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Functional diversity and abundance of nitrogen cycle-related genes in paddy soil

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Abstract

The nitrogen cycle and the associated microbes play an important role in natural ecosystems, including terrestrial habitats; they also have a major effect on climate change. The aim of this study was to explore microbial communities in rice paddy soil by detecting and quantifying some key functional genes involved in the nitrogen cycle using molecular techniques such as conventional polymerase chain reaction (PCR), clone library construction, sequencing, phylogenetic analysis, and real-time PCR. The genes analyzed were as follows: nitrogenase reductase gene (*nif*H), hydrazine synthase gene (*hzs*A), nitrous oxide reductase gene (*nos*Z), copper-containing (*nir*K) and cytochrome cd1-containing (*nir*S) nitrite reductase genes, nitrite oxidoreductase gene (*nxr*B), and ammonium monooxygenase gene (*amo*A). The sequence assessment using the clone library targeting these genes revealed high diversity and dominance of bacterial communities. Furthermore, real-time PCR using SYBR green dye and some primers specific for each gene revealed the high abundance of *nxr*B ($4.1 \times 10^9 \pm 0.4 \times 10^9$ copies g^{-1} soil) and low abundance of *hzs*A ($4.0 \times 10^5 \pm 1.1 \times 10^5$ copies g^{-1} soil). The findings of our study will be useful to explore microbial communities in terrestrial habitats, such as agricultural paddy fields.

Keywords: Clone library, Functional gene, Nitrogen cycle, Real time-polymerase chain reaction, Terrestrial soil

Introduction

Soil is a mixture of organic matter, minerals, gases, water, and microorganisms, which together support life on earth. Soil is considered a major component of the earth's ecosystem as it plays various roles owing to its physiochemical heterogeneity; for example, it acts as a carbon reservoir, water storage system, modifier of earth's atmosphere, and habitat for plants and microorganisms including bacteria and fungi [1]. The high heterogeneity of soil may lead to the heterogeneity of habitats. A small amount of soil can contain up to one billion bacteria [2]. Furthermore, the diversity and composition of soil communities determine the functionality of an ecosystem [3]. Moreover, ecosystems are affected by processes occurring in the soil, especially those driven by microbes, such as the nitrogen cycle. The nitrogen cycle and the associated microbial communities play a crucial role in terrestrial habitats; they also have a major effect on the environment. They have been a subject of interest for researchers and the general public because the nitrogen cycle and the associated microbial communities determine the fate and availability of nitrogen, which is essential to support life [4].

The nitrogen cycle involves the transformation of nitrogen from one form to other. Nitrogen fixation, nitrification or ammonia oxidation, and denitrification are the major processes of the nitrogen cycle. Most of these processes are mediated by microbial communities, which obtain energy or accumulate nitrogen via these processes according to their growth requirements. In nitrogen fixation, nitrogen gas is transformed to ammonium by nitrogen-fixing bacteria. This process involves the nitrogenase enzyme, whose multiple subunits are encoded by *nif*H, *nif*D, and *nif*K (Rubio Lm 2002). Among these three genes, *nif*H has been the most sequenced and extensively used marker gene for studying phylogeny, diversity, and abundance of

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nitrogen-fixing bacteria [5, 6]. In nitrification, ammonia or ammonium is oxidized to nitrite, and this step is catalyzed by the ammonium monooxygenase enzyme encoded by *amo*A [7]. Nitrite is then oxidized to nitrate, which is catalyzed by nitrite oxidoreductases encoded by genes such as *nxr*B [8]. Mostly ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) mediate the nitrification process [7]. Furthermore, with the discovery of anammox bacteria, it has been reported that ammonia oxidation is also anaerobically mediated by hydrazine synthase encoded by *hzs*A [9]. During denitrification, nitrate is reduced ultimately to nitrogen gas via a series of enzymatic reactions by diverse nitrogen oxide reductases encoded by *nir*S, *nir*K, and *nos*Z. Any disturbance in this process due to an imbalance in diverse nitrogen compounds might lead to negative consequences such as global warming and climate change. Therefore, understanding, investigating, and obtaining detailed information about the N cycle-related microorganisms are major requisites for improving the current state of the environment.

Although the nitrogen cycle has been investigated and reported in diverse types of soils such as agricultural field soils [10], acidic forest soils [11], paddy soils [12], intertidal soils [13], and wetland soils [14], information about these processes and probes for detecting specific genes is limited. The most widely used phylogenetic marker for studying microbial communities is the 16S rRNA gene. However, some microorganisms performing a common function are not numerically dominant and are hardly detectable using 16S rRNA gene-based probes. Thus, the use of functional gene markers has been the alternative approach for studying environmental samples. Furthermore, this method provides resolution below species level, because of the high evolutionary rates of the less conserved functional molecules [15, 16]. Considering the advantages of functional genes over the 16S rRNA gene, in the present study, we detected and quantified nitrogen cycle-related genes in a rice paddy field using functional gene markers.

Materials and methods

Rice paddy soil sampling

A rice paddy field site, located in Iksan, South Korea (latitude 35° 49′ 42.4″ N and longitude 127° 02′ 38.4″ E), was selected for soil sampling. Soil samples were collected at 20 cm depth from the surface of the field using a small shovel. Soil slurry with water was prepared at a ratio of 1:5 by shaking for 30 min in a shaker to measure pH and electrical conductivity using portable probes (Thermo Scientific, Pittsburgh, PA, USA).

DNA extraction from soil samples and conventional polymerase chain reaction

Genomic DNA was extracted from 0.25 mg of paddy soil using the MoBio Powersoil DNA kit (Carlsbad, CA, USA), following the manufacturer's protocol. The DNA quantity was measured by fluorometry (Qubit 3.0 Fluorometer, Invitrogen, Pittsburgh, PA, USA). Genomic DNA was diluted with ultra-pure water to $1–5$ ng μL^{-1} for further use. All the primers used in this study are summarized in Additional file 1: Table S1. The PCR mixture of total volume 50 μL contained $1–2$ μL of $1–5$ ng μL^{-1} genomic DNA and 1 μL each of 10 μM forward and reverse primers. The conditions for the PCR cycles for each target gene are summarized in Additional file 1: Table S2. The quality and quantity of the purified DNA were determined by gel electrophoresis and fluorometer, respectively.

Construction of clone libraries and confirmation of positive clones

Clone libraries were constructed by using DNA fragments of specific band sizes after amplification by PCR and purification. DNA amplicons were ligated into pTOP TA V2 (vector) using the TOP Cloner TA Kit (Enzynomics, Daejon, South Korea) or pLUG-Prime TA Cloning Kit II (Intron Biotechnology, Seongnam, South Korea) and transformed into *Escherichia coli* DH5α™ competent cells (Enzynomics, Daejon, South Korea). Blue–white screening was performed using ampicillin (0.1 mg mL^{-1}), IPTG (0.1 mM), and X-gal (40 μg mL^{-1}) in lysogeny broth (LB) agar plates. White colonies were selected and incubated overnight in LB broth supplemented with 100 μg mL^{-1} ampicillin. Plasmid DNA extraction was carried out using the Inclone Mini Plasmid Preparation Kit (Inclone biotech, Yongin, South Korea). The plasmid DNAs isolated from the white colonies were subjected to PCR amplification using the multiple cloning site (MCS) primer sets from the respective vector kits, M13F (-20) and M13R (-40) for pTOP-TA-V2 vector and M13F and M13R for pLUG-prime vector. The PCR product was checked on a 1% agarose gel to confirm positive clones. DNA isolated from the clone library was sequenced at Genotech (Daejeon, South Korea).

Phylogenetic analysis

The forward and reverse nucleotide sequences of cloned functional fragments of each gene were assembled together. The obtained sequences were compared with those in the NCBI GenBank by a BLAST search (https://blast.ncbi.nlm.nih.gov) [17] in order to identify known sequences, for each gene. Phylogenetic trees of the cloned sequences of each gene were constructed along with similar sequences retrieved from the GenBank using Mega 7 [18]. The evolutionary history was inferred using the neighbor-joining

method [19, 20]. All the sequences were deposited in the GenBank with accession numbers as follows, *nif*H: MT109314-MT109333, *amo*A: MT140205-MT140224, *nxr*B: MT140190-MT140204, *hzs*A: MT140225-MT140241, *nos*Z1: MT140150-MT140169, *nos*Z2: MT140170-MT140189, *nir*S: MT140262-MT140281, and *nir*K: MT140242-MT140261, respectively.

Quantitative PCR analysis

qPCR was used to quantify nitrogen-fixing bacterial gene (*nif*H), nitrifying bacterial genes (*nxr*B and *amo*A), denitrifying bacterial genes (*nir*K, *nir*S, and *nos*Z), and anaerobic ammonia oxidizing bacteria-specific gene (*hzs*A). It was performed using the primer sets listed in Additional file 1: Table S1 and Greenstar Accupower Mastermix (Bioneer, Daejeon, South Korea), according to the manufacturer's protocol, on the Biorad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, US). To calculate the gene copy numbers in a known concentration of DNA, Eq. 1 was used.

$$
\begin{aligned}
\text{Gene copy number} = \ & \left(\text{DNA concentration [ng/µL]}\right)\left(1\ \text{g}/1000^3\ \text{ng}\right) \\
& \left(1\ \text{mol bp DNA}/660\ \text{g DNA}\right) \\
& \times \left(6.023 \times 10^{23}\ \text{bp/mol bp}\right)\left(1\ \text{copy/genome or plasmid size [bp]}\right) \\
& \times \left(\text{volume of template [µL]}\right)
\end{aligned}
$$

(1)

Each reaction was carried out with a mixture of total volume 20 µL, containing 10 µL of $2 \times$ Greenstar qPCR master mix, 1 µL of DNA template, and 1 µL each of forward and reverse primer specific for each gene. To prepare the standard, the confirmed clones of each gene after sequencing were used as shown in Additional file 1: Table S3. Serial dilution was performed from 10^{-1} to 10^{-7}. The PCR of the standard and unknown samples of each gene was performed in triplicate. The gene copy number of unknown samples was determined using the standard regression curves of gene copy number.

Results and discussion

Physico–chemical properties of soil

The soil was found to be slightly alkaline with a pH of 7.86. Soil electrical conductivity ($EC_{1:5}$) was 12.7 dS m^{-1} indicating high salinity, as reported for saturated paste equivalent in all soils [21].

Functional gene amplification and clone library construction

Genomic DNA isolated from the paddy soils was used to amplify functional genes using specific primer sets. A comprehensive evaluation was conducted of PCR primers specific for the nitrogen fixation (nitrogenase reductase) gene, that is, *nif*H [6], using the IGK3/DVV primer set, this being the

best with a wide coverage for environmental samples including soil samples. A DNA band of approximately 394 bp was noted. Similarly, primers covering a wide range of samples and with high specificity such as amoA-1F/amoA-2R specific for ammonium monooxygenase gene (*amo*A), nxrB169f/nxrB638r specific for nitrite oxidoreductase gene (*nxr*B), hzsA_382F/hzsA_1857R specific for hydrazine synthase gene (*hzs*A), nirKC2F/nirKC2R specific for copper-containing nitrite reductase gene (*nir*K), nirSC1F/nirSC1R specific for cytochrome cd1-containing nitrite reductase gene (*nir*S), and nosZC1F/nosZC1R specific for nitrous oxide reductase gene (*nos*Z1 and *nos*Z2), yielded DNA bands of approximately 491, 485, 1496, 400–500, 400–500, 450, and 700 bp, respectively. To obtain more details, the PCR products were visualized on an agarose gel (Additional file 1: Fig. S1).

Sequencing and phylogenetic analysis

The cloned fragments after the conventional PCR were sequenced, and the results revealed successful cloning of all the genes. After comparing and analyzing the sequences of the cloned genes with the known sequences of bacterial genes retrieved from the NCBI GenBank, the phylogenetic trees were constructed using the retrieved bacterial genes with high sequence similarities.

Twenty selected isolates with *nif*H (nifH-1 to nifH-20) showed a high similarity, of 90–98%, with bacteria containing known *nif*H sequences. They were closely related to uncultured bacteria, *Asaia bogorensis* [22], *Pseudomonas stutzeri*, *Azotobacter vinelandii*, and *Rhodoblastus acidophilus* carrying *nif*H [23] (Fig. 1). Similarly, 20 isolated clones containing the *amo*A fragment (camoA-1 to camoA-20) showed 96%–100% sequence similarity with bacteria containing *amo*A, and they were closely related to *Nitrospira* sp. [16] and uncultured ammonia oxidizing bacteria carrying *amo*A (Fig. 2). Fifteen clones with *nxr*B gene (cnxrB-1 to cnxrB-20) showed 90–98% similarity with *nxr*B from GenBank database. They were closely related to *Nitrospira moscoviensis* [24], *Nitrospira calida*, 'Candidatus Nitrospira bockiana', and uncultured bacteria carrying *nxr*B (Fig. 3). Similar results were reported by Pester et al. [8], in a microbial community analysis of *nxr*B in different environment samples such as activated sludge and climatically distinct soil samples. Moreover, in 17 isolated colonies with *hzs*A (hzsA-2 to hzsA-7, hzsA-9 to hzsA-16, hzsA-18 to hzsA-20), the sequence similarity ranged from 90 to 98% (Fig. 4), and sequences were closely related to those of the enrichment culture clone, 'Candidatus Brocadia fulgidia' [25], 'Candidatus Brocadia anammoxidans' [26], 'Candidatus Jettenia caeni' [27], 'Candidatus Kuenenia stuttgartiensis' [28], uncultured anaerobic ammonium-oxidizing bacteria, and uncultured anaerobic ammonium-oxidizing bacterial enrichment clones [29].
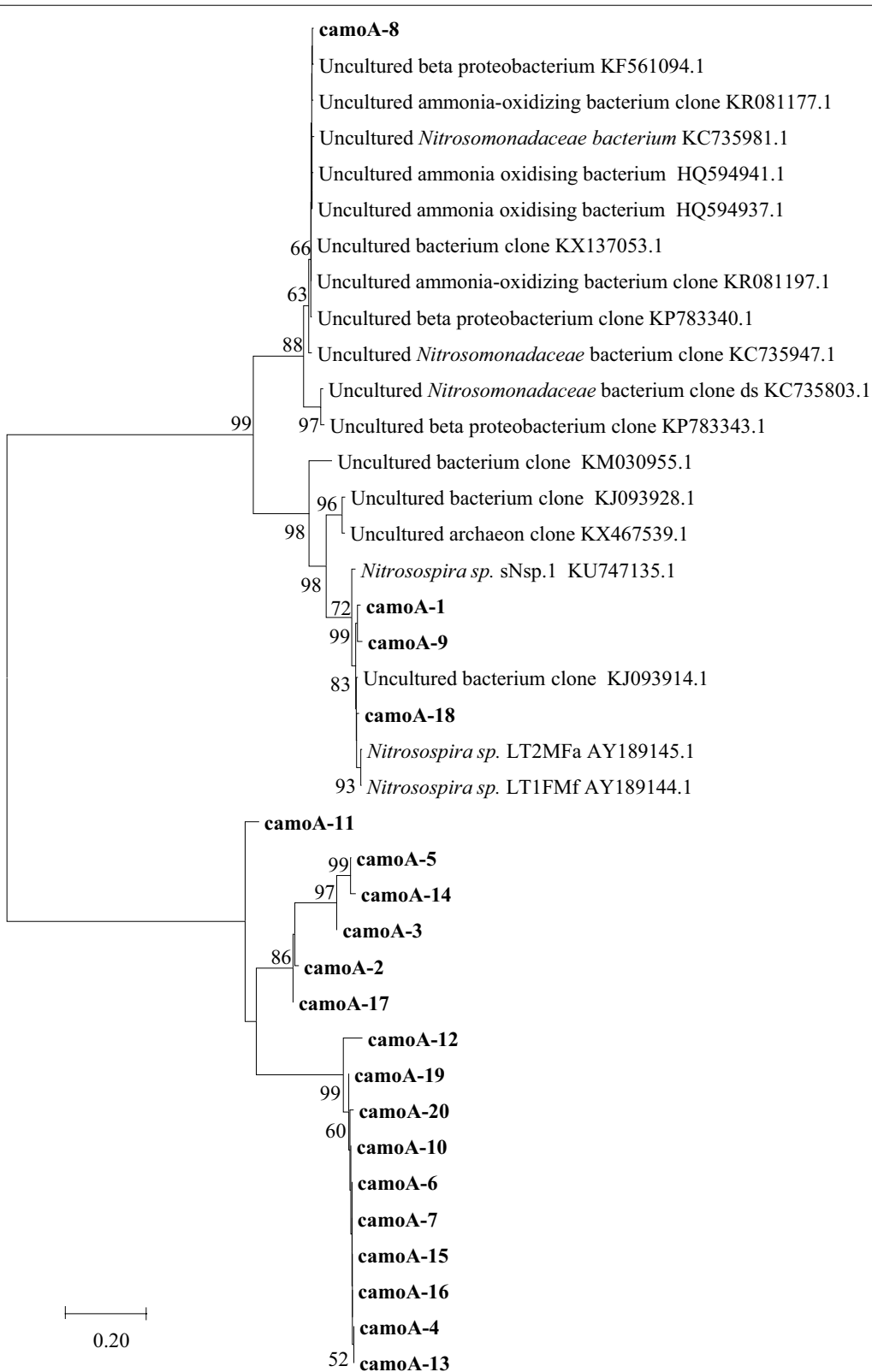
Fig. 1 Neighbor-joining phylogenetic tree of the cloned *nif*H gene sequences

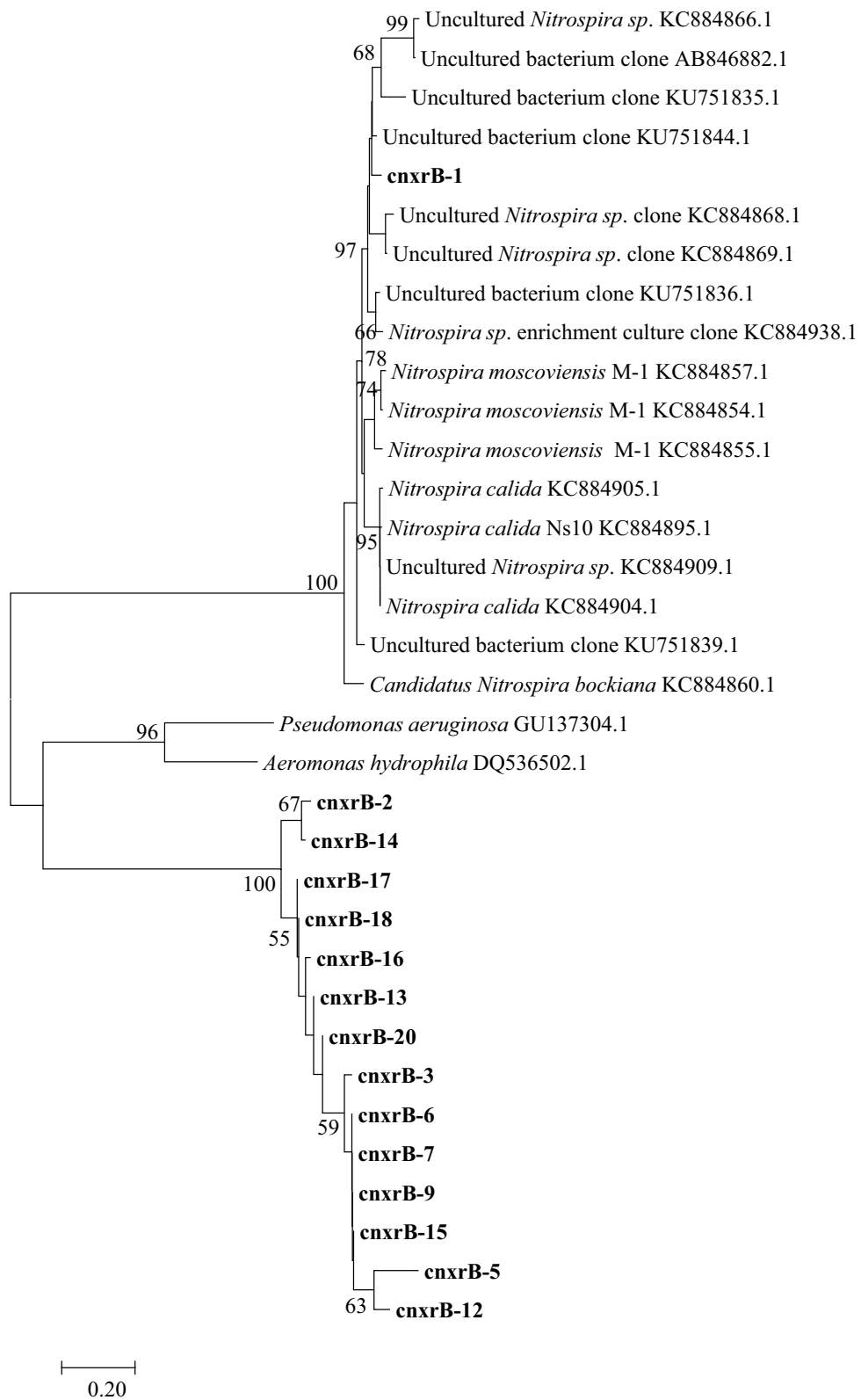**Fig. 2** Neighbor-joining phylogenetic tree of the cloned *amo*A gene sequences

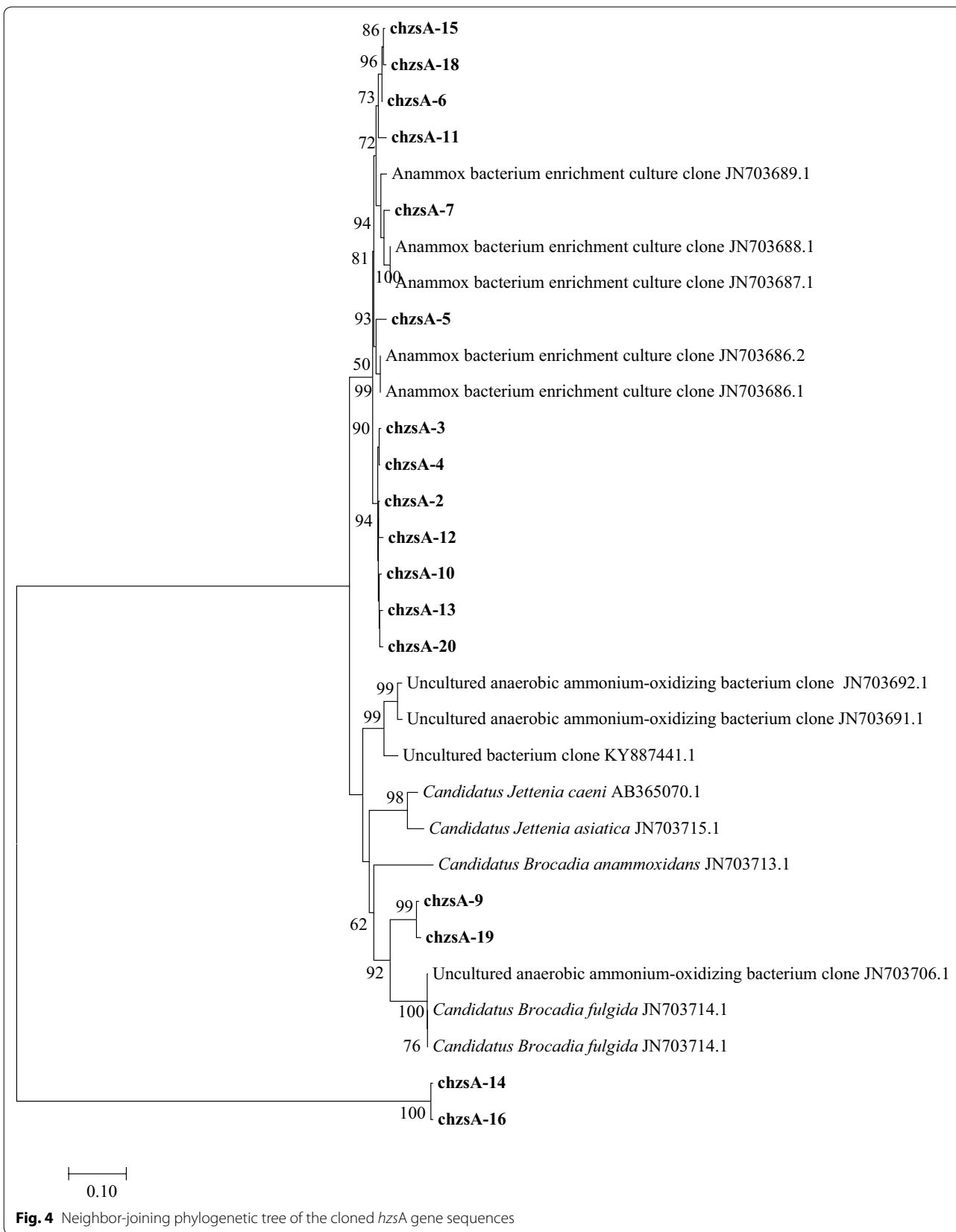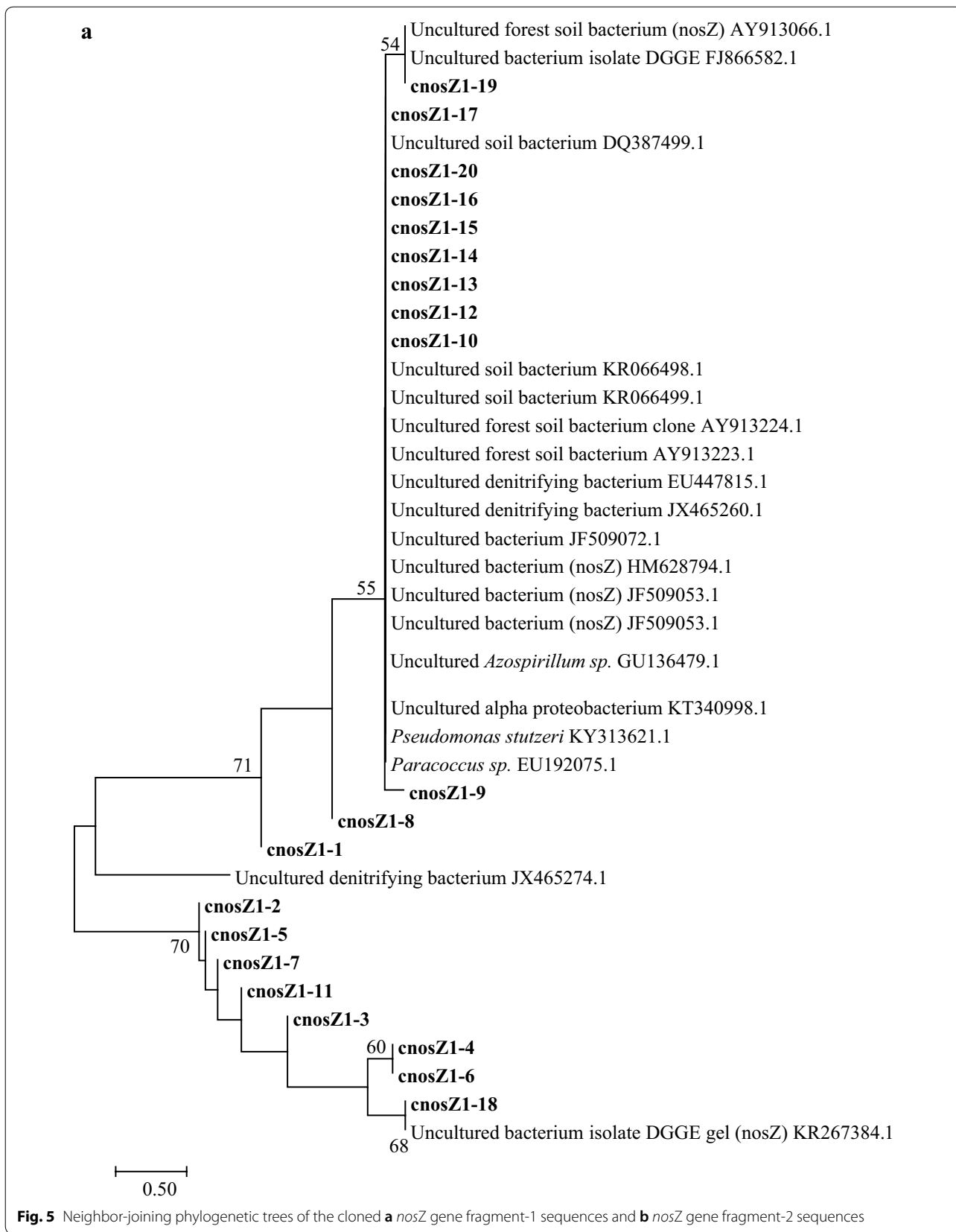**Fig. 3** Neighbor-joining phylogenetic tree of the cloned *nxr*B gene sequences

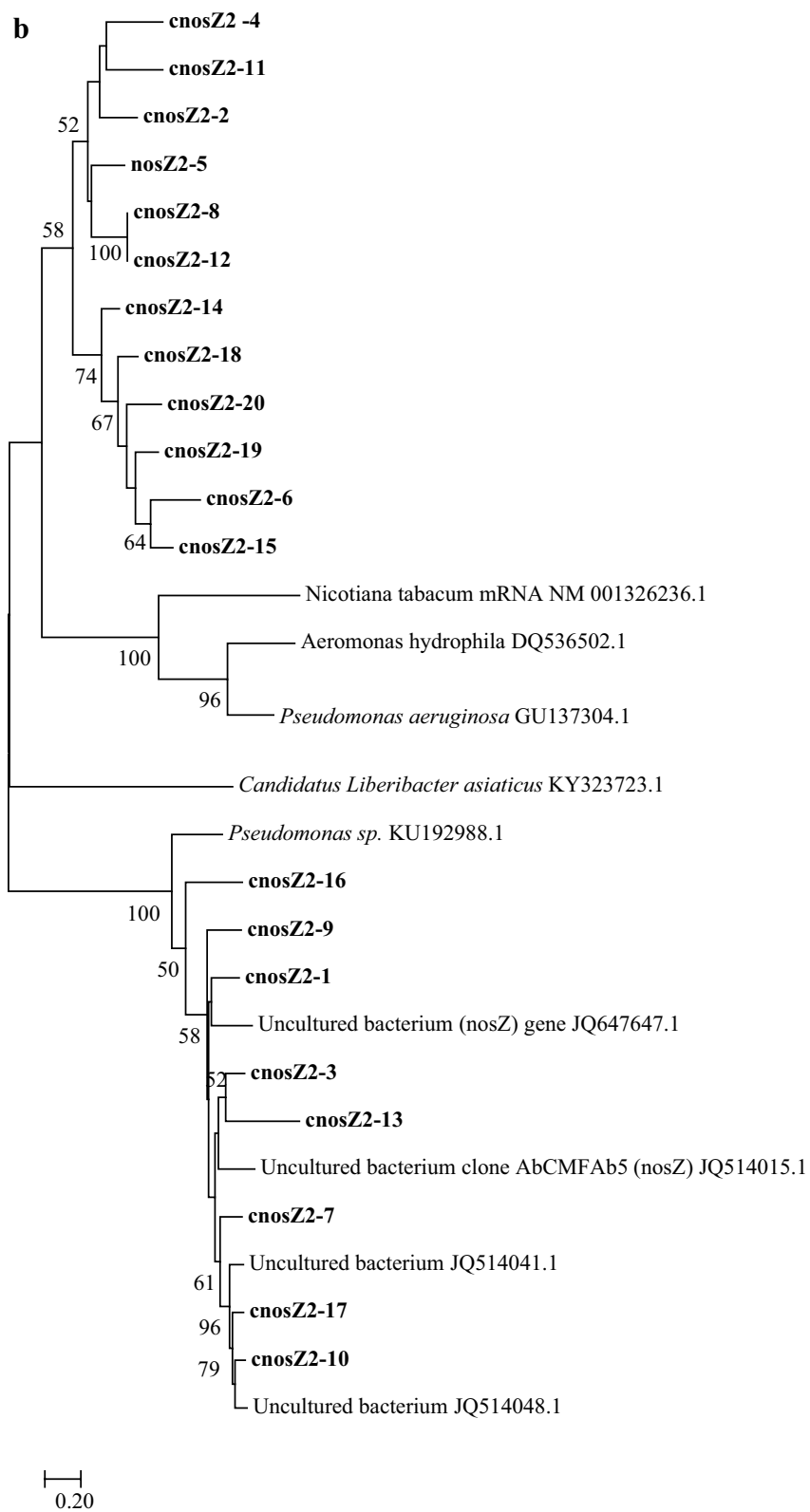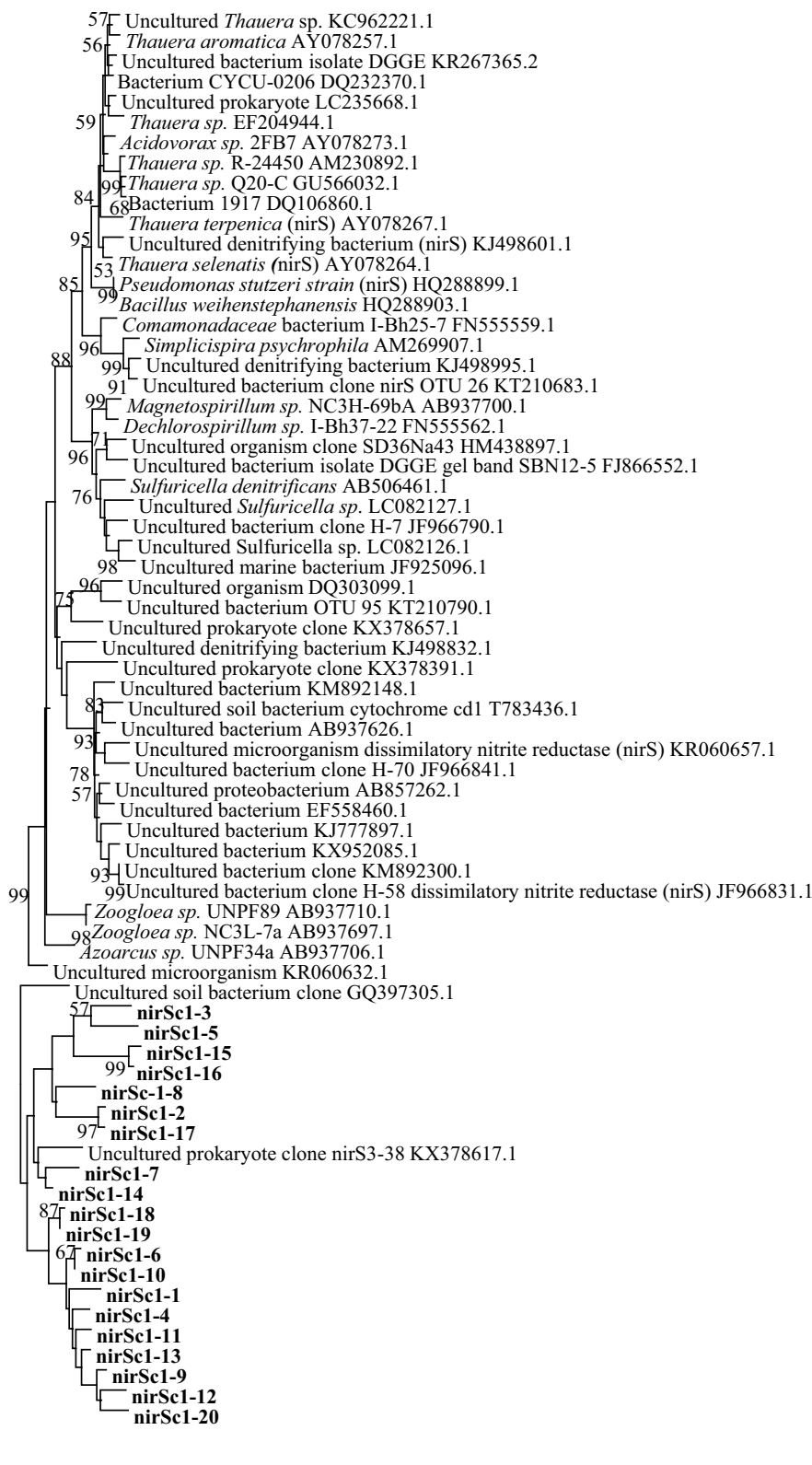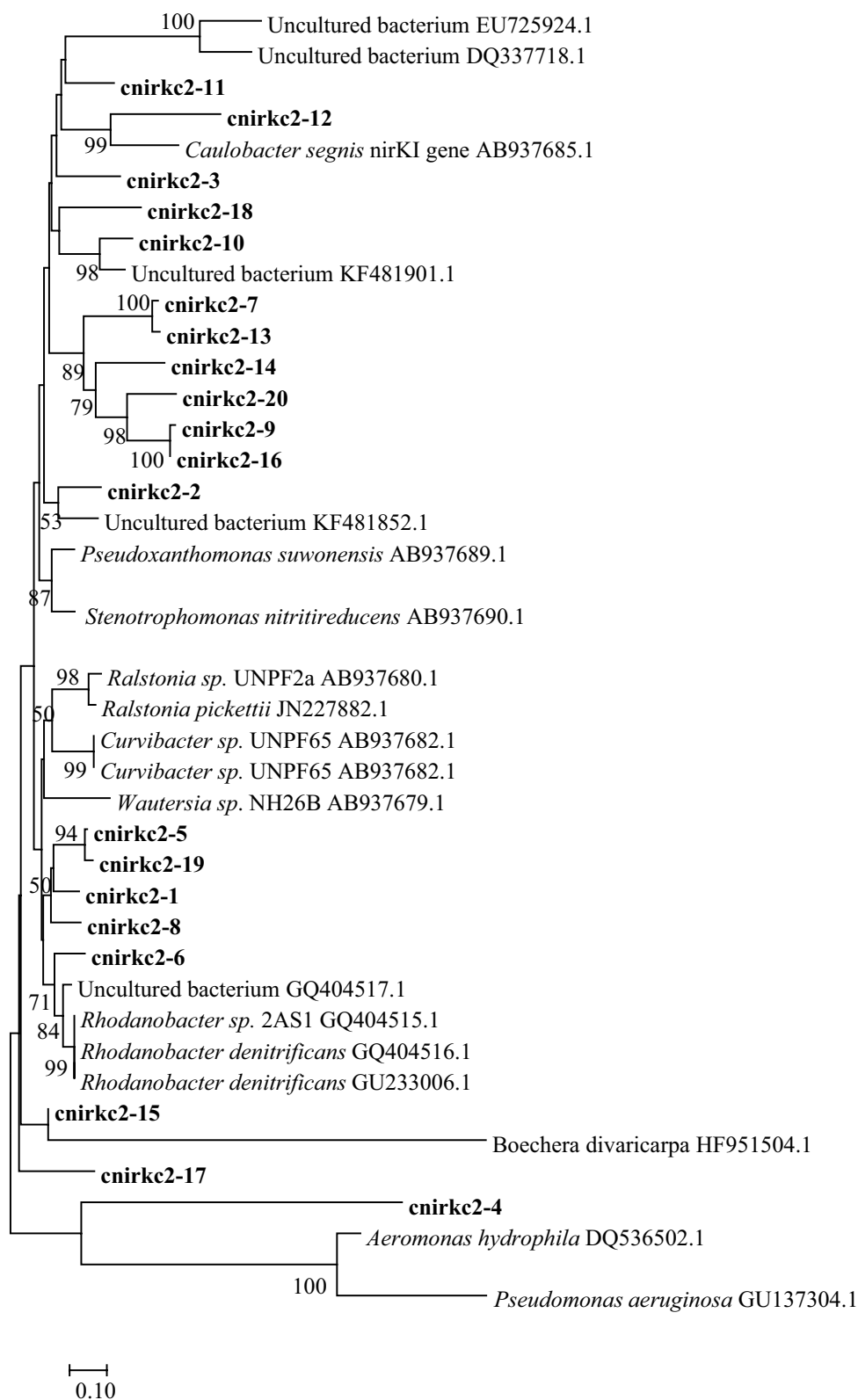**Fig. 4** Neighbor-joining phylogenetic tree of the cloned *hzs*A gene sequences

Fig. 5 Neighbor-joining phylogenetic trees of the cloned **a** *nos*Z gene fragment-1 sequences and **b** *nos*Z gene fragment-2 sequences

Fig. 5 (continued)

Fig. 6 Neighbor-joining phylogenetic tree of the cloned *nir*S gene sequences

Fig. 7 Neighbor-joining phylogenetic tree of the cloned *nir*K gene sequences

Fig. 8 Quantitative PCR result of the copy number of *nif*H, *amo*A, *nxr*B, *hzs*A, *nos*Z1, *nos*Z2, *nir*S, and *nir*K
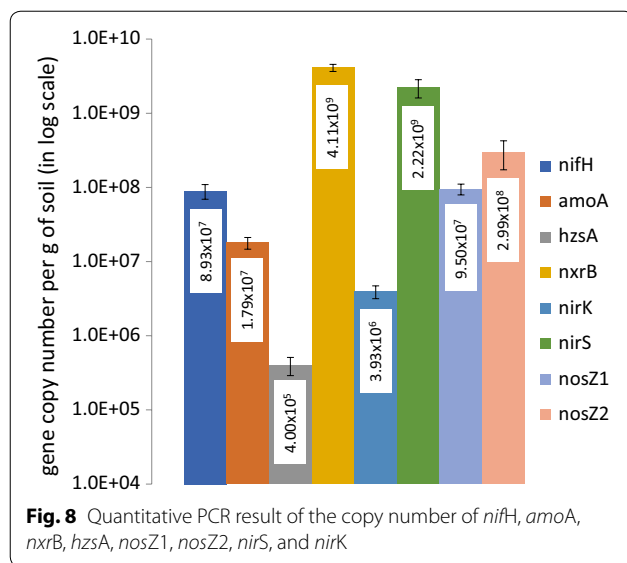
For both fragments of nitrous oxide reductase genes (*nos*Z-1 and *nos*Z-2), 20 colonies were selected for each of the gene clones. The denitrifying gene clones with the *nos*Z-1 gene fragment (cnos*Z*1-1 to cnos*Z*1-20) showed 88–97% sequence similarity (Fig. 5a), and they were found to be closely related to the uncultured denitrifying bacteria, uncultured *Azospirillum* sp. [30], uncultured proteobacteria, *Pseudomonas stutzeri*, and *Paracoccus* sp. Furthermore, the 20 isolated colonies with the *nos*Z-2 gene fragment (cnos*Z*2-1 to cnos*Z*2-20) exhibited 74–100% similarity (Fig. 5b), and they were closely related to uncultured bacterial clones harboring *nos*Z. Both primer pairs used for the identification and determination of *nos*Z functioned well. However, these results suggest that *nos*Z amplified by the primer pair nosZC1F/nosZC1R was more conserved in the soil than *nos*Z amplified by the other primer set nosZC2F/nosZC2R.

All the isolated colonies (20 colonies) from the clone library of denitrifying gene insert (*nir*S gene fragment; cnirSc1-1 to cnirSc1-20) were found to share 96–99% similarity with clones containing known sequences (Fig. 6). The sequenced clone genes were closely related to *nir*S from *Caulobacter segnis*, *Pseudoxanthomonas suwonensis*, *Stenotrophomonas nitritireducens*, *Rhodanobacter denitrificans*, *Ralstonia* sp., *Curvibacter* sp., and *Pseudomonas stutzeri*, and *nir*S uncultured prokaryotic clone.

For the denitrifying gene *nir*K, 20 clones (cnirKc2-1 to cnirKc2-20) showed 73–100% similarity (Fig. 7). The cloned genes were found to be closely related to those of *Thauera* sp. and *Sulfuricella denitrificans*.

Quantitative PCR analysis

Quantitative PCR of all the genes was performed using the absolute quantification method on the basis of the gene copy number. The copy numbers of *nif*H, *amo*A, *hzs*A, *nxr*B, *nir*K, *nir*S, *nos*Z1, and *nos*Z2 were $8.93 \times 10^7 \pm 2.00 \times 10^7$, $1.79 \times 10^7 \pm 0.32 \times 10^7$, $4.00 \times 10^5 \pm 1.10 \times 10^5$, $4.10 \times 10^9 \pm 0.45 \times 10^9$, $3.93 \times 10^6 \pm 0.77 \times 10^6$, $2.22 \times 10^9 \pm 0.61 \times 10^9$, $9.50 \times 10^7 \pm 1.58 \times 10^7$, and $2.99 \times 10^8 \pm 1.26 \times 10^8$ copies/g soil, respectively (Fig. 8). Among the studied nitrifying genes, namely, *nxr*B and *amo*A, the abundance of *nxr*B was two-times higher than that of *amo*A. Similar results were reported by Ramanathan et al. [31], in their study on nitrifier gene abundance in a sediment affected by acid mine drainage. Among various genes used in this study, namely, *nif*H, *amo*A, *nxr*B, *hzs*A, *nir*K, *nir*S, *nos*Z1, and *nos*Z2, the abundance of *nxr*B and *nir*S was relatively higher than that of the others, whereas the abundance of *hzs*A was found to be relatively lower. This suggests the high occurrence of nitrifying microbial communities in the surface environment of paddy soils. As indicated by the relatively high *nir*S abundance, denitrifiers are known to be active in paddy soils [32, 33]. However, under partially oxic conditions, anammox bacteria may not flourish, as indicated by *hzs*A abundance.

Here, we explored the diversity of microbial communities in a rice paddy soil by detecting and quantifying some key functional genes involved in the nitrogen cycle by using molecular techniques. Our study will help better understand the nitrogen cycle and the diversity of associated microbial communities in terrestrial habitats, such as paddy fields. Although we used primers to determine the nitrogen cycle-related genes based on the nitrogen-transforming reactions mediated by bacteria, there are several undiscovered nitrogen-transforming reactions that are thermodynamically feasible and the microorganisms that catalyze these reactions.

Supplementary information

Additional file 1: Table S1. List of primers used in this study. Table S2. PCR conditions used for each gene. Table S3. Positive clones from each gene used to prepare standard for real-time PCR. Figure S1. PCR amplification of functional genes: nifH, amoA, nxrB, hzsA, nosZ, nirK, nirS genes, respectively.

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