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Detection and Potential Abundances of Anammox Bacteria in the Paddy Soil

Anamika Khanal, Seul Lee and Ji-Hoon Lee*

¹Department of Bioenvironmental Chemistry, College of Agriculture & Life Sciences, Jeonbuk National University, Jeonju, Republic of Korea

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ORCID

Ji-Hoon Lee

<https://orcid.org/0000-0001-8916-149X>

Abstract

BACKGROUND: Microbes that govern a unique biochemical process of oxidizing ammonia into dinitrogen gas, such as anaerobic ammonium oxidation (anammox) have been reported to play a pivotal role in agricultural soils and in oceanic environments. However, limited information for anammox bacterial abundance and distribution in the terrestrial habitats has been known.

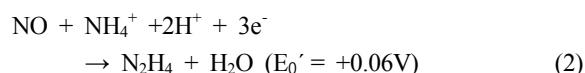
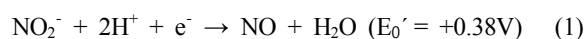
METHODS AND RESULTS: Phylogenetic and next-generation sequencing analyses of bacterial 16S rRNA gene were performed to examine potential anammox bacteria in paddy soils. Through clone libraries constructed by using the anammox bacteria-specific primers, some clones showed sequence similarities with *Planctomycetes* (87% to 99%) and anammox bacteria (94% to 95%). Microbial community analysis for the paddy soils by using Illumina Miseq sequencing of 16S rRNA gene at phylum level was dominated by unclassified *Bacteria* at $33.2 \pm 7.6\%$, followed by *Chloroflexi* at $20.4 \pm 2.0\%$ and *Acidobacteria* at $17.0 \pm 6.5\%$. *Planctomycetes* that anammox bacteria are belonged to was $1.5\% (\pm 0.3)$ on average from the two paddy soils.

CONCLUSION: We suggest evidence of anammox bacteria in the paddy soil. In addition to the relatively well-known microbial processes for nitrogen-cycle, anammox can be a potential contributor on the cycle in terrestrial environments such as paddy soils.

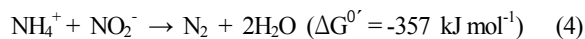
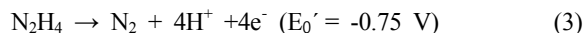
Key words: Anaerobic ammonium-oxidizing bacteria, Clone library, Next-generation sequencing, *Planctomycetes*, Terrestrial soil

Introduction

Anaerobic ammonium oxidation (anammox) process is one of the distinct microbial processes, which is responsible for the release of nitrogen gas to the atmosphere [1]. Even though its molecular mechanism is not completely understood, it has been proposed that the reaction proceeds through hydrazine (N_2H_4), which is produced from an ammonia and nitric oxide (NO). Nitrite is reduced to NO, and then ammonium is oxidized to yield hydrazine (N_2H_4), followed by hydrazine oxidation to N_2 , yielding four electrons [2-4].



*Corresponding author: Ji-Hoon Lee
 Phone: +82-63-270-2546; Fax: +82-63-270-2550
 E-mail: jhlee2@jbnu.ac.kr



Anammox bacteria have been discovered mostly in wastewater sludge since the early 1990s, and have been reported to belong to Planctomycetes [5, 6]. These anammox bacteria have been divided into five genera i.e., *Kuenenia* [4, 7], *Brocadia* [5, 8], *Anammoxoglobus* [9], *Jettenia* [10], and *Scalindua* [11-13]. *Scalindua* has been reported mainly in the marine environment, and some have been also detected in freshwater ecosystems and wastewater treatment plants. Anammox bacteria have been detected also in permafrost and agriculture soils based on 16S rRNA gene probing [14] using different sets of PCR primers for their profiling [15].

The growth rates of these bacteria are slow and doubling times are approximately 2-3 weeks [16, 17]. Many factors are believed to be responsible for their slow growth rates such as ammonium, nitrite, pH, temperature, organic matter, and iron content in natural habitats. Efforts have been made to optimize the growth of the bacteria in laboratory conditions by mimicking similar environments as in the natural habitats using enrichment cultures and bioreactors such as fed-batch reactor and sequencing batch reactor [2, 18].

Anammox bacteria play a prominent role in the nitrogen cycle [19]. They participate in the biogeochemical nitrogen production in paddy soils by 4-37% [20] and 50% of nitrogen loss from marine environment [21, 22]. Besides the ecological aspect, their practical applications in the elimination of ammonia from wastewater sludges, industrial effluents, and leachates suggest sustainable and environment-friendly technological aspects of the microorganism [3].

Even though increased interests on anammox bacteria, their diversity, distribution, and the ecological processes mediated by them have not been well understood over the last two decades, and the information about these is limited, especially on anammox in the terrestrial habitats. It seems that the slow growth rate and the difficult buildup of the biomass of these bacteria contributed to the limited information [23]. The main aim of this work was to find the distribution and abundance of anammox bacteria in the terrestrial niche, particularly of rice paddy soil by using molecular approaches such as clone library and next-generation sequencing (NGS), for the better understanding of these bacteria, in the terrestrial habitat rather than the oceanic and sludge environments.

Materials and Methods

Sampling and analyses of physico-chemical parameters of paddy soil

A rice paddy field site was selected for soil sampling, that is located inside Rural Development Administration, Jeonju, South Korea (latitude 35°49'42.4"N and longitude 127°02'38.4"E; Fig. S1). Soil samples were taken at 30 cm depth (RPS1) from the ground level and from the surface level (RPS2) of a rice paddy field by using a small shovel. Three spots were sampled into individual tubes for each soil depth. Soil slurry with water was prepared in the ratio of 1:5 by shaking for 30 min at shaker to measure the pH and electrical conductivity by using portable probes and meter (Thermo Scientific, Pittsburgh, PA, USA). Measurement of organic carbon was done by detecting the carbon loss on ignition at 360°C [24]. Total organic nitrogen, nitrate, and ammonia were measured by using Dahnke method [25]. Chromic acid digestion and spectrophotometric procedure were used to measure the total organic carbon [26] and total iron was measured by using acid digestion method by inductively coupled plasma-atomic emission spectroscopy.

DNA extraction from soil samples and PCR

Genomic DNA was extracted from the rice paddy soils by using MoBio Powersoil DNA kit (Carlsbad, CA, USA), following the manufacturer protocol using 0.25 mg of the soil samples. The triplicate DNA extracts from each soil depth were pooled into one tube for downstream experiments. The DNA quantity was measured by fluorometry (Qubit 3.0 Fluorometer, Invitrogen, Pittsburgh, PA, USA). Genomic DNA was diluted to 1-5 ng μl^{-1} for further use. Nested PCR was performed in order to amplify 16S rRNA genes of anammox bacteria. Bacterial universal primer set of 27F/1391R was used for first round of PCR, followed by the second round of PCR using the primer sets of Pla46F/Amx820R [7,27], and Amx368F/Amx820R [7, 28]. All the primers used in this study are summarized in Table S1 (Supplementary Information). A total of 50 μl was prepared for the PCR reactions, including 1-2 μl of genomic DNAs (1-5 ng μl^{-1}), and 1 μl of forward and reverse primers (10 pmol). The thermal cycles were operated with an initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and an extension at 72°C for 1 min 30 sec. Final extension was done at 72°C for 5 min. The quality and quantity

of purified DNA were checked, respectively by gel electrophoresis and fluorometry.

Clone library construction and confirmation of positive clones

DNA templates of specific band size after amplification from the nested PCR and purification were ligated into pTOP TA V2 (vector) of TOP cloner TA kit (Enzynomics, Daejon, South Korea) and transformed into *E. Coli* DH5 α TM competent cells (Enzynomics, Daejon, South Korea). Blue-white screening was performed by using ampicillin (0.1 mg ml⁻¹), IPTG (0.1 mM), and X-gal (40 μ g ml⁻¹) in LB (lysogeny broth) agar plate. White colonies were selected and incubated in LB broth containing 0.1 mg ml⁻¹ of ampicillin for overnight. Plasmid DNA extraction was done by using Inclone mini plasmid preparation kit (Inclone biotech, Yongin, South Korea). Plasmid DNAs isolated from the white colonies were subjected to PCR amplification using the multiple cloning site (MCS) primer set of M13F (-20) and M13R (-40) and the PCR products were checked on 1% agarose gel for confirmation of the positive clones. DNAs isolated from the clone library were sequenced at Genotech (Daejeon, South Korea).

Phylogenetic analysis of the clones

The forward and reverse nucleotide sequences of the cloned DNAs of 16S rRNA gene fragments were assembled together. The obtained sequences were compared to NCBI GenBank by using BLAST search [29], in order to find the known sequences so far, particularly for anammox bacteria and clones. Phylogenetic trees for the cloned anammox sequences were constructed together with the similar sequences retrieved from the GenBank by using Mega 7 [30]. The evolutionary history was inferred using the neighbor-joining method [31, 32].

Soil microbial community analysis

Two genomic DNAs isolated from the soil samples (surface and 30 cm-deep soils) were subjected to next-generation sequencing (NGS) for the V3-V4 region of 16S rRNA gene using 341F/805R primer set by Miseq system (Illumina, San Diego, CA, USA), which generated paired-end sequences of approximately 428 bp-long (250 bp x 2). The raw sequence data were analyzed using the Mothur version 1.43.0 [33], referring to the Miseq sequence standard operating procedure [34]. The sequences were contig-assembled, filtered,

and screened for the undesirable sequences such as low-quality reads, sequencing errors, chimeras, archaea, etc. resulting in the total of 122,456 sequences from the initial 508,977 reads. Chimeras were screened by Vsearch [35] and the sequences were aligned with Silva database release v. 132 [36]. The sequences were clustered into 14,403 operational taxonomic units (OTUs) by 3% dissimilarity and classified to taxa by using RDP database [37].

Nucleotide accession Number

All the 16S rRNA gene sequences from clone library related to uncultured bacterial species, *Planctomycetes*, and anammox bacteria were deposited in GenBank with accession numbers of KY883355 to KY883367 respectively for clones PA-1, PA-2, PA-3, PA-4, AA-6, AA-7, PA-9, PA-11, PA-12, PA-13, PA-14, AA-15, and AA-17, and MF171191 and MF171192 for clones AA-5 and AA-16, respectively. The Miseq sequenced data were deposited at NCBI Sequence Read Archive (SRA) with accession number SRR7791714.

Results

There are many microorganisms in the agricultural soils, which contribute to the biogeochemical nitrogen cycle such as anammox bacteria. Although the anammox contribution to nitrogen loss by the generation of N₂ gas in the paddy soils is not as high as in the marine [20, 29], investigating the anammox reaction in the rice paddy soils would provide a better understanding of the reaction in the terrestrial environments and the contribution to the nitrogen cycle. In this study, we have selected the soil samples from the rice paddy field, to study the distribution and abundances of microbial species with more focus on the anaerobic ammonium oxidation bacteria.

Physico-chemical parameters of soil

The measurement, as well as knowledge of physico-chemical parameters of the soil, is very important because it will determine the quality, health of the soil, as well as the diversity of the microorganism [38]. The physico-chemical parameters of the soil (RPS1) such as soil texture, pH, electrical conductivity (EC), ammonia, nitrate, organic matter, total organic carbon, total nitrogen, and total iron is listed in (Table S2). The soil was found to be in good condition for agriculture.

Amplification of 16S rRNA gene from soil sample and clone library construction

Amplification of 16S rRNA gene from the rice paddy soil genomic DNA (RPS1) with 27F/1391R yielded a DNA band of approximately 1.4 kbp fragment (Fig. S2 A) and the subsequent amplification using *Planctomycetes*-specific and anammox-specific primer sets of Pla46F/Amx820R and Amx368F/Amx820R yielded approximately 700 bp - 800 bp and approximately 500 bp fragments, respectively (Fig. S2 B and C).

After a series of procedures for clone library construction such as the purification of amplicons, ligation, and transformation, among the white colonies by the blue-white screening, 15 colonies were taken and subjected to plasmid extraction. Positive clones were confirmed by PCR using the MCS primer set of M13F and M13R and the amplicons of cloned gene fragments were sequenced.

Sequencing and phylogenetic analysis

Among the white colonies, 15 selected clones (PA-1,

PA-2, PA-3, PA-4, AA-6, AA-7, PA-9, PA-11, PA-12, PA-13, PA-14, AA-15, and AA-17 with accession numbers KY883355 to KY883367; AA-5, and AA-16 with accession numbers MF171191 and MF171192) showed sequence similarities ranging from 94% to 99% with an uncultured bacterium. Some clones showed sequence similarities with *Planctomycetes* (PA-4 at 98%; PA-12, PA-13, and PA-14 at 97 %; PA-1 at 87 %) and 4 clones with anaerobic ammonium oxidation bacteria (PA-4 at 94%, PA-12, PA-13, and PA-14 at 95%). Rest of the clones showed sequence similarities with other microorganisms such as salt marsh clones, *Proteobacteria* and *Firmicutes*. The Phylogenetic trees for the evolutionary relationship on the basis of sequence similarities with the uncultured bacterium, *Planctomycetes*, anammox bacteria, and other microbial species like *Firmicutes*, salt-marsh clones etc. are shown in Figs. 1, 2, and 3. We have identified clues of *Planctomycetes* in the rice paddy fields by clone library construction, sequencing, and phylogenetic analysis.

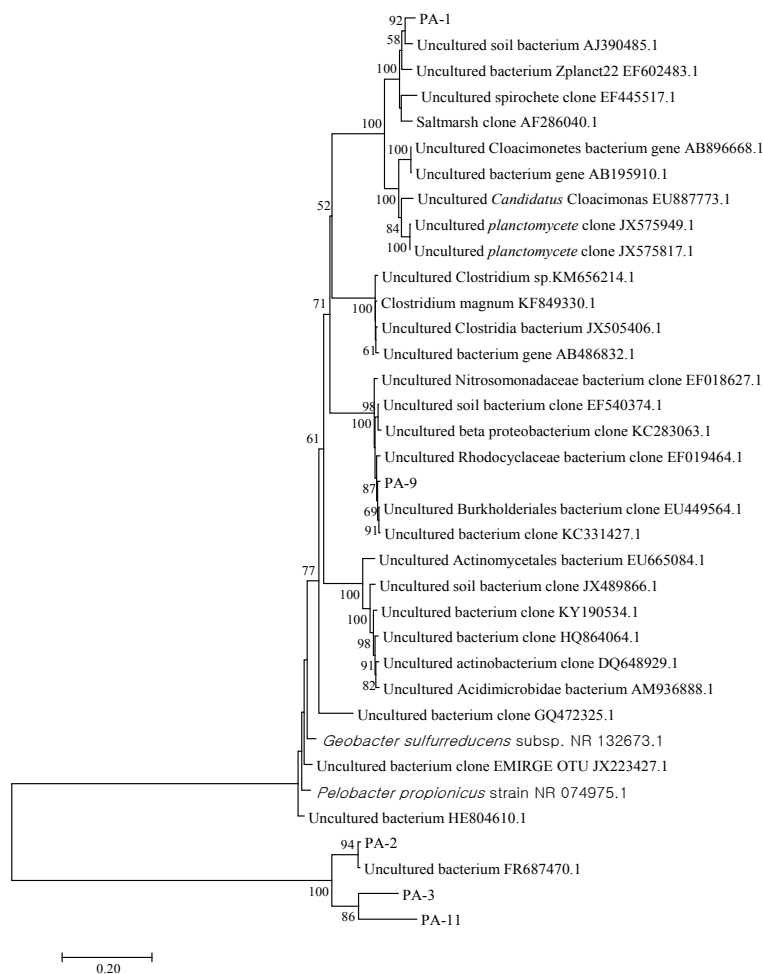


Fig. 1. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences of clones PA-1, PA-2, PA-3, PA-9, and PA-11 obtained from the clone library with known anammox bacterial 16S rRNA gene sequences.

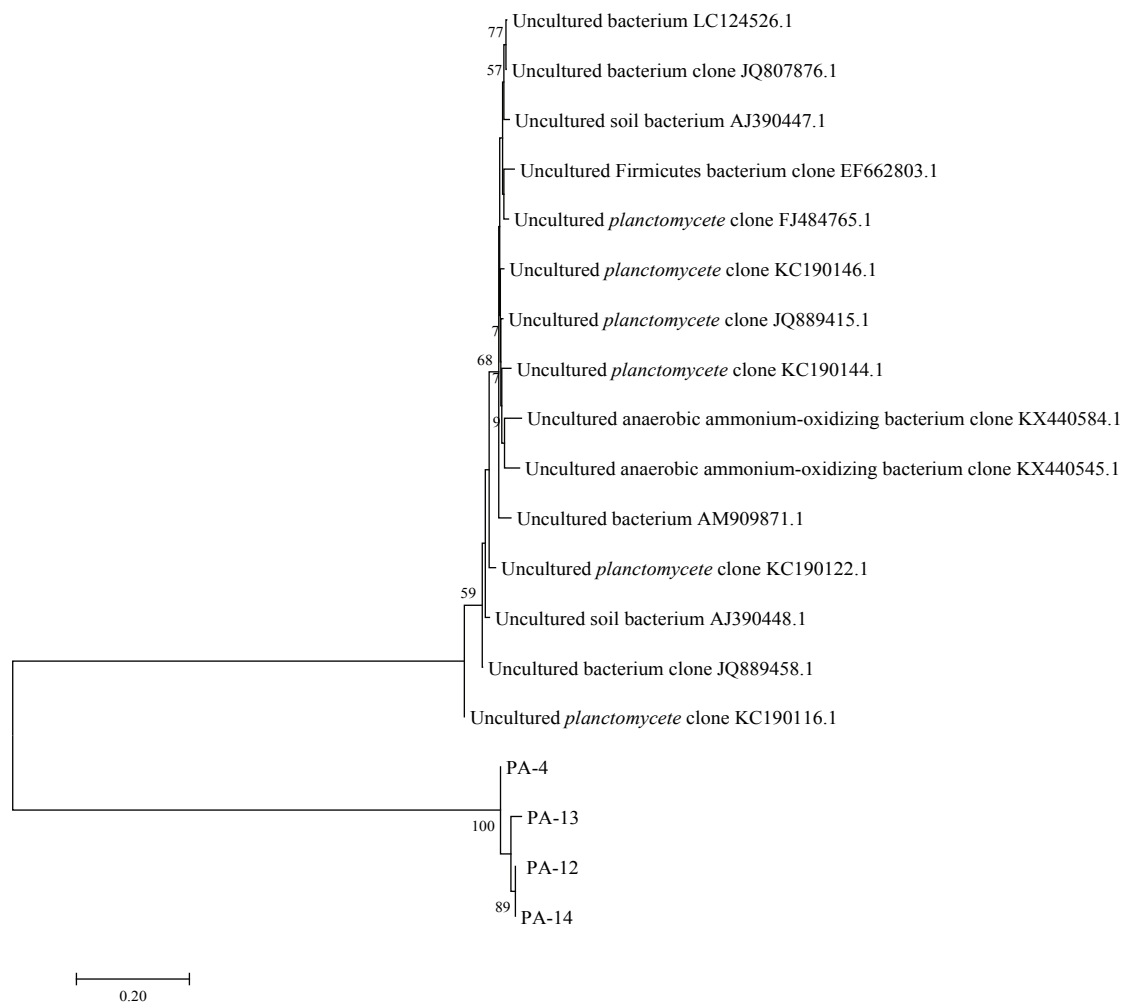


Fig. 2. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences of clones PA-4, PA-8, PA-12, PA-13, and PA-14 obtained from the clone library with known anammox bacterial 16S rRNA gene sequences.

Microbial community analysis by Miseq sequences

To examine diversity of microorganism including anammox bacteria in the particular field samples, microbial community analysis was conducted. The numbers of nucleotide sequences of the two samples (RSP1 from 30 cm- depth and RPS2 from the surface) were normalized to 53,532 to perform alpha-diversity analysis, which showed Good's coverage values of 91.2% and 91.5% respectively, for RPS1 and RPS2 (Table S3), indicating sufficient resolution of the microbial communities. Bacterial species richness, which is a count of species, calculated by Chao1 was found to be 23,146 and 26,638, suggesting higher bacterial counts at the surface soil (RPS2) (Table S3). The bacterial diversity indices of Shannon and inverse Simpson, reflecting both the richness and species evenness showed opposite patterns of the values, while inverse Simpson index was in consistent pattern

with Chao1 index, indicating higher bacterial diversity in RPS2.

Excluding the most abundant unclassified *Bacteria* (27.8% and 38.5%, respectively for RPS1 and RPS2) at phylum level, the distribution of bacteria showed that *Chloroflexi* was abundant at 21.7% and 19.0%, respectively for soils RPS1 and RPS2, followed by *Acidobacteria* at 12.4% and 21.6%, respectively (Fig. 4). Phylum *Planctomycetes*, where the anammox bacteria belong was at 1.7% and 1.2%, respectively for the 30-cm depth (RPS1) and the surface (RPS2) soils. The similar type of result was reported by [39], where no difference in abundance of anammox was detected between the surface (0-5 cm) and subsurface (20-25 cm) soils. The community composition at genus level suggested as shown by sequence abundance revealed that unclassified *Bacteria* were the most abundant at 27.8% and 38.5%, respectively for the RPS1 and RPS2,

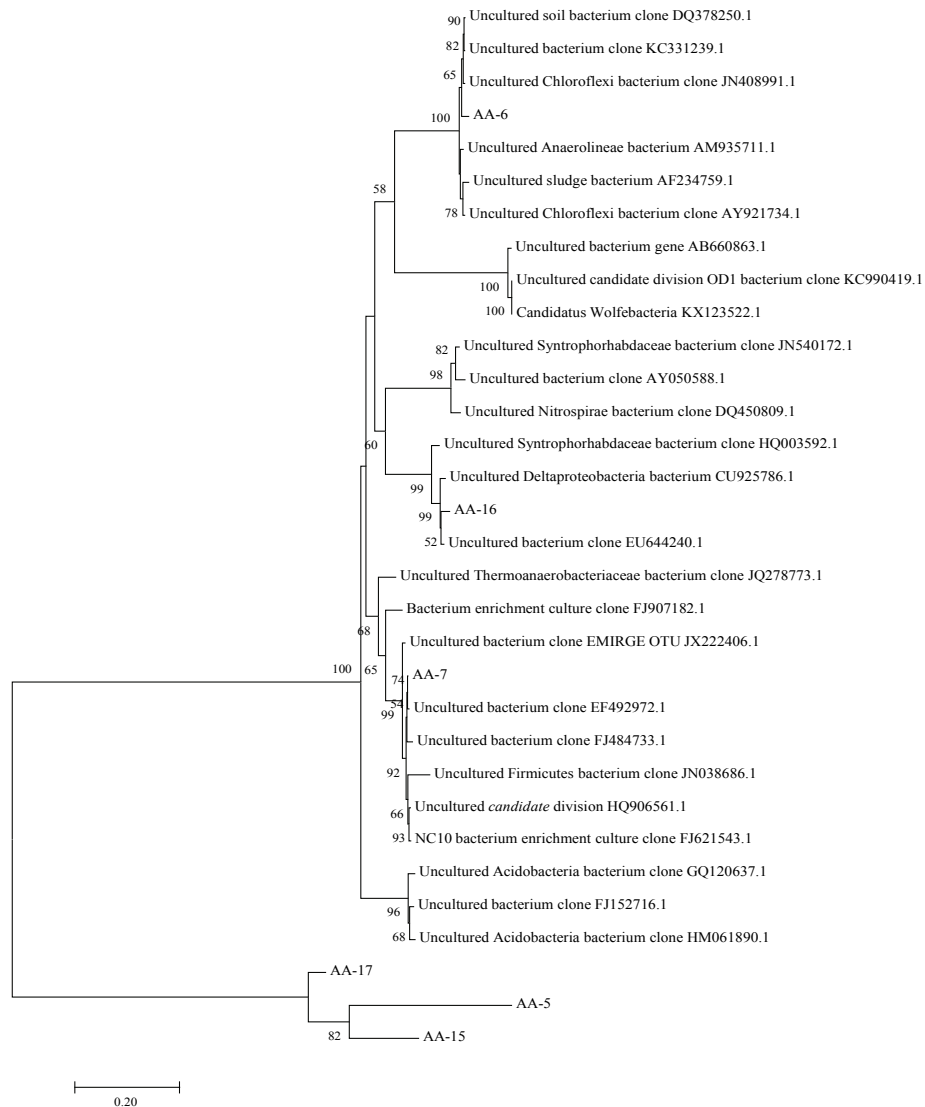


Fig. 3. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences of clones AA-5, AA-6, AA-7, AA-15, AA-16, and AA-17 obtained from the clone library with known bacterial 16S rRNA gene sequences.

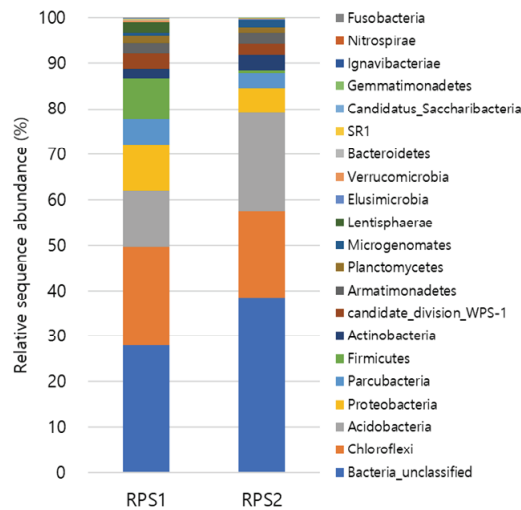


Fig. 4. Relative sequence abundance of 21 bacterial phyla out of total 29, based on OTUs clustered at 97% similarity (excluding phyla less than 0.01%).

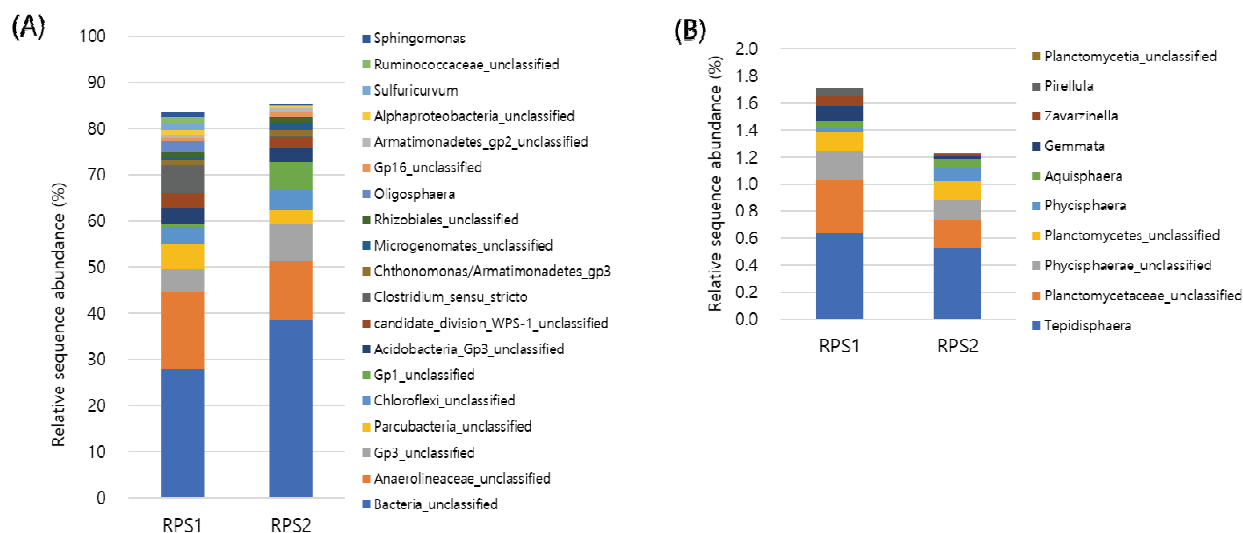


Fig. 5. Relative sequence abundance of bacterial genera. (A) 19 out of total 349 genera with exclusion of genera whose sequence abundances were less than 1% in both the samples. (B) 10 genera classified as *Planctomycetes*.

followed by unclassified *Anaerolineaceae* (16.7% and 12.8%, respectively) and unclassified Gp3 (4.9% and 7.8%) (Fig. 5). We detected the anammox bacterial 16S rRNA genes, but their abundances were found to be low which was supported by the microbial community data by Miseq sequencing. Although relatively high abundances of the phylum *Planctomycetes* were found with 1.7% and 1.2% for two soil samples, anammox bacterial sequences were very minor.

Discussion

Based on the above-presented results, we could suggest that there were very low abundances of anammox bacteria in the paddy soils. Although the paddy field is not a completely natural environment, it is also very different from an engineered environment of wastewater sludges from which most anammox bacteria have been enriched. Various factors such as excessive use of fertilizers, oxygen availability, pH and/or total organic carbon in the soil environment, could be responsible for low abundance of these bacteria [40]. In addition, several studies have shown that the commonly used 16S rRNA gene primers could not extend the diversity of these anammox bacteria, and analysis on functional genes could be a better option for future [16]. Rice paddy fields have been focused as a model for habitats of diverse microorganisms [41]. Still many aspects of questions, however, are remained about the indigenous microorganisms, especially on the anammox bacteria in the paddy soils, and their potential roles on the bio-

geochemical nitrogen cycle.

Bacteria-mediated anaerobic ammonium oxidation (anammox) resulting in conversion of ammonium and nitrite to dinitrogen gas is one of the important processes in environments such as wastewater, ocean, freshwater, and terrestrial habitat. Researchers have identified and reported anammox reaction and anammox bacteria in the above-mentioned specific environments, but still there is only limited information regarding their diversity and roles in terrestrial habitats. This study will help to contribute towards understanding and exploring the anammox bacteria in terrestrial habitat.

Note

The authors declare no conflict of interest.

Acknowledgement

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References

- Shi Y, Hu SH, Lou JQ, Lu PL, Keller J, Yuan ZG (2013) Nitrogen removal from wastewater by coupling anammox and methane-dependent denitrification in a membrane biofilm reactor. *Environmental Science & Technology*, 47, 11577-11583. <https://doi.org/10.1021/>

- es402775z.
- deGraaf AAV, deBruijn P, Robertson LA, Jetten MSM, Kuenen JG (1996) Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology-UK*, 142, 2187-2196. <https://doi.org/10.1099/13500872-142-8-2187>.
 - Kartal B, Maalcke WJ, de Almeida NM, Cirpus I, Gloerich J, Geerts W, den Camp H, Harhangi HR, Janssen-Megens EM et al. (2011) Molecular mechanism of anaerobic ammonium oxidation. *Nature*, 479, 127-130. <https://doi.org/10.1038/nature10453>.
 - Strous M, Pelletier E, Mangenot S, Rattei T, Lehner A, Taylor MW, Horn M, Daims H, Bartol-Mavel D et al. (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature*, 440, 790-794. <https://doi.org/10.1038/nature04647>.
 - Strous M, Fuerst JA, Kramer EHM, Logemann S, Muyzer G, van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MSM (1999) Missing lithotroph identified as new planctomycete. *Nature*, 400, 446-449. <https://doi.org/10.1038/22749>.
 - Fuerst JA, Sagulenko E (2011) Beyond the bacterium: planctomycetes challenge our concepts of microbial structure and function. *Nature Reviews Microbiology*, 9, 403-413. <https://doi.org/10.1038/nrmicro2578>.
 - Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten M, Metzger JW, Schleifer K-H, Wagner M (2000) Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Systematic and Applied Microbiology*, 23, 93-106. [https://doi.org/10.1016/S0723-2020\(00\)80050-8](https://doi.org/10.1016/S0723-2020(00)80050-8).
 - Kartal B, van Niftrik L, Rattray J, de Vossenberg J, Schmid MC, Damste JSS, Jetten MSM, Strous M (2008) Candidatus 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. *FEMS Microbiology Ecology*, 63, 46-55. <https://doi.org/10.1111/j.1574-6941.2007.00408.x>.
 - Kartal B, Kuypers MMM, Lavik G, Schalk J, den Camp H, Jetten MSM, Strous M (2007) Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. *Environmental Microbiology*, 9, 635-642. <https://doi.org/10.1111/j.1462-2920.2006.01183.x>.
 - Quan ZX, Rhee SK, Zuo JE, Yang Y, Bae JW, Park JR, Lee ST, Park YH (2008) Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor. *Environmental Microbiology*, 10, 3130-3139. <https://doi.org/10.1111/j.1462-2920.2008.01642.x>.
 - Kuypers MMM, Sliemers AO, Lavik G, Schmid M, Jørgensen BB, Kuenen JG, Sinninghe Damsté JS, Strous M, Jetten MSM (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature*, 422, 608-611. <https://doi.org/10.1038/nature01472>.
 - van de Vossenberg J, Rattray JE, Geerts W, Kartal B, van Niftrik L, van Donselaar EG, Damste JSS, Strous M, Jetten MSM (2008) Enrichment and characterization of marine anammox bacteria associated with global nitrogen gas production. *Environmental Microbiology*, 10, 3120-3129. <https://doi.org/10.1111/j.1462-2920.2008.01643.x>.
 - Hong YG, Yin B, Zheng TL (2011) Diversity and abundance of anammox bacterial community in the deep-ocean surface sediment from equatorial Pacific. *Applied Microbiology and Biotechnology*, 89, 1233-1241. <https://doi.org/10.1007/s00253-010-2925-4>.
 - Humbert S, Tarnawski S, Fromin N, Mallet MP, Aragno M, Zopfi J (2010) Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. *The ISME Journal*, 4, 450-454. <https://doi.org/10.1038/ismej.2009.125>.
 - Sonthiphand P, Neufeld JD (2013) Evaluating primers for profiling anaerobic ammonia oxidizing bacteria within freshwater environments. *PLoS ONE*, 8, e57242. <https://doi.org/10.1371/journal.pone.0057242>.
 - Harhangi HR, Le Roy M, van Alen T, Hu BL, Groen J, Kartal B, Tringe SG, Quan ZX, Jetten MSM et al. (2012) Hydrazine synthase, a unique phylogenetic marker with which to study the presence and biodiversity of anammox bacteria. *Applied and Environmental Microbiology*, 78, 752-758. <https://doi.org/10.1128/aem.07113-11>.
 - Lindsay MR, Webb RI, Strous M, Jetten MS, Butler MK, Forde RJ, Fuerst JA (2001) Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. *Archives of Microbiology*, 175, 413-429. <https://doi.org/10.1007/s002030100280>.
 - Kartal B, Koleva M, Arsov R, van der Star W, Jetten MSM, Strous M (2006) Adaptation of a freshwater anammox population to high salinity wastewater.

- Journal of Biotechnology, 126, 546-553. <https://doi.org/10.1016/j.jbiotec.2006.05.012>.
19. Li M, Gu JD (2011) Advances in methods for detection of anaerobic ammonium oxidizing (anammox) bacteria. *Applied Microbiology and Biotechnology*, 90, 1241-1252. <https://doi.org/10.1007/s00253-011-3230-6>.
 20. Zhu GB, Wang SY, Wang WD, Wang Y, Zhou LL, Jiang B, Op den Camp HJM, Risgaard-Petersen N, Schwark L et al. (2013) Hotspots of anaerobic ammonium oxidation at land-freshwater interfaces. *Nature Geoscience*, 6, 103-107. <https://doi.org/10.1038/ngeo1683>.
 21. Arrigo KR (2005) Marine microorganisms and global nutrient cycles. *Nature*, 437, 349-355. <https://doi.org/10.1038/nature04159>.
 22. Nie SA, Li H, Yang XR, Zhang ZJ, Weng BS, Huang FY, Zhu GB, Zhu YG (2015) Nitrogen loss by anaerobic oxidation of ammonium in rice rhizosphere. *The ISME Journal*, 9, 2059-2067. <https://doi.org/10.1038/ismej.2015.25>.
 23. Kuenen JG (2008) Anammox bacteria: from discovery to application. *Nature Reviews Microbiology*, 6, 320-326. <https://doi.org/10.1038/nrmicro1857>.
 24. Schulte E, Hopkins B (1996) Estimation of soil organic matter by weight loss-on-ignition. In: *Soil organic matter: analysis and interpretation*. pp 21-31.
 25. Dahnke W, Johnson GV (1990) Testing soils for available nitrogen. In: *Soil Testing and Plant Analysis*. pp 128-139.
 26. Heanes DL (1984) Determination of total organic-C in soils by an improved chromic-acid digestion and spectrophotometric procedure. *Communications in Soil Science and Plant Analysis*, 15, 1191-1213. <https://doi.org/10.1080/00103628409367551>.
 27. Neef A, Amann R, Schlesner H, Schleifer KH (1998) Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology-UK*, 144, 3257-3266. <https://doi.org/10.1099/00221287-144-12-3257>.
 28. Schmid M, Walsh K, Webb R, Rijpstra WIC, van de Pas-Schoonen K, Verbruggen MJ, Hill T, Moffett B, Fuerst J et al. (2003) *Candidatus "Scalindua brodae"*, sp. nov., *Candidatus "Scalindua wagneri"*, sp. nov., two new species of anaerobic ammonium oxidizing bacteria. *Systematic and Applied Microbiology*, 26, 529-538. <https://doi.org/10.1078/072320203770865837>.
 29. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology*, 215, 403-410. <https://doi.org/10.1006/jmbi.1990.9999>.
 30. Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33, 1870-1874. <https://doi.org/10.1093/molbev/msw054>.
 31. Saitou N, Nei M (1987) The neighbor-joining method—A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406-425. <https://doi.org/10.1093/oxfordjournals.molbev.a040454>.
 32. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 11030-11035. <https://doi.org/10.1073/pnas.0404206101>.
 33. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, 75, 7537-7541. <https://doi.org/10.1128/aem.01541-09>.
 34. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013) Development of a dual-Index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology*, 79, 5112-5120. <https://doi.org/10.1128/aem.01043-13>.
 35. Rognes T, Flouri T, Nichols B, Quince C, Mahe F (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, 4, e2584. <https://doi.org/10.7717/peerj.2584>.
 36. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, 41, D590-D596. <https://doi.org/10.1093/nar/gks1219>.
 37. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR et al. (2014) Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, 42, D633-D642. <https://doi.org/10.1093/>

- nar/gkt1244.
38. Faoro H, Alves AC, Souza EM, Rigo LU, Cruz LM, Al-Janabi SM, Monteiro RA, Baura VA, Pedrosa FO (2010) Influence of Soil Characteristics on the Diversity of Bacteria in the Southern Brazilian Atlantic Forest. *Applied and Environmental Microbiology*, 76, 4744-4749. <https://doi.org/10.1128/aem.03025-09>.
 39. Wang J, Gu JD (2013) Dominance of *Candidatus Scalindua* species in anammox community revealed in soils with different duration of rice paddy cultivation in Northeast China. *Applied Microbiology and Biotechnology*, 97, 1785-1798. <https://doi.org/10.1007/s00253-012-4036-x>.
 40. Jin RC, Yang GF, Yu JJ, Zheng P (2012) The inhibition of the anammox process: A review. *Chemical Engineering Journal*, 197, 67-79. <https://doi.org/10.1016/j.cej.2012.05.014>.
 41. Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, von Mering C, Vorholt JA (2012) Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *The ISME Journal*, 6, 1378-1390. <https://doi.org/10.1038/ismej.2011.192>.