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Short communication

Development of PCR primers targeting fungal *nirK* to study fungal denitrification in the environment

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ABSTRACT

Fungal denitrification in soils is receiving considerable attention as one of the dominant N_2O production processes. However, because of the lack of a methodology to detect fungal denitrification-related genes, the diversity and ecological behavior of denitrifying fungi in soil remains unknown. Thus, we designed a primer set to detect the fungal nitrite reductase gene (*nirK*) and validated its sensitivity and specificity. Through clone library analyses, we identified congruence between phylogenies of the 18S rRNA gene and *nirK* of denitrifying fungal isolates obtained from the surface-fertilized cropland soil and showed that fungi belonging to Eurotiales, Hypocreales, and Sordariales were primarily responsible for N_2O emissions in the soil.

Nitrous oxide (N_2O) is a potent greenhouse gas (IPCC, 2007) and is involved in stratospheric ozone depletion (Ravishankara et al., 2009). It is produced through microbial denitrification, in which nitrate and nitrite are reduced to gaseous N_2O (Isobe and Ohte, 2014). Fungal denitrification in soil has recently received considerable attention as an N_2O production process. We previously performed an antibiotic assay and isolated denitrifying fungi to demonstrate the dominance of fungal denitrification in a surface-fertilized cropland soil (Wei et al., 2014). This has also been reported in grassland and forest soils using the same methodology (Laughlin and Stevens, 2002; Blagodatskaya et al., 2010). Many fungal species are known to produce N_2O (Shoun et al., 1992; Wei et al., 2014); however, the diversity and ecological behavior of denitrifying fungi in soil, unlike denitrifying bacteria, remains unknown, probably because of the lack of a methodology to detect fungal denitrification-related genes. In addition, the ability to denitrify varies at the species level (Wei et al., 2014), making it difficult to identify denitrifying fungi based on their taxonomic position. Previous studies revealed that *Fusarium oxysporum* and *Cylindrocarpon tonkinese*, the most thoroughly characterized denitrifying fungi (Nakanishi et al., 2010), use copper-containing nitrite reductase (NirK) to reduce nitrite to nitric oxide, bearing a close resemblance to its bacterial counterpart (Kobayashi and Shoun, 1995; Kim et al., 2010). Additionally, fungal cytochrome cd1-type nitrite reductase remains undiscovered. Thus, developing a methodology to detect fungal *nirK* should lead to the precise identification of denitrifying fungi and elucidation of their ecological behavior.

Consequently, the objectives of this study were to design suitable PCR primers to detect fungal *nirK* and use these primers to investigate the diversity of fungal *nirK* and identify the denitrifying fungi in surface-fertilized cropland soil where fungal denitrification is dominant (Wei et al., 2014).

We searched full-length *nirK* fungal sequences from the public databases, NCBI Microbial Genomes (http://www.ncbi.nlm.nih.gov/genomes) and Functional Gene Repository (http://fungene.cme.msu.edu/index.spr) and obtained 15 sequences belonging to Ascomycota. We also obtained the representative sequences of *nirK* from diverse bacterial phyla and Euryarchaeota from database. Then, we generated the phylogenetic tree of *nirK*

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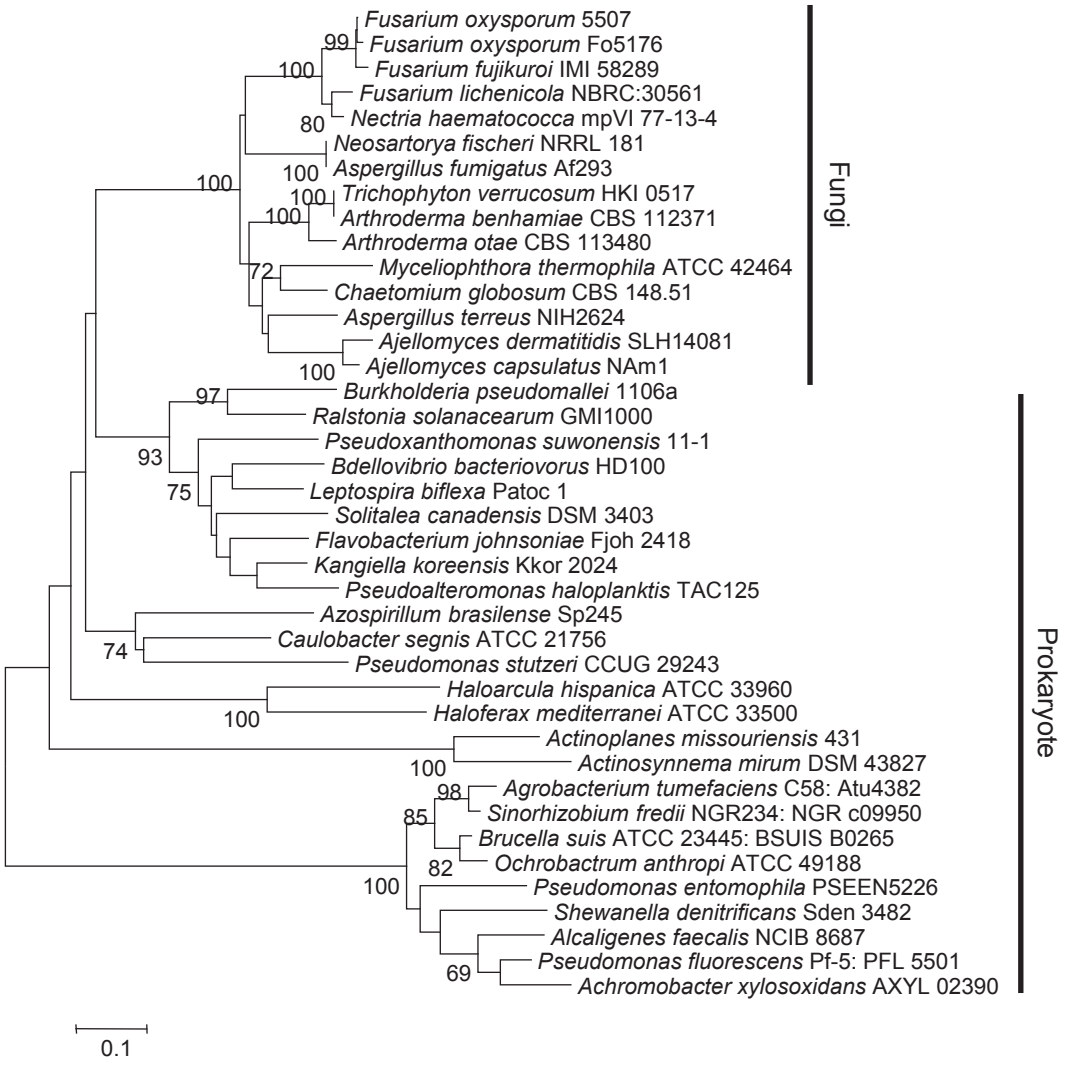

Fig. 1. Tree of maximum likelihood phylogeny of database-retrieved full-length NirK amino acid sequences of fungi, bacteria, and archaea. Bootstrap values (500 replicates) greater than 70% are denoted above the branches and branch lengths correspond to sequence differences, which are indicated by a scale bar.

(Fig. 1), and found that fungal *nirK* formed a monophyletic cluster distinct from the prokaryotic *nirK* with 100% bootstrap support.

NirK is a two-domain enzyme including two copper centers, types 1 and 2 (Sakurai and Kataoka, 2007). We designed the primer sets based on homologs of the copper center type 1 domain (Fig. S1). Because widely used primers for bacterial *nirK* (such as primer set F1aCu/R3Cu and nirK2F/nirK5R, Braker et al., 1998; Hallin and Lindgren, 1999) also target this region, we can compare fungal *nirK* sequences with the large quantity of bacterial *nirK* sequences. We designed the forward primer to anneal with four conserved amino acids (tyrosine, valine, glutamine, and proline) and reverse primer to anneal with four conserved amino acids (aspartic acid, lysine, glycine, and alanine; Fig S1). Most of these amino acids were not conserved in prokaryotic *nirK* (Fig S1). The primer set specific to fungal *nirK* sequences, nirKfF (5′-TACGGGCTCATGtaygtnsarcc-3′) and nirKfR (5′-AGGAATCCCACAscnccyttntc-3′), were designed based on the Consensus–Degenerate Hybrid Oligonucleotide Primer (CODEHOP) algorithm (Rose et al., 1998). Both primers consist of the degenerate core region (lowercase letters) corresponding to all possible codons specifying the conserved amino acids and consensus clamp region (uppercase letters) containing a single most common (consensus) nucleotide sequence derived from the rest of amino acids in the primer-designed region (Fig S1).

We validated the specificity and sensitivity of the designed primer set, nirKfF/nirKfR, using fungal and prokaryotic strains. We used seventeen denitrifying and three nondenitrifying fungal strains isolated from the collected organic fertilizer (COF) and residual soil (RS) from a surface-fertilized cropland soil where fungal denitrification predominates (Wei et al., 2014). We also used ten prokaryotic strains (nine bacterial and one archaeal) obtained from culture collections (Japan Collection of Microorganisms (JCM), Koyadai, Japan or the Biological Resource Center (NBRC), Kazusakamatari, Japan; Table 1 and Fig. 1). Their abilities to produce N_2O were confirmed as described previously (Isobe et al., 2011). Genomic DNA was extracted as described previously (Wei et al., 2014), and PCR was performed with the designed primers. The PCR mixture and conditions are described