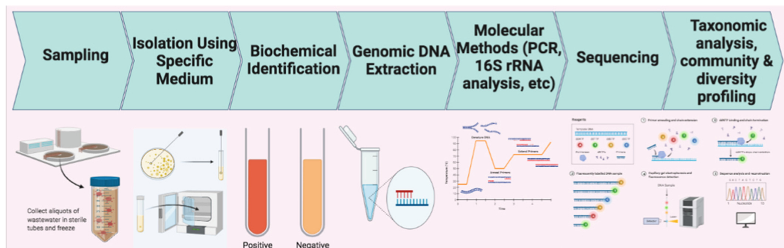
Abstract. East Kolkata Wetland (EKW) is an *"International Ramsar Site",* famous for broad biodiversity and insightful use of sewage for aquaculture. Native nitrifying bacteria of EKW play a significant role in maintaining water quality and controlling environmental pollution by converting ammonia into nitrate in wastewater. Therefore, the characterization of nitrifying bacteria is important in EKW. Thus, the main focus of this research was to identify and characterize the nitrifying bacteria, investigating their phylogeny and diversity in EKW. 16S rRNA and functional genes analysis may help in the proper evaluation of composition and distribution of nitrifying bacteria in some water bodies in EKW, which has not yet been explored. Molecular and phylogenetic characterization was targeted and achieved through 16S rRNA and functional gene analysis, followed by computational estimation. Resulted sequences were analysed to gain insight into the knowledge for global and local taxonomic orientation. Hence, a model can be created for characterizing the dynamics of nitrifying bacteria in wastewater treatment and sustainable aquaculture in different water bodies of EKW.

Graphical Abstract



Keywords: 16S rRNA gene · functional gene · Nitrifying bacteria · Phylogenetic tree · East Kolkata Wetlands

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Abbreviations

EKW	East Kolkata Wetlands
***amo*A**	ammonia monooxygenase
***nxr*A**	nitrite oxidoreductase
AOB	Ammonia oxidizing bacteria
NOB	nitrite oxidizing bacteria
MEGA	Molecular Evolutionary Genetics Analysis software
NJ method	Neighbour-joining method

Highlights

1. Identification and phylogenetic analysis of nitrifiers in EKW.
2. Analysis of 16S rRNA gene sequencing as a tool for identifying bacterial species level.
3. Functional genes are important molecular markers.
4. Characterization of AOBs and NOBs in the EKW indicate themselves as bioremediator. This strategy can be used in other wetlands and pollutant water bodies.

1 Introduction

East Kolkata Wetlands (EKW) is an *"International Ramsar Site"* as per Ramsar Convention, November 2002 (Saha et al. 2021). A huge cluster of sewage-fed bheries (a local term of the fish pond) is located in the eastern periphery of Kolkata, West Bengal, India. EKW is a prominent example of rich biodiversity and the inventory of bacteria, which has immense ecological, biotechnological and commercial applications (Ray Chaudhuri and Thakur 2006). Ammonia and nitrite are important water quality parameters for fish farming and they must be within the safe tolerance limit depending on various environmental conditions (Bhatnagar and Devi 2013). High levels of ammonia or nitrites are toxic for the fish health, growth, and lead to fish mortalities (Makori et al. 2017). Nitrification is an integral part of the nitrogen (N) cycle and environmental sustainability. Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) arechemolithoautotrophic bacteria that catalyse the nitrification process by converting ammonia to nitrate (Daims et al. 2016). *Nitrosomonas* sp. belongs to ß-proteobacteria of the AOB genuswhile *Nitrobacter* sp. is common under α-proteobacteria and belong to NOB (Zhao et al. 2018; Baskaran et al. 2020; Sharif Shourjeh et al. 2021). *Nitrosomonas* sp. and *Nitrobacter* sp.are the two prominent genera of AOB and NOB respectively (Gonzalez-Silva et al. 2021). They are the interdependent group that regulates the level of ammonia and nitrite in aquaculture farms. They are involved in wastewater bioremediation.Evidence from various experiments stated that the use of nitrifying bacteria in aquaculture farms helps in the improvement of water quality, fish health and disease control (Hasan and Banerjee 2020). Thus, investigating the contribution of nitrifying bacteria in EKW is significantly important.

Unique and conserved functional genes in nitrifying bacteria are often used as genetic markers to define bacterial diversity. *amo*A gene encodes ammonia monooxygenase which is a membrane-bound enzyme, responsible for the oxidation of ammonia to hydroxylamine, which is further oxidized to nitrite by the enzyme hydroxylamine oxidoreductase (Mohanty et al. 2019). *amo*A is the most relevant gene of AOB, thus they are widely used in community analysis research of water bodies and soil (He et al. 2018; Malinowski et al. 2020). *nxr*A gene is present in NOB and codes for the alpha subunit of nitrite oxidoreductase (Rani et al. 2016) and helps in the oxidation of nitrite to nitrate (Khanal and Lee 2020). *nxr*A gene is widely used for the identification of NOB from different environmental samples (Ouyang and Norton 2020). Highly conserved regions in the 16S rRNA gene made them an efficient and effective tool for identifying evolutionary divergence at various levels (Clarridge 2004). Similarly, this 16S rRNA sequence is used as a marker to explore the phylogenetic orientation of nitrifying bacterial community in water and soil samples. 16S rRNA genes analysis is a reliable tool for bacterialidentification (Khanal and Lee 2020). Capabilities of the 16S rRNA gene to establish the intragenomic diversity among bacteria were explored in this present study to establish the phylogenetic orientation. In this experimental work, the prospects of the 16S rRNA gene as a marker were explored to identify and reveal the nitrifying bacteria in wastewater ponds.

2 Materials and Methods

Water samples were collected in different seasons and processed for further experiments from different areas of EKW. Different physicochemical properties like pH, temperature, TDS, and DO were measured using a multi-parameter portable water quality testing kit (350i- Merck, Germany). Ammonia (NH_3) and nitrate (NO_{-3}) concentrations were measured as per the method followed by APHA. The data were simultaneously analysed and interpreted statistically using ANOVA (Saha et al. 2021). Winogradsky phase I and phase II medium were used for isolation and initial identification of *Nitrosomonas* sp. and *Nitrobacter* sp. respectively. Gram staining was done followed by a series of biochemical identification tests using Nessler's reagent, Trommsdrof's reagent and diphenylamine reagent were used to detect the presence of ammonia, nitrites and nitrate in the medium for initial confirmation as *Nitrosomonas* sp. and *Nitrobacter* sp. (Saha et al. 2021). Genomic DNA of the isolated nitrifying bacteria samples was extracted using the Phenol-Chloroform extraction method and quantified according to the standard protocol. 27F and 1492R, universal primers (Eurofins, Bengaluru, India) were used for PCR amplification of the 16S rRNA gene of the isolated strains. Amplified PCR products were stained with EtBr (Sigma, USA) and visualized in 1.5% agarose gel electrophoresis. Similarly, *amoA*-3F and *amoB*-4R primers were used for amplification of *amo*A fragments from AOB. F1*nor*A and R1*nor*A primers were used for *nxr*A gene amplification from NOB.Visualization of the amplified *amo*Aand *nxr*Agene products was carried out using 2.0% agarose gel electrophoresis. A series of PCR reactions have been carried out for standardizing experiments and the primer list is given in Table 1. Further, PCR purification and sequencing of the amplified products were done. The taxonomic relationships between all isolates and the phylogenetic tree were generated by Mega v5.05

(Tamura et al. 2011). The evolutionary history was inferred using the Neighbour-joining (NJ) method. The phylogenetic trees were constructed by Mega v5.05 using 16S rRNA global sequences of nitrifying bacteria for diversity study of nitrifiers. The maximum composite likelihood method was used to determine the evolutionarydistance. Clustered taxa were associated together in the bootstrap test. The tree was drawn with the same scale unit of branch length and evolutionary distance to summarize the respective phylogenetic tree.

3 Result and Discussion

Nitrosomonas sp. and *Nitrobacter* sp. were isolated using specific Winogradskyphase I and phase II medium. Pure colonies were initially characterized and confirmed by microscopy (Zeiss). Biochemical tests using Nessler's reagent, Trommsdrof'sand diphenylamine reagent confirms the isolates as nitrifying bacteria (Saha et al. 2021). Further analysis was done to measure and interpret the nitrifying bacterial diversity by using conventional techniques along with advanced molecular tools. Genomic DNA of the chosen isolates were extracted and high molecular weight DNA bands were visualized through 0.8% agarose gel electrophoresis. Based on the most conserved domain hypothesis, 16S rRNA-PCR amplification was done using 16S rRNA universal primers for the genus identification in molecular level. ~1.5 Kb amplified band was visualized and purified for the sequencing. Selected sequences were checked and further analysed for nitrifying bacterial community structure profiling. Out of the 07 nitrifying strains, 04 of them was clustered with *Nitrosomonas* sp. and 03 of them was clustered with *Nitrobacter* sp. Initial biochemical tests for identification of *Nitrosomonas* sp. and *Nitrobacter* sp. were consistent with the result of 16S rRNA sequence analysis. Homology profiling and phylogenetic orientation were done based on a 16S rRNA sequence. Phylogenetic trees were constructed and analysed the taxonomic position of the isolates from the experimental sites. All the 16S rRNA sequences were compared and evaluated with the sequenced available in the NCBI-GenBank database. Thus, 16S rRNA gene sequences confirmed the genus of our isolated strains at the molecular level. Evolutionary relationships of experimented taxa with the other taxa from databank were initiated by phylogenetic tree construction based on the NJ method by Mega v5.05 and presented in Fig. 1A. The optimal tree with the sum of branch length was 120.76440006. The analysis involved 57 nucleotide sequences. A total of 333 positions were shown in the final dataset. Taxonomic classification was also done separately between all experimental *Nitrosomonas*isolates with others from the database following the same tree construction method and outlined in Fig. 1B. 190.07822561 is the sum of the branch length of the constructed tree. The analysis involved 32 nucleotide sequences and 333 positions were present in the final dataset. Similar attempts were targeted for *Nitrobacter* sp. and shown in Fig. 1C. The sum of branch length was 84.29577718 for the tree and the analysis involved 29 nucleotide sequences. A total of 1010 positions were found in the final dataset. Distance tree displays the evolutionary origin of *Nitrosomonas* sp. and *Nitrobacter* sp. within a lineage shared by the different strains between reference sequences from the NCBI database and our experimental dataset.

Assessment of AOB and NOB in the present study through 16S rRNA analysis was done and few satisfactory results were obtained. The phylogenetic tree (Fig. 1A) based

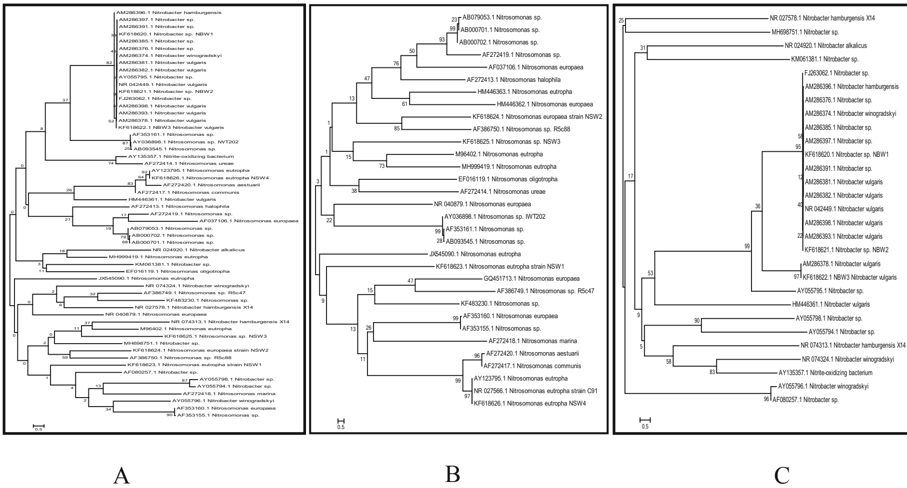

Fig. 1. Phylogenetic tree construction using Mega v5.05. The scale bar indicates 0.5 nucleotide substitutions per nucleotide positions. Accession numbers are presented on the right side with organism's name. 1000 replicates were taken for the bootstrap test. **A.** Construction of the phylogenetic tree on the basis of NJ method using the 16S rRNA sequences of all the seven experimental isolates with other sequences from databank; **B.** Representation of phylogenetic tree (NJ method) with 16S rRNA sequences of all the four *Nitrosomonas* sp. with global sequences; **C.** NJ method was used for construction of the phylogenetic tree of the local and global 16S rRNA sequences of *Nitrobacter* sp.

Table 1. List of primers with their sequences used in this experimental study

Sl. No	Name of Primer	Sequence (5′ to 3′)	Reference
1.	27F	AGAGTTTGATCCTGGCTCAG	Lane (1991)
2.	1492R	GGTTACCTTGTTACGACTT	Lane (1991)
3.	*amoA*-3F	CGTGAGTGGGYTAACMG	Purkhold et al. (2000)
4.	*amoB*-4R	GCTAGCCACTTTCTGG	Purkhold et al. (2000)
5.	F1norA	CAGACCGACGTGTGCGAAAG	Poly et al. (2008)
6.	R1norA	TCYACAAGGAACGGAAGGTC (Y = Cor T)	Poly et al. (2008)

on the global 16S rRNA sequences of AOB and NOB predicted the taxonomic orientation of local isolates with their respective genus. Figure 1B represents the position between local and global *Nitrosomonas* strains, where NSW4 and NSW1 were clustered with *Nitrosomonas eutropha.* NSW2 was clustered with *Nitrosomonas europaea* as expected, whereas NSW3 was grouped with different species of *Nitrosomonas* sp. Fig. 1C showed the phylogenetic cluster of NBW1 and NBW2 within the same group of different *Nitrobacter* sp. However, the NBW3 strain was grouped with *Nitrobacter*

vulgaris. PCR amplified band of *amo*A gene and *nxr*A gene of our isolates were checked and visualized on 2.0% agarose gel. Amplified band of *amo*A gene produced 491 bp fragment size was produced by NSW1 and NSW4. NSW2 and NSW3 produced a 415 bp band after PCR amplification. Similarly, PCR amplification of *nxr*A gene for NBW1and NBW2 resulted in 322bp whereas NBW3 showed 318 bp band respectively in 2.0% agarose gel.

Microorganisms play an important role in the purification system of EKW (Sarkar et al. 2016). Advanced molecular biology techniques and bioinformatics tools have opened new avenues in evaluating the ecological aspects and community survey of nitrifying bacteria (Guo et al. 2020). The present study identifies a few nitrifying bacteria in EKW using molecular and computational techniques. A similar experiment was conducted by Khanal and Lee (2020), and established that these attempts provide better potential for analysis and assessment of AOB and NOB. In our study, we found nitrifying bacteria in EKW by analysing the phylogenetic relationship of 16S rRNAgene clusters. This result was supported by the study of Zhao et al. (2018), where *Nitrosomonas* sp. and *Nitrobacter* sp. were proven as the major AOB and NOB. The diverse bacterial communities in wastewater can be analysed using advanced molecular-based markers, genomic tools and *in-silico* approaches (Ahmad et al. 2021). 16S rRNA gene sequences of all the isolates were used for phylogenetic tree construction and intra-genomic diversity assay. Similar reports regarding the phylogenetic conception of nitrifying bacteria were published by Ouyang and Norton 2020. Research conducted by different groups also reported the 16S rRNA sequences to help in complete abundance and community studies of AOB and NOB biomass in the environment (Takahashi et al. 2020; Poly et al. 2008; Purkhold et al. 2000).

From the above results, it could be inferred that the investigation of the 16S rRNA gene helps in the identification of nitrifying bacteria in EKW. 16S rRNA PCR and gene sequences, established as a reliable molecular tool for the identification of the genus AOB/*Nitrosomonas* and NOB/*Nitrobacter*. The *in-silico* approach with molecular analysis of bacterial isolates suggested the phylogenetic relationship of the nitrifying taxa in EKW.16S rRNA sequences of the experimental nitrifying bacteria were found to be similar with the above studies and reports. Considering all the above points and discussion, it can be conjectured and concluded that all the isolates from the water sample of EKW belong to AOB and NOB groups.

4 Conclusion

Development and application of molecular techniques help in the worthy observation of the relation between nitrifying bacteria, their composition and habitats in an ecosystem. We targeted to develop and validate a strategy to classify/identify nitrifying bacteria based on sequences of the 16S rRNAgene. Phylogenetic orientation and functional diversity of respective genes and their interactions in ecology can be easily established. Large reference libraries can be created of the new strains and can be assessed to monitor bacterial diversity in ecological communities. In the future, species and strain differentiation, studies of molecular taxonomy of a wide variety of bacteria can be achieved from the present research along with the involvement of modern molecular and computational

tools. However, more elaborative and further deep research is required to explore the AOB and NOB community in water bodies of EKW and other wastewater systems.

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Author Contribution. Mousumi Saha – Conceptualization, Formal Analysis and Investigation, Writing – Original Draft Preparation; Agniswar Sarkar – Writing – Review and Editing; BidyutBandyopadhyay- Supervision.

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