

ORIGINAL ARTICLE

Higher diversity and abundance of denitrifying microorganisms in environments than considered previously

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Denitrification is an important process in the global nitrogen cycle. The genes encoding NirK and NirS (*nirK* and *nirS*), which catalyze the reduction of nitrite to nitric oxide, have been used as marker genes to study the ecological behavior of denitrifiers in environments. However, conventional polymerase chain reaction (PCR) primers can only detect a limited range of the phylogenetically diverse *nirK* and *nirS*. Thus, we developed new PCR primers covering the diverse *nirK* and *nirS*. Clone library and qPCR analysis using the primers showed that *nirK* and *nirS* in terrestrial environments are more phylogenetically diverse and 2–6 times more abundant than those revealed with the conventional primers. RNA- and culture-based analyses using a cropland soil also suggested that microorganisms with previously unconsidered *nirK* or *nirS* are responsible for denitrification in the soil. PCR techniques still have a greater capacity for the deep analysis of target genes than PCR-independent methods including metagenome analysis, although efforts are needed to minimize the PCR biases. The methodology and the insights obtained here should allow us to achieve a more precise understanding of the ecological behavior of denitrifiers and facilitate more precise estimate of denitrification in environments.

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Introduction

Denitrification is a microbial dissimilatory process where nitrate and nitrite are reduced stepwise to gaseous compounds, such as nitric oxide (NO), nitrous oxide (N₂O) and dinitrogen (N₂) (Knowles, 1982; Zumft, 1997). Denitrification causes nitrogen (N) loss from natural ecosystems and agricultural fields, as well as N removal during wastewater treatment (Tiedje, 1988; Conrad, 1996; Martin *et al.*, 1999; Bouwman *et al.*, 2002), and it can also facilitate the anaerobic degradation of organic pollutants (Leahy and Olsen, 1997). One of the end products of denitrification, N₂O, is a potent greenhouse gas and ozone-depleting substance (Davidson, 2009; Ravishankara *et al.*, 2009).

Therefore, denitrification has attracted much attention from the scientific, agricultural and industrial fields for a long time. Recent studies have demonstrated that ecological traits such as the diversity, abundance and ability to produce N₂O/N₂ of denitrifying microbial communities could reflect denitrification in environments (Philippot *et al.*, 2009; Morales *et al.*, 2010; Wei *et al.*, 2014); thus, the importance of a precise understanding of these traits is widely recognized by many fields where major research efforts are ongoing.

The reduction of nitrite to NO is catalyzed by two structurally different but functionally equivalent nitrite reductases, that is, copper-containing reductase (NirK) and cytochrome *cd1*-containing reductase (NirS) (Hochstein and Tomlinson, 1988; Cutruzzola *et al.*, 2001; Sakurai and Kataoka, 2007), and this is a key step in the denitrification process because dissolved N is converted into gaseous N during this step. Taxonomically diverse microorganisms have the ability to denitrify (Tiedje, 1994), and phylogenies based on the *nir* and 16S rRNA genes are incongruent (Jones *et al.*, 2008).

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Therefore, *nirK* and *nirS* are used as marker genes to study the ecological behavior of denitrifying microorganisms in environments. Many attempts have been made to design and modify the polymerase chain reaction (PCR) primers required to amplify *nirK* and *nirS* (Braker *et al.*, 1998; Hallin and Lindgren, 1999; Braker *et al.*, 2000; Michotey *et al.*, 2000; Throbäck *et al.*, 2004). These approaches were conducted based on the available *nirK* and *nirS* sequences from cultivable denitrifying bacterial strains, most of which belong to the classes of alpha-, beta- and gamma-proteobacteria.

Recent developments in the genome analysis of cultured and uncultured strains have shown that many diverse microorganisms possess *nirK* or *nirS*, including the phyla Nitrospirae, Actinobacteria, Bacteroidetes, Spirochetes, Chloroflexi and Firmicutes, and even the phyla Euryarchaeota and Crenarchaeota in Archaea (Cantera and Stein, 2007; Nolan *et al.*, 2009; Bartossek *et al.*, 2010; Moir, 2011; Mardanov *et al.*, 2012; Murawska *et al.*, 2013; Nishizawa *et al.*, 2013). Furthermore, as demonstrated by Jones *et al.* (2008), these *nirK* and *nirS* sequences were not considered during the design and modification of the *nirK* and *nirS* primers described above. However, even after the report of Jones *et al.* (2008), the conventional primers are still widely used to illustrate the ecological traits of denitrifying microorganisms in the environment (for example, Katsuyama *et al.*, 2008; Yoshida *et al.*, 2012; Li *et al.*, 2014). This suggests that the diversity, abundance and functional importance of denitrifying microorganisms in the environment are underestimated at present.

Thus, the objective of this study was to determine the overall diversity, abundance and functional importance of denitrifying microorganisms in environments. To achieve this objective, we performed a phylogenetic analysis of the sequence diversity of all currently available *nirK* and *nirS* sequences, designed multiple primer sets to cover these diverse *nirK* and *nirS*, examined the diversity, abundance and distribution of *nirK* and *nirS* in various terrestrial environments using the newly designed primers, and assessed the presence of microorganisms with previously unconsidered *nirK* or *nirS* using cropland soil.

Materials and methods

Selection, alignment and analysis of the sequences of NirK and NirS genes

The full-length nucleotide sequences of *nirK* and *nirS* were obtained from the Kyoto Encyclopedia of Genes and Genomes Repository (<http://www.genome.jp/kegg>) and the Functional Gene Repository (<http://fungene.cme.msu.edu/index.spr>). All of the reference sequences were downloaded in June 2012. Thus, genomes of some of the denitrifying microorganisms that have been deposited in

database after the month of download are not included in this study, such as *nirK* of *Bacillus thuringiensis* in Firmicutes (Murawska *et al.*, 2013) and *nirS* of *Pyrobaculum* sp. in Archaea (Mardanov *et al.*, 2012). The *nirK* and *nirS* sequences were aligned by amino acids using ClustalW2 (Larkin *et al.*, 2007). The homologs of the copper center type 1 domain in *nirK*, which contained a type 1 copper ligand (that is, two His and one Met) and a type 2 copper ligand (that is, two His), were retained for analysis (Supplementary Figures S1a and S1b). The homologs of the cytochrome d1 domain in *nirS*, which bound and reduced the nitrite substrate, were retained for analysis (Supplementary Figures S1c and S1d). The phylogenetic trees based on the amino-acid sequences were generated using MEGA 5 with the maximum likelihood algorithm (Tamura *et al.*, 2011), and node support was determined using 500 bootstrap replicates.

Primer design for the detection of diverse NirK and NirS genes

We designed forward primers for *nirK*, which can anneal with the sequences around the conserved methionine from the type 1 copper ligand, and reverse primers, which can anneal with sequences that contain conserved histidine from the type 2 copper ligand (Supplementary Figures S1a and S1b). We also designed forward primers for *nirS*, which can anneal with sequences around conserved glycine, and reverse primers, which can anneal with the sequences that contain two consecutive conserved glycines (Supplementary Figures S1c and S1d). Several sets of degenerate primers specific to the *nirK* and *nirS* sequences in each cluster were designed using the COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) algorithm (Rose *et al.*, 1998). The degenerate primers of each *nirK* and *nirS* cluster consisted of a 3' degenerate core region with an 11-bp length across four highly conserved amino-acid codons and a 5' consensus non-degenerate clamp region with an 11- to 14-bp length (Supplementary Figures S1 and S2, Supplementary Table S1). In particular, the sequence of widely used conventional primers that had been designed to amplify the *nirK* and *nirS* in Cluster I showed mismatches with the sequences described in Supplementary Tables S2. Therefore, we designed new primers for the *nirK* and *nirS* in Cluster I. The 3' end amino acid of the newly designed forward primer for *nirK* in Cluster I was a highly conserved proline, which was different from the non-conserved end amino acid (that is, lysine or arginine) of the conventional primer F1aCu (Supplementary Figure S1a) (Hallin and Lindgren, 1999). The 3' end amino acid of the newly designed reverse primer for *nirK* in Cluster I was a highly conserved histidine, which was different from the non-conserved end amino acid (that is, serine, asparagine or threonine) of the widely used primer

R3Cu (Supplementary Figure S1b) (Hallin and Lindgren, 1999). The 3' end amino acid of the newly designed reverse primer for *nirS* in Cluster I was a highly conserved leucine, which was different from the non-conserved end amino acid (that is, isoleucine, valine or leucine) of the conventional primer R3cd (Supplementary Figure S1d) (Throback *et al.*, 2004).

Validation of the designed primers using the denitrifying strains

Eighteen bacterial strains obtained from the culture collection of the Japan Collection of Microorganisms (JCM, Tsukuba, Ibaraki, Japan) or the Biological Resource Center (NBRC, Kisarazu, Chiba, Japan), which were expected to belong to each cluster in the phylogenetic trees (Figure 1) according to their 16S rRNA-based taxonomy, were used to validate the coverage and specificity of all designed primer candidates (Supplementary Table S2). In addition, forty-four strains of denitrifying bacteria, which had been isolated from rice field soil in Niigata, Japan (Ashida *et al.*, 2010; Nishizawa *et al.*, 2012; Nishizawa *et al.*, 2013), were used (Supplementary Table S2). The denitrification abilities of all bacterial strains were analyzed using ^{15}N -labeled NaNO_3 (99.5 atom% ^{15}N , SI Sciences, Japan) and a gas chromatography-mass spectrometry (GC-MS) system (GCMS-QP2010Plus, Shimadzu, Kyoto, Japan) as previously described (Isobe *et al.*, 2011). Genomic DNA was extracted from the single colonies as previously described (Wei *et al.*, 2014). Genomic DNA from a non-denitrifying bacterium, *Geobacillus kaustophilus*, which does not possess the *nirK* or *nirS*, was used as a negative control.

PCR conditions were optimized for *nirK* and *nirS* in each cluster that used the strains. All reactions

were performed as described in Supplementary Table S1. The PCR product purification, cloning and sequencing were performed as described elsewhere (Wei *et al.*, 2014). According to the obtained *nirK* or *nirS* sequence, each denitrifier isolate was determined to belong to any cluster of *nirK* or *nirS* except for *nirS* Cluster III. On the basis of these results, the best primers were selected, and their sequences and conditions for conventional PCR and quantitative PCR (qPCR) were optimized as shown in Supplementary Table S1.

Diversity of *NirK* and *NirS* genes in various environments

Clone library analyses were performed using the environmental samples to determine the diversity of the *nirK* and *nirS* sequences in various environments. We used cropland soil (Takahashi *et al.*, 1991), rice paddy soil (Itoh *et al.*, 2013), forest soils (Urakawa *et al.*, 2015) and lake sediment (Abe *et al.*, 2000) as described below. All soil samples were collected in triplicate at 5–10 cm depth from each experimental site, and lake sediment was sampled in triplicate at 10 cm depth from the sediment surface using an Ekman sampler. Environmental DNA was extracted using an ISOIL kit (Nippon Gene, Toyama, Japan). The PCR conditions were the same as previously described except that the MgCl_2 concentration was reduced to 2.0 mM. Three hundred fifty-two sequences of *nirK* and 228 sequences of *nirS* were obtained after the removal of poor-quality reads (that is, low-quality base calling and frame-shift errors) and potential chimeric sequences. The sequences from each library were then clustered into operational taxonomic units with 3% differences using the Mothur program (Schloss *et al.*, 2009). The final set of 195 sequences for *nirK* and 102 sequences for *nirS* was aligned by translating them to amino-acid sequences as

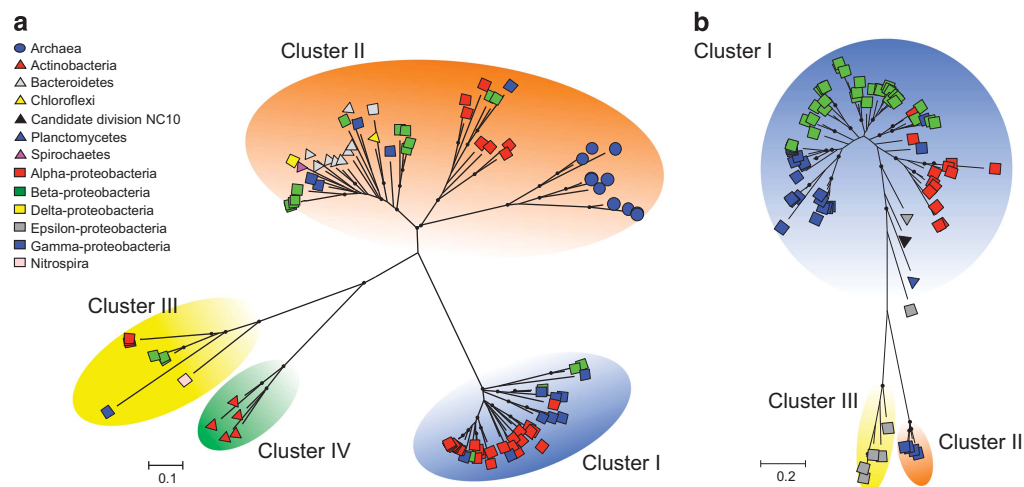


Figure 1 Unrooted maximum likelihood phylogeny of partial *nirK* (a) and *nirS* (b) amino-acid sequences obtained from genomes. The *nirK* type 1 copper center and *nirS* cytochrome d1 heme were detected for each cluster. Bootstrap values (500 replicates) >70% are denoted by dots above the branches, and the branch lengths correspond to sequence differences, which are indicated by the scale bar. Symbols on tree tips indicate the taxonomic affiliations of reference sequences.

previously described, and the amino-acid sequences from the selected microbial genomes and denitrifying cultured collections were used as a reference alignment.

Abundance and distribution of NirK and NirS genes in various environments

The abundance of *nirK*, *nirS* and 16S rRNA genes in the environmental samples was determined by qPCR. Various environmental samples from terrestrial habitats were used, including a cropland soil (Andosol) applied with an organic fertilizer (Wei *et al.*, 2014), a cropland soil (Gray Lowland Soil, Eutric Fluvisol) applied with an organic or urea fertilizer (Takahashi *et al.*, 1991), paddy soils from flooded and non-flooded seasons (Gray Lowland Soil, Eutric Fluvisol) (Itoh *et al.*, 2013), two natural forest soils (Brown Forest soil and Andosol) (Urakawa *et al.*, 2015), a planted forest soil (Brown Forest soil) (Oda *et al.*, 2009) and a lake sediment (Abe *et al.*, 2000).

Environmental DNA was extracted as previously described. The qPCR was conducted using a StepOne real-time PCR system (Applied Biosystems, Warrington, UK) and a KOD SYBR qPCR Mix kit, which was suitable for the degenerate primers that had long sequences and high T_m values. The quantification of the 16S rRNA gene was performed with the primers 357F/520R as previously described (Itoh *et al.*, 2013). The quantification of *nirK* and *nirS* in each cluster was performed with the designed primers. We also utilized the widely used conventional primers F1aCu/R3Cu for *nirK* in Cluster I and cd3aF/R3cd for *nirS* in Cluster I for comparison. All reactions of qPCR were performed as described in Supplementary Table S1.

The standard curves in the qPCR analyses of the 16S rRNA, *nirK* and *nirS* genes were generated using linearized plasmids that contained the following: the cloned 16S rRNA gene from *Pseudomonas stutzeri* JCM-5965; *nirK* from *Ochrobactrum anthropi* JCM21032 in Cluster I, which can be amplified by the designed primers nirKC1F and nirKC1R, as well as by the widely used primers F1aCu/R3Cu; *nirK* from *Azospirillum lipoferum* NBRC-102290 in Cluster II; *nirK* from *Actinosynnema mirum* NBRC-10460 in Cluster III; *nirK* from *Nitrobacter winogradskyi* NBRC-14297 in Cluster IV; *nirS* from *Denitratisoma oestradiolicum* JCM12830 in Cluster I, which can be amplified by the designed primers nirSC1F and nirSC1R, as well as the widely used conventional primers cd3aF and R3cd; and *nirS* from *Methylomonas koyamae* NBRC-105905 in Cluster II. The absence of the PCR inhibitors in soil DNA was confirmed by mixing a known amount of standard DNA with environmental DNA in a qPCR reaction. The amplification efficiencies, R^2 of the standard curve and T_m value of the melting curve in the qPCR assay for the 16S rRNA gene, *nirK*

in Clusters I–IV and *nirS* in Clusters I and II were estimated as shown in Supplementary Table S1.

Response of NirK and NirS genes under denitrification-induced conditions

The expression levels of *nirK* and *nirS* in soil were analyzed using the soil microcosm under denitrification-induced conditions. We used the Gray Lowland Soil (Eutric Fluvisol) of cropland in Niigata, Japan. This soil is the same as the soil from which forty-four bacterial strains have previously been isolated (Supplementary Table S2). Forty grams of the non-fertilized soil were placed in 80-ml glass bottles, mixed with 0.5 g of granular organic fertilizers and incubated for 20 days as previously described (Wei *et al.*, 2014). The soil without fertilizer was also incubated as a control. The N_2O flux was measured every 2 days, and the highest N_2O emission rate was observed on the eighth day. The soil RNA and DNA were extracted on the eighth day using an RNA PowerSoil Total RNA Isolation Kit and DNA Elution Accessory Kit (MO BIO Laboratories, Carlsbad, CA, USA). Total RNA and DNA were extracted from 1.2 g of soil according to the manufacturer's protocol. The concentrations of the extracted RNA and DNA were determined using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Digestion of the residual DNA in RNA solution was performed using the Ambion TURBO DNA-free Kit (Life Technologies). RNA was transcribed into complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The absence of residual DNA was confirmed in the PCR without reverse transcription. The DNA and cDNA of 16S rRNA gene and *nirK* and *nirS* in soils were quantified as previously described. DNA-based clone library analyses of *nirK* and *nirS* in the soil on the eighth day after fertilization were simultaneously performed as previously described.

Nucleotide sequence accession numbers

The nucleotide sequences of partial *nirK* and *nirS* from the environmental samples in this study have been deposited in the DDBJ/EMBL/GenBank databases with accession numbers AB936839 to AB937093 and AB937560 to AB937661, respectively. The nucleotide sequences of partial *nirK* and *nirS* from the denitrifying isolates have been deposited in the databases with accession numbers AB937662 to AB937717.

Results

Phylogeny of NirK and NirS genes

Ninety-seven full-length *nirK* sequences that belong to the bacterial phyla Actinobacteria, Bacteroidetes, Chloroflexi, Nitrospirae, Proteobacteria and Spirochetes and the archaeal phyla Euryarchaeota were

obtained from a public database after selecting a representative sequence with high similarity (>99% of amino-acid sequence) from one species. We constructed a phylogenetic tree based on the sequences for the electron entry site (including type I copper center; 480–560 bp) in NirK. All widely used conventional primer sets, such as F1aCu/R3Cu and nirK2F/nirK5R (Braker *et al.*, 1998; Hallin and Lindgren, 1999), were also designed to amplify the sequence for this site. The tree was divided into four clusters (that is, Clusters I–IV) with high bootstrap support (>70%) (Figure 1a; see Supplementary Figure S2 for species names). We determined that the sequences that can be amplified with the conventional primers are located in Cluster I, which contains *nirK* from the class alpha-, beta- and gamma-proteobacteria, but not in Clusters II–IV (Supplementary Figures S1 and S2). Cluster II contains *nirK* from the class alpha-, beta-, gamma-, delta- and epsilon-proteobacteria, and the phyla Bacteroidetes, Chloroflexi and Spirochetes. The *nirK* from the halophilic archaeal family, Halobacteriaceae, formed a distinct clade, but it had similar sequences in the primer region to all bacterial phyla in Cluster II (Supplementary Figure S1). Cluster III consisted entirely of the *nirK* from the phylum Actinobacteria. Cluster IV consisted entirely of *nirK* from nitrifiers, including the genera *Nitrospira*, *Nitrosococcus*, *Nitrosomonas* and *Nitrobacter*; however, additional *nirK* sequences from other nitrifiers were also assembled in Clusters I and II (Supplementary Figure S1).

Seventy-five full-length *nirS* sequences that belong to the bacterial phyla Bacteroidetes, NC10, Planctomycetes and Proteobacteria were obtained with the same approach as the *nirK* sequences. We constructed the phylogenetic tree based on the sequences of the catalytic site (including cytochrome heme d1; 530–570 bp) in NirS. All widely used conventional primers, such as cd3aF/R3cd and nirS2F/nirS4R (Braker *et al.*, 1998; Michotey *et al.*, 2000; Throback *et al.*, 2004), were also designed to amplify the sequence of this site. The tree was divided into three clusters (that is, Clusters I–III) with high bootstrap support (>87%) (Figure 1b; see Supplementary Figure S2 for species names). We determined that the sequences that can be amplified with conventional primers are located in Cluster I, but not in Clusters II and III (Supplementary Figures S1 and S2). Cluster I contained the *nirS* from the class alpha-, beta-, gamma- and epsilon-proteobacteria, and the phyla Bacteroidetes, Planctomycetes and NC10. Cluster II consisted entirely of the *nirS* from methane-oxidizing bacteria, including the genera *Methylobacter*, *Methylomonas* and *Methylomicrobium*. Cluster III consisted entirely of the epsilon-proteobacterial *nirS* from deep-sea sediments in hydrothermal fields. These phylogenetic analyses indicate that *nirK* and *nirS* are distributed among taxonomically diverse microorganisms, and a considerable proportion (that is, primarily

nirK in Clusters II, III and IV and *nirS* in Clusters II and III, Figure 1) cannot be detected by the widely used conventional primers; thus, they represent the previously unconsidered *nirK* and *nirS* sequences.

New primer design and its validation using denitrifying strains

We designed seven sets of primers that can potentially amplify *nirK* or *nirS* located in each of the clusters shown in Figure 1 (nirKC1F/nirKC1R, nirKC2F/nirKC2R, nirKC3F/nirKC3R and nirKC4F/nirKC4R for Clusters I–IV in the phylogenetic tree of *nirK* and nirSC1F/nirSC1R, nirSC2F/nirSC2R and nirSC3F/nirSC3R for Clusters I–III in the phylogenetic tree of *nirS*, Supplementary Table S1). The amplified position and expected amplified fragment size (~430–468 bp for *nirK* and 410–420 bp for *nirS*) were approximately the same as the widely used conventional primers (for example, F1aCu/R3Cu or nirK2F/nirK5R for *nirK* and cd3aF/R3cd for *nirS*) (Supplementary Figures S1 and S2). This enables the comparative analysis of the *nir* sequences obtained via the newly designed primers with the massive store of *nir* sequences previously obtained using the conventional primers.

All primer sets successfully generated single amplified fragments with the expected size from the genomic DNA of the denitrifying bacteria obtained from the culture collection and isolates from the rice paddy soil. A sequence analysis of the amplified fragments confirmed that each primer set amplified the *nirK* or *nirS* belonging to each target cluster. No amplification product was generated from non-denitrifying bacteria (Supplementary Table S2). For comparison, the amplification of *nirK* and *nirS* from the denitrifier strains using the most widely used conventional primers (F1aCu/R3Cu for *nirK* and cd3aF/R3cd for *nirS*) was tested. These primers amplified the *nirK* and *nirS* in Cluster I, but failed to amplify them in the other Clusters. Non-specific amplifications were also observed (Supplementary Tables S2). These results indicate the superior coverage and specificity of the newly designed primer sets compared with the conventional primers.

Diversity of NirK and NirS genes in various environments

To examine the diversity of *nirK* and *nirS* in the environment, including previously unconsidered *nirK* and *nirS*, we performed a DNA-based clone library analysis using the newly designed primers. The *nirK* clones in Clusters I–IV and the *nirS* clones in Clusters I and II were obtained from all environmental samples examined, that is, cropland, rice paddy, forest soils and lake sediment (Figures 2 and 3) (Supplementary Table S3). The *nirS* clones in Cluster III, which contained the entire *nirS* sequence from deep-sea sediments in hydrothermal fields (Takai *et al.*, 2006), were not obtained from these environmental samples.

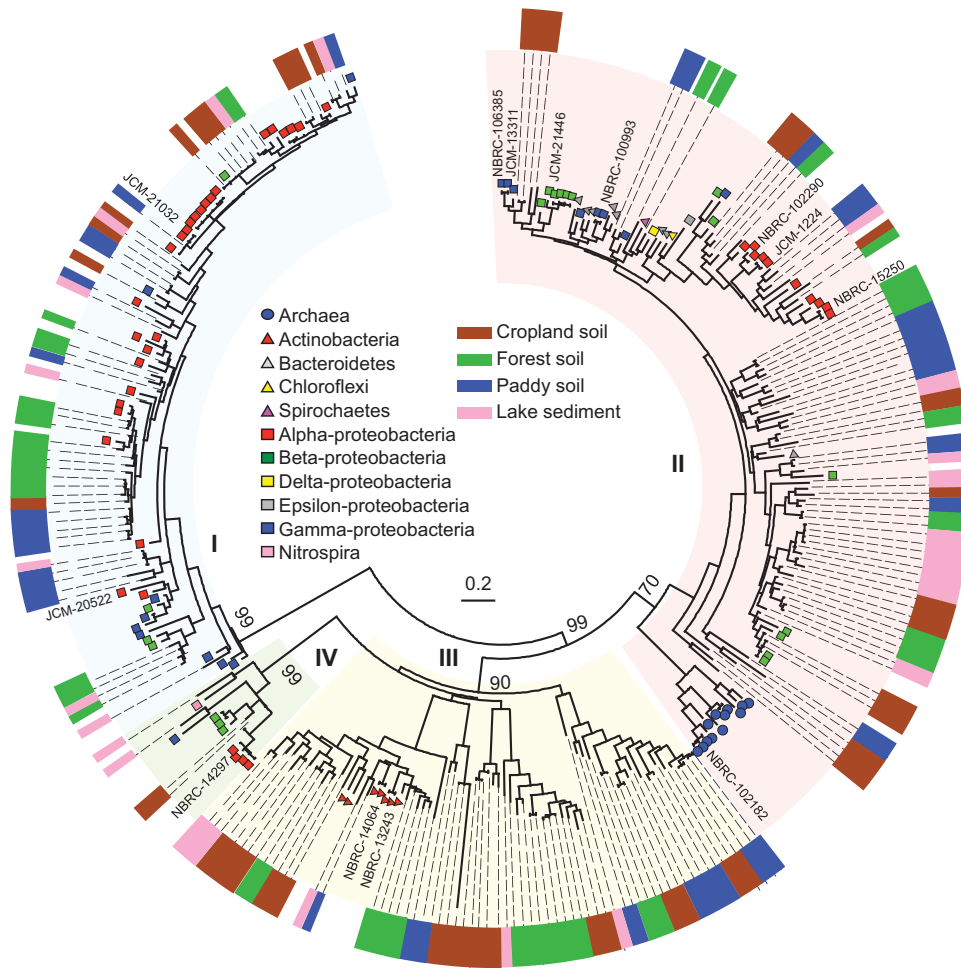


Figure 2 Maximum likelihood phylogeny of the four clusters of *nirK* amino-acid sequences obtained from the environmental samples listed in Supplementary Table S3 and the reference sequences from the genomes and denitrifying strains listed in Supplementary Table S2. Bootstrap values (500 replicates) of each cluster are denoted above the branches. Non-coded and coded symbols on tree tips indicate the taxonomic affiliations of the reference sequences from genomes and denitrifying strains, respectively. The outer color strip shows the source of environmental clones. The branch lengths correspond to sequence differences, which are indicated by the scale bar.

A comparative sequence analysis of the obtained environmental clones with the *nir* sequences of known denitrifier strains in the public database was conducted. The *nirK* sequences of all clones in Cluster I showed the highest similarities (>70% of amino-acid sequence) with those of the denitrifiers that belong to class alpha-, beta- and gamma-proteobacteria. The *nirK* sequences of 90, 13 and 5 clones of a total of 112 clones in Cluster II exhibited the highest similarities (64–99%, 68–87% and 72–76%, respectively) with those of the denitrifiers that belong to the phyla Proteobacteria, Bacteroidetes and Spirochetes, respectively. The other four clone sequences did not exhibit similarities with those of the known denitrifier strains or genomes. The *nirK* sequences from 34 of a total of 103 clones in Cluster III showed the highest similarity (60–90%) with those of the phylum Actinobacteria. The remaining clones did not show substantial similarity (<50%) with those of any of

the known Actinobacterial strains or genomes. Moreover, 26 sequences formed an unexpected clade distant to Cluster III. However, these sequences were amplified with the primers for Cluster III; thus, we included this clade in Cluster III (Figure 2). The *nirK* sequences of all 31 clones in Cluster IV showed the highest similarities (60–90%) with those from the nitrifying genera, including *Nitrobacter*, *Nitrosomonas*, *Nitrosococcus* and *Nitrospira*.

The *nirS* sequences from 96 of 99 clones in Cluster I showed the highest similarities (71–95%) with those of the denitrifiers that belong to class alpha-, beta- and gamma-proteobacteria. The other three clone sequences showed the highest similarity (77–80%) with those from the phylum Chloroflexi. The *nirS* sequences of all 65 clones in Cluster II showed the highest similarities (82–99%) with those from the methane oxidizing genera *Methylomicrobium*, *Methylobacter* and *Methylomonas*.

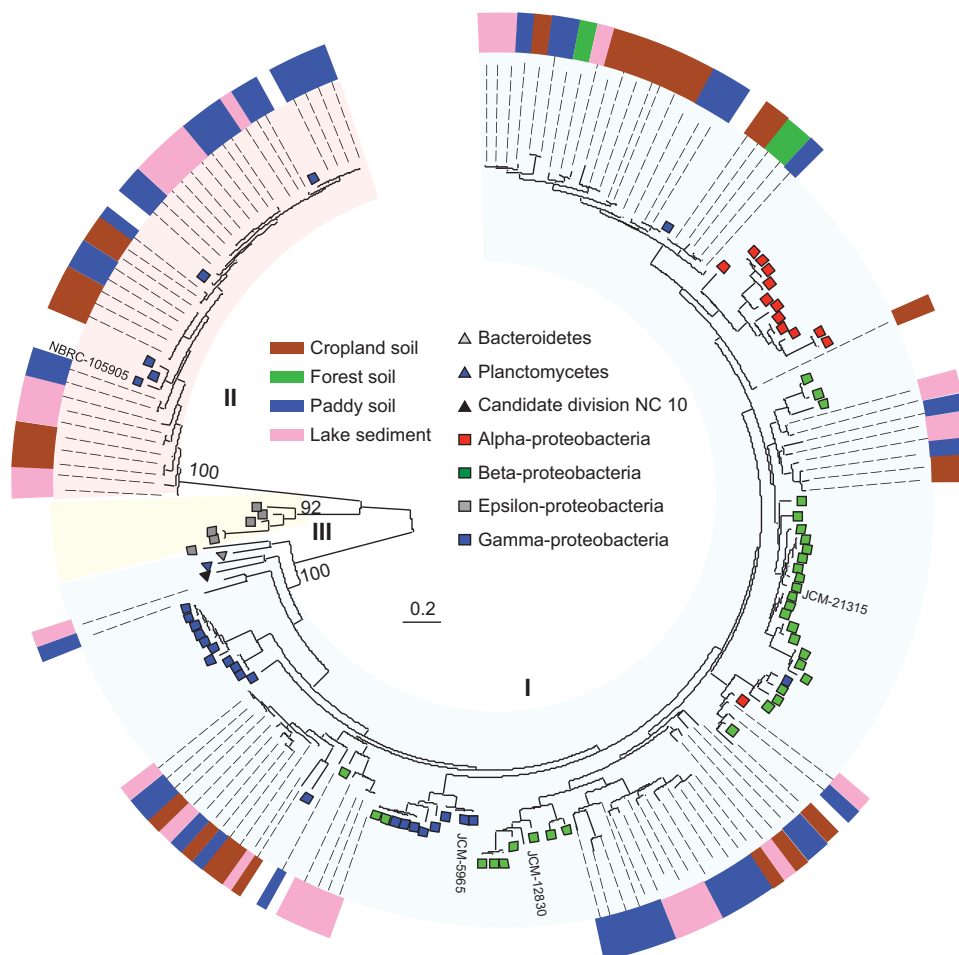


Figure 3 Maximum likelihood phylogeny of the three clusters of *nirS* amino-acid sequences obtained from the environmental samples listed in Supplementary Table S3 and the reference sequences from the genomes and denitrifying strains listed in Supplementary Table S2. Bootstrap values (500 replicates) of each cluster are denoted above the branches. Non-coded and coded symbols on tree tips indicate the taxonomic affiliations of the reference sequences from genomes and denitrifying strains, respectively. The outer color strip shows the source of environmental clones. The branch lengths correspond to sequence differences, which are indicated by the scale bar.

Abundance of *NirK* and *NirS* genes in various environments

We performed a quantitative PCR study using the newly designed primers to examine the abundance and distribution of *nirK* and *nirS* sequences, including previously unconsidered sequences, in various terrestrial environmental samples: cropland soils with different soil types or fertilizations, rice paddy soils in water-flooding and non-flooding seasons, natural and planted forest soils and a lake sediment. For comparison, *nirK* and *nirS* in Cluster I were quantified using the widely used conventional primers F1aCu/R3Cu for *nirK* and cd3aF/R3cd for *nirS*. The abundance of the *nirK* and *nirS* in each cluster was normalized by the abundance of the 16S rRNA gene to standardize the unit.

First, we determined that the *nirK* sequence in Clusters I–IV and the *nirS* sequence in Clusters I–II were present in abundance in all environmental samples (Figure 4). The *nirS* sequence in Cluster III, which contained only epsilon-proteobacteria *nirS*

sequences from hydrothermal regions, was not detected in the tested samples. Previous studies also showed that the abundance of epsilon-proteobacteria was quite low in soils and sediments, as examined by their 16S rRNA and *nosZ* genes (Roesch *et al.*, 2007; Jones *et al.*, 2013). Many studies have used the conventional primer sets F1aCu/R3Cu and cd3aF/R3cd for the quantification of *nir* in environments, despite the possibility of nonspecific amplification previously described. The abundances of *nirK* and *nirS* in Cluster I that were quantified by the newly designed primers were similar to or higher than those quantified by the conventional primers, with the exception of *nirK* in Cropland-3. The total abundances of *nirK* in Clusters I–IV and *nirS* in Clusters I–II quantified with the newly designed primers were ~2–6 times larger compared with the primers F1aCu/R3Cu and cd3aF/R3cd (Supplementary Table S4).

The distribution of *nirK* and *nirS* differed in the terrestrial environments (Figure 4). For example,

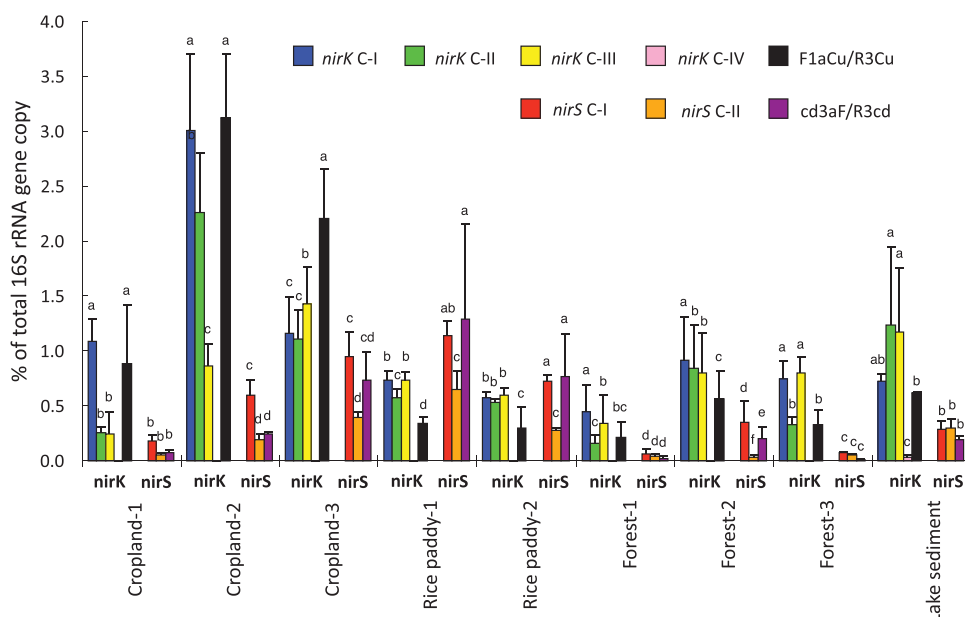


Figure 4 Relative abundance of each *nirK* and *nirS* cluster of gene copies from the different environmental samples listed in Supplementary Table S4, calculated as a proportion of the total number of bacterial 16S rRNA gene copies (for the mean and s.d. of replicates by site, see Supplementary Tables S4). Relative abundance of *nirK* cluster IV and *nirS* cluster I genes detected by the widely used primer sets F1aCu/R3Cu and cd3aF/R3cd are shown as a reference. Different small letters indicate the significant difference among *nir* clusters in each environmental sample according to the least significant difference test at $P < 0.05$.

Table 1 The relative abundance of the nitrite reductase gene and transcript responsible for N_2O emission in a soil microcosm

	Treatments ^a	<i>nirK</i> in Cluster I ^b	<i>nirK</i> in Cluster II	<i>nirK</i> in Cluster III	<i>nirK</i> in Cluster IV	Total <i>nirK</i>	<i>nirS</i> in Cluster I	<i>nirS</i> in Cluster II	Total <i>nirS</i>	Total Nitrite reductase gene
Gene	NF-8	0.506	0.717	2.191	0.027	3.441	1.863	0.498	2.36	5.802
	OF-8	5.631	5.272	1.282	0.014	12.199	1.344	0.275	1.619	13.818
Transcript	NF-8	0.002	0.003	0.001	— ^c	0.006	—	—	—	0.006
	OF-8	0.010	0.049	0.002	—	0.062	0.001	—	0.001	0.063

^aNF-8 and OF-8 represent the control and organic fertilized soil on the eighth day of N_2O flux in the soil microcosm, which corresponds to the curve in Supplementary Figure S3.

^bRelative abundance of the nitrite reductase gene or transcript copies calculated as a percentage of the total bacterial 16S rRNA gene copies (for the mean and s.d. of replicates by treatment see Supplementary Table S4).

^cNot detected.

cropland soils (Fluvisols) that received organic fertilization (Cropland-2) had more *nirK* in Clusters I and II but less *nirS* in Clusters I and II compared with cropland soils (Fluvisols) that received urea fertilization (Cropland-3). Water flooding increased *nirK* and *nirS* in paddy soils (Rice paddy-1 and 2). In addition, the abundance of *nirS* in Cluster II, which contains *nirS* sequences only from methane oxidizers, was high in rice paddy soils (10–17% of total *nir* gene) but low in cropland, forest soil and lake sediment (1–3% of total *nir* gene), which indicated a higher abundance or contribution to the denitrification of methane oxidizers in rice paddy soil. The total abundance of *nirK* fragments (Clusters I, II, III and IV) was highest in cropland soils (Fluvisols), whereas the total abundance of *nirS* fragments (Clusters I and II) was highest in flooded paddy soils (Fluvisols).

Involvement of microorganisms with *NirK* and *NirS* genes in the denitrification in soil

We performed RNA- and culture-based analyses using a soil microcosm system to examine the involvement in denitrification and taxonomic positions of microorganisms with *nirK* or *nirS* detected using the newly designed primers. First, we performed gene expression analyses of *nirK* and *nirS* in the soil microcosm. The soil was incubated in a vial after the application of the N fertilizer to induce denitrification. Control soil without fertilizer application was also prepared. N_2O emissions due to denitrification peaked at 8 days after the application of the fertilizer (Supplementary Figure S3). The DNA and RNA transcripts of *nirK* and *nirS* were quantified in both soils using the newly designed primers (Supplementary Table S4), and their abundances were normalized based on the 16S rRNA gene abundance (Table 1). We found that *nirK* in

Clusters I–III and *nirS* in Clusters I–II were abundant in both soils, and the abundance of *nirK* in Clusters I–II was higher in the denitrification-induced soil compared with the control soil. The *nirK* transcripts in Clusters I–III and the *nirS* transcripts in Cluster I were detected in both soils, and the abundances of these transcripts were higher in the denitrification-induced soil compared with the control soil (Table 1).

We analyzed the homology between the *nirK* and *nirS* sequences obtained from the soil microcosm study (Supplementary Table S3) and those from the denitrifying strains (Supplementary Table S2), which had been isolated previously from the same soil using a single-cell isolation method (Ashida *et al.*, 2010). The strains were isolated in conditions where the activity and growth of denitrifiers were enhanced (Ashida *et al.*, 2010; Nishizawa *et al.*, 2012); thus, they were considered to be the dominant and active denitrifying populations in the soil. Among the 44 denitrifying isolates, 24 isolates possessed *nirK* or *nirS* that could be detected using the newly designed primers, but they could not be detected with the conventional primers (Supplementary Table S2). The *nir* sequences of 45 of 111 soil clones obtained from the microcosm experiment that belonged to Clusters I–III for *nirK* and Cluster I for *nirS* shared high homology (>80% of the amino-acid sequence) with the sequences of the denitrifying isolates (Supplementary Figure S4). The *nir* sequences from 26 of these 45 clones shared high homology with the *nir* sequences of the denitrifying isolates that could not be detected with the conventional primers, which included *nirK* of *Enterobacter* sp. and *Sinorhizobium* sp. in Cluster I; *nirK* of *Ralstonia* sp., *Curvibacter* sp., *Wautersia* sp. and *Yersinia* sp. in Cluster II; *nirK* of *Streptomyces* sp. and *Micromonospora* sp. in Cluster III; and *nirS* of *Dechloromonas* sp. in Cluster I. All of these denitrifying isolates exhibited a high capacity to produce N₂O as the end product of denitrification (that is, the ratio of N₂O production relative to N₂O and N₂ production was >80%).

Discussion

Previous studies utilized conventional primer sets to analyze *nirK* and *nirS* of denitrifiers that belonged primarily to alpha-, beta-, and gamma-proteobacteria from various environmental samples (Braker *et al.*, 2000; Heylen *et al.*, 2006; Smith *et al.*, 2007; Ishii *et al.*, 2011; Palmer *et al.*, 2012). However, our phylogenetic analysis of the available *nirK* and *nirS* showed that a greater diversity of microorganisms possess *nirK* or *nirS*, including Actinobacteria, Bacteroidetes, Chloroflexi, Nitrospirae, Spirochetes, Planctomycetes, NC10, and even the archaeal phylum Euryarchaeota (Figure 1). Their *nirK* and *nirS* were not detected using the conventional primers (Supplementary Figures S1 and S2). The clone library analysis verified that the previously

unconsidered *nirK* and *nirS* are distributed in many terrestrial environments, including cropland and paddy soils, natural and planted forest soils, and lake sediments (Figures 2 and 3). We also found that the microorganisms with *nirK* or *nirS* were 2–6 times more abundant than those determined using conventional primer sets in all of the terrestrial environments tested (Figure 4). These results strongly suggest that denitrifying microorganisms with *nirK* or *nirS* are more diverse and abundant than considered previously, and they are widely distributed in terrestrial environments.

The distributions of *nirK* and *nirS* differed in the terrestrial environments that we tested (Figure 4). Habitat-specific factors may have a crucial effect on the abundance and diversity of denitrifying microorganisms with *nirK* or *nirS* in different environments as suggested by Jones and Hallin (2010). In particular, the high abundance of *nirS* compared with *nirK* in rice paddy soils, where denitrification is generally active, may be attributed to the high availability of soluble iron (Yamazaki *et al.*, 1995; Einsle *et al.*, 2002). Soluble iron is used for the production of NirS, but it is generally limited by the insolubility of iron under aerobic conditions above pH 4 (Kraemer 2004). However, soluble iron can be seasonally available in paddy field soils because of water flooding. In contrast, Enwall *et al.* (2010) noted that copper availability might be a strong driving factor that shapes the abundance of *nirK* because NirK is a multicopper protein. The different distributions of *nirK* and *nirS* in each cluster also highlight the different physiological features of the total denitrifying communities in these environments as the results indicated a higher abundance or contribution to the denitrification of methane oxidizers in rice paddy soil. The different distributions also may affect the specific rates or end products of denitrification. Determination of the relationships between the phylogenies of *nirK* and *nirS* and the physiological features or responses to environmental gradients of denitrifying microorganisms will be important for understanding the spatial and temporal dynamics of denitrification (Philippot *et al.*, 2009; Morales *et al.*, 2010).

RNA- and culture-based analyses using a cropland soil also suggested that previously unconsidered microorganisms that possess *nirK* and *nirS* are responsible for N₂O emissions. We performed RNA-based analyses to investigate their involvement in denitrification because microorganisms with *nirK* and *nirS* generally have life strategies other than denitrification, such as oxygen respiration, nitrification and methane oxidation. The abundances of transcripts of *nirK* in Clusters I–III and *nirS* in Cluster I were clearly higher in the denitrification-induced soil compared with the non-induced soil (Table 1). In addition, we found that many soil clones with *nirK* in Clusters I–III and *nirS* in Cluster I shared similar sequences with *nirK* or *nirS*, which could not be detected by the

conventional primers, of the denitrifying strains isolated from the same soil (Supplementary Table S2 and Supplementary Figure S4). These strains exhibited a high capacity to produce N₂O as the end product of denitrification. We also showed that the microorganisms in the soil microcosm tested were affiliated to *Enterobacter* sp., *Sinorhizobium* sp., *Ralstonia* sp., *Curvibacter* sp., *Wautersia* sp., *Yersinia* sp., *Streptomyces* sp., *Micromonospora* sp. and *Dechloromonas* sp.

To summarize, our new primers showed that microorganisms with *nirK* or *nirS* are phylogenetically more diverse and abundant than previously considered and are involved in denitrification in terrestrial environments. Although denitrifying microorganisms have been studied for a long time in various environments using culture- and PCR-based techniques with *nirK* and *nirS* as marker genes, these genes from many of the denitrifying strains that we isolated could not be amplified using the conventional primers. The methodology developed in the present study and the insights obtained should allow us to achieve a more precise understanding of the spatial and temporal dynamics of denitrification and facilitate more precise estimates of denitrification in different environments. It should be noted that the number of well-defined *nirK* and *nirS* is increasing. For example, since our collection of *nirK* and *nirS* sequences from databases, sequences have been obtained from more diverse microorganisms, such as *nirK* from Firmicutes (Murawska *et al.*, 2013) and *nirS* from Crenarchaeota (Mardanov *et al.*, 2012). Some groups of fungi also have *nirK* (Wei *et al.*, 2015). PCR techniques still have a greater capacity for the deep analysis of target genes, although efforts are needed to minimize the PCR biases. The currently unknown diversity detected using metagenomic analyses and obtained by sophisticated isolation techniques will allow us to design primers with greater coverage.

Conflict of Interest

The authors declare no conflict of interest.

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