Detection of N₂O-producing fungi in environment using nitrite reductase gene (*nirK*)-targeting
primers

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Abstract

Fungal denitrification has been increasingly investigated, but its community ecology is still poorly understood due to the lack of culture-independent tools. In this work, four pairs of nitrite reductase gene (*nirK*)-targeting primers were designed and evaluated for primer specificity and efficiency using thirty N₂O-producing fungal cultures and an agricultural soil. All primer sets amplified *nirK* from fungi and soil, but their efficiency and specificity were different. A primer set, FnirK_F3/R2 amplified ~ 80% of tested fungi, including *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma*, as compared to ~ 40-70% for other three primers. Rarefaction analysis from clone libraries of soil DNA also showed that FnirK_F3/R2 amplified more diverse *nirK* from environment than other primer sets. The *nirK* fragments of fungal and soil DNA amplified by FnirK_F3/R2 were phylogenetically related to known denitrifying fungi in the orders Eurotiales, Hypocreales, and Sordariales; and clone sequences were also distributed in the clusters of *Chaetomium*, *Metarhizium*, and *Myceliophthora* that were not isolated from soil in our previous work. This proved the wide-range capability of primers for amplifying diverse denitrifying fungi from environment. However, our primers and recently-developed other primers amplified some bacterial *nirK* from soil and this co-amplification of fungal and bacterial *nirK* was theoretically discussed. The FnirK_F3/R2 was further compared with other recently-published primers for primer specificity and coverage. Results from the clone libraries of soil DNA demonstrated that FnirK_F3/R2 was more specifically targeted on fungi and had broader taxonomical coverage than some others.

Keywords: fungi; bacteria; denitrification; *nirK*; nitrous oxide; soil

1. Introduction

Fungal denitrification, a sequential reduction of NO_2^- and/or NO_3^- to N_2O , has been progressively recognized as an important biological process for soil N_2O emissions (Laughlin and Stevens 2002; Yanai et al., 2007; Crenshaw et al., 2008; Laughlin et al., 2009; Jirout et al., 2013; Mothapo et al., 2013; Wei et al., 2013; Chen et al., 2014; Jirout 2015). Though in a rudimentary state, knowledge on the relative significance of fungi versus bacteria to soil N_2O emissions in terms of soil moisture, pH, and quality of organic C has been generated (Seo and DeLaune 2010; Herold et al., 2012; Rütting et al., 2013; Wei et al., 2013; Chen et al., 2015a, b). N_2O -producing fungi of different taxa have been isolated from soils across diverse ecosystems and geographic regions (Jirout et al., 2013; Mothapo et al., 2013; Wei et al., 2013; Jirout 2015; Maeda et al., 2015). To date, 90% of N_2O -producing fungal isolates are in the phylum Ascomycota; 41% are in the class Sordariomycetes; and isolates with a high frequency of occurrence usually belong to *Fusarium*, *Trichoderma*, *Penicillium*, and *Aspergillus* (Mothapo et al., 2015). This “apparently-low” diversity of denitrifying fungi is likely due to limitation and biases of culture-dependent approach, e.g., selection of rapidly growing fungi, and therefore culture-based studies may underestimate the diversity of N_2O -producing fungal community.

Microbial diversity and abundance are vital traits of soil microbial community that may facilitate a better understanding of ecosystem functions and services (Shade et al., 2012). Recent work has demonstrated that community traits of denitrifying bacteria can be good metrics to predict N_2O production (Philippot et al., 2009; Morales et al., 2010). The emphasis has been particularly placed on the abundance of denitrifying bacteria that do not possess a full set of enzymes for catalyzing the complete reduction of NO_3^- to N_2 (Philippot et al., 2011). While only a fraction of denitrifying bacteria are lacking the genetic ability of synthesizing N_2O reductase

and thus mediate a truncated pathway of denitrification with N_2O as the end product, almost all the denitrifying fungi do not have this enzyme synthesis ability (Zumft 1997; Shoun et al., 2012). As such, understanding the abundance and distribution of denitrifying fungi may provide new insight into soil N_2O emissions under various environmental settings.

Similar to bacterial counterparts, denitrifying fungi also possess a Cu-containing NO_2^- reductase (NirK) that catalyzes the reduction of NO_2^- to NO for the production of ATP (Nakanishi et al., 2010). Fungal NirK has been purified and characterized from a few denitrifying fungi, such as *Fusarium oxysporum* and *Aspergillus oryzae* (Kobayashi and Shoun 1995; Kim et al., 2009; Nakanishi et al., 2010). In addition, genome analyses have provided strong evidence that genes that are homologous to bacterial NO_2^- reductase gene (*nirK*) are widely distributed among many fungi (Kim et al., 2009; Shoun et al., 2012). Phylogenetic trees constructed with full-length NirK amino acid sequences of fungi, bacteria, protozoa, and algae indicate that NirK of prokaryotic and eukaryotic microorganisms share the same ancestry (Kim et al., 2009; Shoun et al., 2012; Wei et al., 2015b). Fungal NirK and homologs appear to originate from proto-mitochondria, the ancestral bacterial endosymbionts from which all mitochondria are derived (Kobayashi and Shoun 1995). NirK has been characterized into two classes; class I NirK is mainly associated with prokaryotes, whereas recently-identified class II NirK appears to come from both prokaryotes and eukaryotes (Boulanger and Murphy 2002, Kim et al., 2009). Although the domain of fungal NirK has similarity of up to 50% with bacterial and other eukaryotic NirK in class II, fungal NirK formed a monophyletic cluster (Kim et al., 2009; Wei et al., 2015b). This suggests that fungal *nirK* can be used as a marker gene to examine the community structure of denitrifying fungi.

The objective of this study was initially set for developing a pair of *nirK*-targeting primers to detect N₂O-producing fungi in environmental samples. During this study, however, several primer sets for examining N₂O-producing fungi were published (Long et al., 2015; Maeda et al., 2015; Wei et al., 2015b). When applied to environmental samples, these published primers were found to amplify both denitrifying fungi and bacteria, indicating that primer specificity was moderate (Long et al., 2015; Maeda et al., 2015). Therefore, we designed several more primers targeting fungal *nirK* to produce amplicons of different sizes and determined if these new primers could be more specific for denitrifying fungi. Efficiency and specificity of designed primers were evaluated against fungal species/strains of different taxa that were previously isolated from diverse agroecosystems and proven to be capable of N₂O production (Mothapo et al., 2013). Designed primers were also tested using metagenomic DNA extracted from an agricultural soil. Further, our primers were compared with recently-developed other primers (Long et al., 2015; Maeda et al., 2015; Wei et al., 2015b) for primer coverage and efficiency.

2. Materials and Methods

2.1. Primer Design

Degenerate primers were designed and optimized based on fungal *nirK* retrieved from the National Center for Biotechnology Information (NCBI)-Nucleotide, NCBI-Blast, and European Nucleotide Archive (ENA). The 1st database of fungal *nirK* was established by searching NCBI-Nucleotide and included three complete coding DNA sequences (CDS) of *Aspergillus oryzae*, *Fusarium oxysporum*, and *Fusarium lichenicola* (Supplementary Table S1). The 2nd database included 34 DNA sequences homologous to fungal *nirK* in the 1st database. By using NCBI-Blast, we collected the regions of sequences with > 90% query coverage and 85% identities from

fungal whole genome shotgun contigs. The 3rd database of fungal *nirK* was made by searching
 HMMER (Finn et al., 2011) to find proteins homologous to fungal NirK in the 1st database and
 then corresponding complete CDS of fungal species other than *A. oryzae*, *F. oxysporum*, and *F.*
lichenicola were retrieved from ENA. To further aid *in silico* validation of degenerate primers,
 we also created the 4th database of non-fungal *nirK*, including complete CDS of a protozoan, a
 eukaryotic alga, and 12 bacteria that were retrieved from ENA and were most homologous to
 fungal NirK based on the HMMER search. It should be noted that the limited number of class II
 NirK in the public database might constrain the diversity of references in the 4th database.
 However, if these references could not be *in silico* amplified, perhaps no other sequences from
 the public database could, given that other sequence similarity with fungal NirK is lower than
 that of reference sequences in the 4th database.

Fungal *nirK* in the 1st, 2nd, and 3rd databases were aligned using Clustal Omega (McWilliam
 et al., 2013), a multiple sequence alignment tool to find the “relatively” conservative regions of
 DNA, RNA, and protein sequences. Then, NCBI Primer-Blast (Ye et al., 2012) was used to
 design forward and reverse primers of PCR from the conservative DNA sequence regions, using
Aspergillus oryzae as the template. Thus-designed primers were manually modified to create
 degenerate primers through multiple alignments with fungal *nirK* in the 1st, 2nd and 3rd databases.
 Degenerate primers were further modified and validated against *nirK* in the 1st, 2nd, 3rd and 4th
 databases using De-MetaST for an *in silico* amplification (Gulvik et al., 2012). Four primer pairs
 were able to *in silico* amplify diverse fungal *nirK*, but were unlikely for *nirK* of bacteria, algae
 and protozoa, if allowing maximal 3-bp mismatches (Table 1). The corresponding positions of
 degenerate primers on the *nirK* of *Aspergillus oryzae* are given in Fig. 1.

To better validate the degenerate primer pairs in the following experimental tests, several other primer pairs were also included as different types of positive controls (Table 1). A primer pair, 5.8S and ITS1f (Fierer et al., 2005) targeting eukaryotic rDNA was used to confirm that fungal DNA extracts could be amplified under experimental conditions. A recently-developed primer pair, nirKfF and nirKfR, for amplifying fungal *nirK* (Wei et al., 2015b) was also included to determine the efficiency and specificity of the degenerate primers. Further, a primer pair, nirK876 and nirK1040 (Henry et al., 2004) targeting bacterial *nirK*, was used to examine if bacterial cultures (see below) could be amplified by bacterial *nirK* primers but not by the designed degenerate primers.

2. 2. Fungal Isolates and Soil Sample

Degenerate primers were examined against 30 fungal isolates, including spp. of *Actinomucor*, *Aspergillus*, *Fusarium*, *Gibberella* (teleomorph of *Fusarium*), *Hypocrea* (teleomorph of *Trichoderma*), *Ophiocordyceps*, *Penicillium*, *Phoma*, *Purpureocillium*, and *Trichoderma*, which were collected from diverse agroecosystems and proven to be of N₂O-producing capability (Mothapo et al., 2013). The purity of these fungal isolate cultures was assured by (1) using streptomycin to inhibit bacterial growth, (2) observing fungal hyphae and spores under a scanning electron microscopy, and (3) amplifying DNA extracted from fungal cultures using ITS- and/or 16S-specific primers (Mothapo et al., 2013). Degenerate primers were also tested against a soil sample that was collected from a conventional cash cropping system at the Center for Environmental Farming Systems (35°22'48'' N, 78°02'36'' W), Goldsboro, North Carolina, USA (Chen et al., 2015a). This soil was previously examined for fungal contribution to soil N₂O production and used for collecting fungal isolates (Mothapo et al., 2013; Chen et al.,

2014). Furthermore, six cultures of bacterial denitrifiers (*Actinoplanes missouriensis* ATCC 31681, *Alcaligenes faecalis* ATCC 8750, *Ochrobactrum anthropi* ATCC BAA-749, *Paracoccus pantotrophus* ATCC 35512, *Pseudoalteromonas haloplanktis* ATCC 14393, and *Pseudomonas stutzeri* ATCC 53819) were included to determine whether the degenerate primers were specific to amplify fungal denitrifiers rather than bacterial denitrifiers.

2.3. DNA Extraction from Fungal Isolates and Soil Sample

After two-week growth in a liquid Czapek medium at 30 °C (Mothapo et al., 2013), mycelia of fungal isolates were collected and then extracted for DNA using FastDNA Spin Kit (MP Bio, Solon, OH) according to the manufacturer's instructions. Metagenomic DNA was also extracted from 0.5 g soil sample using FastDNA Spin Kit and then column-purified using OneStep PCR Inhibitor Removal Kit (Zymo Research, Orang, CA). DNA concentrations were determined ($> 50 \text{ ng } \mu\text{L}^{-1}$) and purity was confirmed by the ratio of absorbance at 260 and 280 nm ($260/280 = 1.70\text{-}1.90$) using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE).

2.4. PCR Amplification

A 20 μL PCR reaction was comprised of 10 μL 1X Quick-Load Taq Master Mix (New England Biolabs (NEB), Ipswich, MA), 1 μL of template DNA ($4\text{-}20 \text{ ng } \mu\text{L}^{-1}$), 1 μL of 10 mM of each primer, and 7 μL of nuclease-free water. PCR was carried out using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA) and reaction was initiated at 95 °C for 30 sec, followed by 30 cycles of 30 sec at 95 °C, 30 sec at annealing temperature specified in Table 1, and 1 min at 68 °C, followed by a final elongation of 5 min at 68 °C. To increase product yield and eliminate heteroduplexes (Thompson et al., 2002), reconditioning PCR (i.e., a dilution of

amplification product into fresh reaction mixture followed by second amplification) was performed for soil DNA. The reconditioning PCR reaction and thermal cycling conditions were the same as described above except for 18 cycles for the first PCR. Then, the PCR product was diluted 10 times and run as template with fresh PCR reagent for 20 more cycles for the second PCR. This PCR reaction protocol was also used to amplify *nirK* from the soil DNA by recently-developed other primers (Long et al., 2015; Maeda et al., 2015; Wei et al., 2015b), so that fungal *nirK* diversities of clone libraries constructed with different primer sets could be compared. PCR products were analyzed by gel electrophoresis using 1.5% low-melting agarose. Bands of desired size were excised and purified using the PureLink Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA).

2.5. Cloning and Sequencing

For fungal isolates, gel-purified PCR products were directly sequenced by the Sanger sequencing method on an ABI 96-capillary 3730 sequencer (Eton Bioscience Inc., Research Triangle Park, NC). For soil DNA, however, gel-purified reconditioning PCR products were first ligated into pMiniT vector and cloned by NEB 10-beta competent *E. coli* using the NEB PCR Cloning Kit (NEB, Ipswich, MA). At least 20 white colonies were randomly picked with sterile pipette tips and placed into PCR tubes for amplification with primers for cloning analysis using the same PCR reaction and conditions as described above. PCR products were then sequenced by the Sanger sequencing method. For comparing fungal *nirK* diversities of clone libraries constructed with different primer sets, the same cloning protocol was used except that > 65 white colonies were randomly chosen for sequencing.

2.6. Data Analyses

Sequences were trimmed and quality was manually checked. Chimeric sequences were identified using UCHIME (Edgar et al., 2011) on the MOTHUR platform (<http://www.mothur.org/wiki/Chimera.uchime>). To find homologies, sequences were blasted against NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST/>). Thus-obtained high quality sequences were then subjected to phylogenetic analysis. Phylogenetic trees were constructed with nucleotide-based maximum likelihood method using Mega 6.06. References for the assemblage of phylogenetic trees were the sequences of fungal isolates as well as sequences chosen from the four databases (Supplementary Table S1). The MOTHUR was also used to assign clone sequences to operational taxonomic units (OTUs) and conduct rarefaction analysis at 3% sequence divergence (Schloss et al., 2009). The sequences of amplicons obtained from fungal cultures and the agricultural soil were submitted to the NCBI database (accession numbers KT462468-KT462537, KX272972-KX273057).

3. Results and Discussion

3.1. Quality of DNA Extracts from Microbial Cultures and Soil Sample

DNA samples of all the 30 fungal isolates were amplified to the desired size by the primer pair 5.8S and ITS1f (data not shown), indicating that DNA extraction and purification were effective to generate samples of high quality. DNA samples of bacterial cultures were amplified by the primer pair nirK876 and nirK1040, despite that two (*Paracoccus pantotrophus* ATCC 35512 and *Pseudomonas stutzeri* ATCC 53819) out of the six tested strains produced multiple bands rather than single desired band (data not shown), confirming that at least four bacterial

cultures possessed *nirK*. Further, soil DNA extraction was of high quality as verified by the amplification using the primer pair 5.8S and ITS1f (data not shown).

3.2. Fungal *nirK* Primer Specificity and Efficiency in Amplifying Fungal Cultures

Primer specificity is defined as the proportion of negative controls (i.e., bacterial cultures) that were amplified correctly. Neither our primers nor the primer set designed by Wei et al. (2015b) were able to amplify bacterial cultures (Table 2), indicating that fungal *nirK* primers were of good specificity.

Here, we use the ratio of fungal cultures amplified by fungal *nirK* primers to total fungal cultures to determine primer efficiency. To ensure the reliable tally of positive fungal cultures in terms of amplification, the sequences of amplicons from the same fungal cultures were aligned between different primer sets, including *nirKf* designed by Wei et al. (2015b). Only amplicons having high similarity (i.e., $\geq 97\%$ identities) in aligned regions between different primers were confirmed as positives. Thus, although bands of desired size were obtained, *Aspergillus versicolor*, *Hypocrea intricata*, *H. lixii*, *Penicillium citrinum*, *Phoma macrostoma*, and *Trichoderma piluliferum* were interpreted as “incongruent” due to poor sequence quality/similarity between primers (Table 2). Of total 30 fungal culture strains representing 10 genera and 22 species, F*nirK*_F3/R2 was able to amplify 83%, 80% and 77% of culture strains, genera, and species, respectively (Table 2). The corresponding percentages were reduced to 70%, 60%, and 68% for F*nirK*_F2/R2, and 40%, 40%, and 27% for F*nirK*_F1/R1 and F*nirK*_F1/R2. Apparently, the two primer sets, F*nirK*_F3/R2 and F*nirK*_F2/R2 could cover a broad range of fungal genera and species and the coverage was comparable to or greater than the coverage of *nirKf* designed by Wei et al. (2015b), showing 73%, 80%, and 64% of culture strains, genera,

and species being amplified, respectively (Table 2). Our primer coverage seemed to be comparable to the coverage of another primer set, designed by Maeda et al. (2015), who reported that 64% of 70 fungal cultures could be amplified. However, we should realize that only a fraction of tested fungal cultures were common among individual studies, and therefore cautions need to be taken with such comparative information.

A number of factors may affect the efficiency of PCR amplification, including amplicon size and primer-template mismatches (Kwok et al., 1990; Arnheim and Erlich 1992; Cha and Thilly 1993; Smith et al., 2002). While amplicons of large size could reduce amplification efficiency, the size perhaps was not the major cause for different efficiencies among primers since low efficiency was observed for both small (~ 331 bp for FnirK_F1/R1) and large (~ 544 bp for FnirK_F1/R2) amplicons. As shown in Table 1, FnirK_F1 has the highest number of mismatches with fungal *nirK* genes. Therefore, it is not surprising to see relatively low efficiency and coverage of primer sets with FnirK_F1 as the forward primer.

Similar to primers designed by Maeda et al. (2015) and Wei et al. (2015b), our primers amplified diverse fungal cultures belonging to the phylum Ascomycota, e.g., *Fusarium*, *Aspergillus*, *Penicillium*, and *Trichoderma*. Our primers FnirK_F3/R2 and FnirK_F2/R2 also worked for a fungal culture, *Actinomucor elegans* of the phylum Zygomycota (Table 2). We noted from the work of Wei et al. (2015b) that nirKf failed to amplify a culture of *Actinomucor elegans*; however, this primer set was proven to be successful to the Zygomycota culture in this study. Such phenomenon that primers might not amplify all the strains of the same species was also observed for *Trichoderma asperellum* in this study and *Fusarium oxysporum* in the work of Wei et al. (2015b). Nonetheless, our primers, together with primers designed by Maeda et al.

(2015) and Wei et al. (2015b), could detect *nirK* from a broad taxonomic group of denitrifying fungi.

3.3. Phylogeny of Denitrifying Fungal Cultures

The full-length sequences of amplicons by FnrK_F3/R2 were used to construct a phylogenetic tree to infer evolutionary relationships among various denitrifying fungal cultures (Fig. 2). In agreement with other reports (Kim et al., 2009; Maeda et al., 2015; Wei et al., 2015b), *nirK* of all the fungal cultures were well separated from the *nirK* of bacterial references and clustered with references of known denitrifying fungi. Sequences of all the *Fusarium* and *Gibberella* cultures resided in the clade of references, *Fusarium oxysporum* and *F. lichenicola* with 89% bootstrap confidence, further confirming their identities as the member of the family Nectriaceae. Sequences of *Trichoderma*, *Ophiocordyceps*, and *Purpureocillium* cultures also formed a single cluster with 79% bootstrap support. Clearly, members of the order Hypocreales (i.e., species of *Fusarium*, *Gibberella*, *Trichoderma*, *Ophiocordyceps*, *Purpureocillium* and *Metarhizium*) were distributed in different clades, suggesting some distant relatedness. However, the inferences based on *nirK* were consistent with those derived from ITS, showing a great dispersal of the order Hypocreales within the phylogenetic tree (Mothapo et al., 2015).

Most *Penicillium* and *Aspergillus* cultures were clustered with respective references at genus levels, except *Aspergillus sydowii* and *Aspergillus fischerianus* (Fig. 2). Particularly, *A. fischerianus* Suc-5625 showed closer relatedness to members of the order Sordariales rather than members of its taxonomically-accepted order Eurotiales. Further, *Actinomucor elegans* Con-1417, a fungal culture of the phylum Zygomycota was not separated from the phylum Ascomycota to form its own cluster, as is the case of ITS phylogeny (Mothapo et al., 2015; Wei

et al., 2015b). Despite some contradictions between *nirK* and ITS trees, phylogenies were generally congruent between most *nirK* and ITS (Maeda et al., 2015; Mothapo et al., 2015; Wei et al., 2015b), which is in sharp contrast to non-congruence between bacterial *nirK* and 16S rRNA genes (Jones et al., 2008). The congruence might imply vertical inheritance of *nirK* in fungi (Maeda et al., 2015).

However, cautions need to be taken when making any inferences on patterns of genetic inheritance from *nirK* and ITS phylogenies. First, the number of fungal *nirK* in public databases is much lower than that of bacterial *nirK*. We should not rule out the possibility that inclusion of additional fungal *nirK* from taxonomically different groups could dramatically shift the structure of phylogenetic trees. Second, ITS as the fungal barcoding genome region may be insufficient to resolve some fungal species, and a fungus at different morphological states could be separated into different clusters (O'Donnell and Cigelnik 1997; Geiser et al., 2004; O'Donnell et al., 2013; Mothapo et al., 2015). Therefore, it is suggested that data from multiple loci need to be used to draw conclusions on the processes driving the evolutionary of denitrifying fungi (Mothapo et al., 2015).

3.4. Fungal *nirK* from the Environment

All our primers were able to amplify soil DNA with a single band of desired size. Clone libraries contained 16 clones by F_{nirK}_F3/R2, 19 clones by F_{nirK}_F2/R2, 27 clones by F_{nirK}_F1/R1 and 15 clones by F_{nirK}_F1/R2, leading to 11, 14, 7 and 6 OTUs, respectively (Fig. 2, Fig. 3). Clearly, PCR of soil DNA supported the results made from fungal cultures that F_{nirK}_F3/R2 and F2/R2 were most effective for amplifying diverse *nirK*-containing microorganisms.

Phylogenetic tree (Fig. 2) showed that soil fungi amplified by FnirK_F3/R2 were closely related to species of *Fusarium*, *Aspergillus*, *Chaetomium* and *Metarhizium*. A quarter of total clones were related to members of the order Sordariales, suggesting greater abundance of this group of fungi in soil. Seven of total 16 clones were clustered with denitrifying bacteria, suggesting that this primer set could amplify genes related to bacterial *nirK* (Fig. 2). Indeed, Blast analysis showed that some sequences of our bacterial clones were homologous to the sequences of uncultured bacteria (Wei et al., 2015a). For example, the sequence of OTU-5 (KT462503) was 90% similar to the sequence of bacterial clone C-2-13 (AB936921.1) from the work of Wei et al. (2015a). It should be noted that amplicon size for fungal clones was 236 bp, whereas amplicon size for bacterial clones was 218 or 221 bp.

Compared to FnirK_F3/R2, The primer set FnirK_F2/R2 generated even more bacterial *nirK*-containing clones (Fig. 2). Sixteen of total 19 clones were clustered with bacterial references, whereas only three clones were clustered with fungal references. Again, bacterial clones (290-302 bp) were smaller than fungal clones with 323 bp. Further, fungal clones amplified by this primer pair was related to member of the order Sordariales.

All 27 clones generated by FnirK_F1/R1 belonged to *nirK*-containing fungi (Fig. 3). Clones were well clustered with *Fusarium oxysporum*, *Myceliophthora thermophila*, *Chaetomium globosum*, and *Metarhizium acridum*. FnirK_F1/R2 generated clones belonging to both fungi and bacteria, but 13 of 14 clones resided in the cluster of fungal references (Fig. 3). Here, bacterial clone was 517 bp and fungal clones were 550 – 568 bp. We found that clones amplified by FnirK_F1/R1 and FnirK_F1/R2 were mainly clustered with reference fungi in the orders Hypocreales and Sordariales. Thus, in terms of primer specificity, results made by soil samples

and pure cultures were inconsistent, as PCR of soil DNA demonstrated that primer sets tested in this study could amplify *nirK* from bacteria.

The inconsistency between DNA samples from pure cultures and soil sample is likely because bacteria tested in pure cultures only represented a small fraction of diverse bacteria in the environment. There are two major groups of NirK, classes I and II (Boulanger and Murphy 2002). While class I includes only bacterial NirK, class II contains both eukaryotic and prokaryotic NirK (Kim et al., 2009). Kim and colleagues (2009) documented that fungal NirK had high similarities with bacterial NirK in class II, but not in class I. Because only one (*Pseudoalteromonas haloplanktis* ATCC 14393) of the six bacterial cultures used as negative controls likely contained class II NirK, the chance for observing positive results from negative controls should be low. This perhaps explains the strong primer specificity from pure culture experiment. In contrast, soil always harbors extremely diverse microorganisms, thereby increasing the chance for primers targeting fungal *nirK* to amplify bacterial *nirK* in the same class. In fact, some bacterial clones detected by our primers were of high similarity with bacterial *nirK* in class II, corresponding to the clusters II of a prokaryotic phylogenetic tree made by Wei et al. (2015a).

Despite differences in specificity and coverage, all our primers amplified fungi belonging to the order Sordariales from the soil samples. However, our previous study did not isolate any fungal strains of the order Sordariales from the same environment; and instead, majority of isolates belonged to the orders Hypocreales and Eurotiales (Mothapo et al., 2013). Wei and colleagues (2015b) also showed that half of OTUs was closely related to the order Sordariales, although they did not isolate fungal cultures of this order from the environment (Wei et al.,

2013). These findings indicate that culture-dependent methods for characterizing denitrifying fungal community have severe limitation in detecting dominant species in the environment.

We realized that our clone library size was too small to reliably estimate the diversity of denitrifying fungi in soil samples. As shown by the rarefaction analysis (Supplementary Fig. S1), the number of clone sequences was far less than the number required for achieving maximal OTUs. Still, rarefaction analysis could help identify primer efficiency and coverage. Almost linear increase of OTUs with the number of clone sequences for FnirK_F3/R2 and F2/R2 confirmed that the two primer sets could cover more diverse denitrifying-fungi and bacteria in the environment.

3.5. Sequence Comparison of Clone Libraries Made by Different Primer Sets

Our primer pair, FnirK_F3/R2 was further evaluated against recently-developed other primers (Fig. 1). Fungal *nirK* primer pair developed by Long et al. (2015) did not produce a single and clear band, but rather a vague smear. Thus, clone libraries of soil DNA were only made for the primer pair FnirK_F3/R2 and primers developed by Wei et al. (2015b) and Maeda et al. (2015). The number of OTUs in Maeda's clone library increased linearly with the number of clones (Supplementary Fig. S2); however, by blasting against the NCBI nucleotide collection all the clone sequences were found to be closely related to bacterial *nirK* (Table 3). In contrast, ~76-84% of the OTUs in Chen's and Wei's clone libraries belonged to fungal *nirK*. It should be mentioned that % of individual clone sequences belonging to fungal *nirK* was greater for Wei's primers than for Chen's primers, since one bacterial *nirK* OTU in Chen's clone library included 20 individual clone sequences (Fig. 4).

Of total 16 fungal-*nirK* OTUs in Wei's clone library (Fig. 4), 12 were related to *Chaetomium globosum*; only one related to *Fusarium oxysporum* and *Aspergillus oryzae*, respectively. By contrast, of total 21 fungal-*nirK* OTUs in Chen's clone library, seven were related to *Chaetomium globosum*, five to *Fusarium oxysporum*, four to *Aspergillus oryzae*, and one to *Penicillium rubens*. The more even distribution among fungal genera/species of clone sequences in Chen's over Wei's clone library suggested that our primers were able to detect more diverse fungal *nirK* from the environment. This implication was further confirmed by the rarefaction curves (Supplementary Fig. S2).

Our results indicate a great challenge for designing a primer set that can amplify fungi with great coverage and specificity. The results also suggest that conserved oligonucleotide regions of fungal *nirK*, the suitable candidates for primers based on melting temperatures, 3'-complementaries, and secondary structures were highly similar to bacterial *nirK*. Following this suggestion, we further conducted theoretical analyses to estimate the possibility of amplifying both fungal and bacterial *nirK* by our primers and other recently-published primers for fungal *nirK* and bacterial *nirK* in class II (Long et al., 2015; Maeda et al., 2015; Wei et al., 2015a; Wei et al., 2015b). A total of 24 NirK and corresponding *nirK* in the class II were used for such analyses, including 12 fungi, 10 bacteria, a protozoan and a eukaryotic alga (Supplementary Fig. S3a). As expected from other findings (Kim et al., 2009; Shoun et al., 2012), fungal NirK formed a monophyletic cluster and were well separated from bacterial and other eukaryotic NirK (Supplementary Fig. S3a). However, oligopeptides, the regions for all the forward and reverse primers in the analyses were of high similarity between fungi and other organisms containing class II NirK (Fig. 5). Here, we showed that oligopeptide regions for priming bacterial NirK in class II designed by Wei et al. (2015a) were conserved for bacteria as well as fungi, suggesting

this primer set having great possibility to amplify fungal *nirK*. Given that most amino acids correspond to more than one codon, primer-template oligonucleotide mismatches were also examined following the alignment of full-length *nirK* (Supplementary Fig. S2b). This mismatch diagram further demonstrated that all primers targeting fungal *nirK* in this and other studies (Long et al., 2015; Maeda et al., 2015; Wei et al., 2015b) had some chances to amplify bacterial *nirK* in class II, and vice versa for bacterial primers (Wei et al., 2015a). For example, primers for bacterial cluster II (Wei et al., 2015a) appeared to be able to amplify fungi because several species of fungi, such as *Myceliophthora*, *Chaetomium*, and *Ajellomyces* showed mismatch rates as low as bacterial species. Surprisingly, fungal primers developed by Maeda et al. (2015) that appeared to have greatest specificity among all the examined primers amplified no single fungal *nirK*, but all bacterial *nirK* from the soil DNA (Table 3, Fig. 4). Perhaps, length of target sequence and preferential amplification of bacterial *nirK* limited the specificity of primers developed by Maeda et al. (2015). Nonetheless, primers showing similar mismatch rates between fungi and bacteria amplified more bacteria than fungi from the environment, with ~ 89% of clones being closely related to bacteria for FnirK_F2/R2 in this study and ~ 92% for fungal primers designed by Long et al. (2015). This is perhaps due to much higher abundance of bacteria than fungi in soil. Based on NirK alignment (Fig. 5), amino acid fragments between forward and reverse primers, were generally shorter by a few amino acids for bacteria than for fungi and the deletion of amino acids were mainly located in the linker loop and tower loop regions. This explained why sequences of bacterial clones were often shorter by up to 30 bp than fungal clones. Nonetheless, such theoretical analyses did support that FnirK_F3/F2 was superior to FnirK_F2/R2 in selectively amplifying fungal *nirK*.

4. Conclusions

Four new primer sets targeting *nirK* were developed to detect denitrifying fungi in environmental samples. Experimental evidence from microbial cultures and a soil sample supported that all the four primer sets were able to detect denitrifying fungi in the environment, but efficiency and specificity differed. The primer set FnirK_F3/R2, which generated *nirK* fragments of ~ 233 bp, was proven to be more efficient since it amplified ~ 80% of tested fungal genera and species, including *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma*. Further, this primer pair, together with FnirK_F2/R2 could amplify more diverse *nirK* genes from the environment than the other two primer sets, as verified by clone libraries-based rarefaction analysis. Phylogenetic trees confirmed that a large fraction of clone sequences were closely related to *nirK* of the genera *Chaetomium* and *Metarhizium* that failed to be isolated and cultured previously from the same environment, further indicting that our new primers were able to detect diverse denitrifying fungi.

Primer sets, particularly FnirK_F2/R2 could also amplify genes homologous to bacterial *nirK* in class II, suggesting primer specificity was not strong. Sequence comparison of clone libraries made by the primer pair FnirK_F3/R2 and recently-developed other primers also showed that bacterial *nirK* was amplified more or less for all tested primer sets. Theoretically, the four new primers designed in this study as well as recently-published primers targeting fungal *nirK* or bacterial *nirK* in class II (Long et al., 2015; Maeda et al., 2015; Wei et al., 2015a, b) could amplify non-target organisms, due to high similarity of conserved regions of NirK and/or *nirK* between denitrifying fungi and bacteria. Additionally, higher abundance of bacteria than fungi in soil might result in a high background level of unwanted bacterial *nirK*. Nonetheless, denitrifying bacteria amplified by our primers from the environment were generally

related to those detected recently (Wei et al., 2015a). Further construction of clone libraries from soil DNA using the primer pair in this study and other recently-published primers showed that FnirK_F3/R2 was more specifically targeted on fungal *nirK* and had broader taxonomical coverage of fungal *nirK* in the environment than others. As such, our primers, particularly FnirK_F3/R2 were effective and specific to class II *nirK* gene-containing microorganisms that were rarely detected from the environment previously.

Acknowledgements

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References

- Arnheim N, Erlich H (1992) Polymerase chain reaction strategy. *Annu Rev Biochem* 61: 131-156
- Boulanger MJ, Murphy ME (2002) Crystal structure of the soluble domain of the major anaerobically induced outer membrane protein (*AniA*) from pathogenic *Neisseria*: a new class of copper-containing nitrite reductases. *J Mol Biol* 315: 1111-1127
- Cha RS, Thilly WG (1993) Specificity, efficiency, and fidelity of PCR *Genome Research* 3: S18-S29

- 470 Chen H, Mothapo NV, Shi W (2014) The significant contribution of fungi to soil N₂O
 471 production across diverse ecosystems. *Appl Soil Ecol* 73: 70-77
- 472 Chen H, Mothapo NV, Shi W (2015a) Fungal and bacterial N₂O production regulated by soil
 473 amendments of simple and complex substrates. *Soil Biol Biochem* 84: 116-126
- 474 Chen H, Mothapo NV, Shi W (2015b) Soil moisture and pH control relative contributions of
 475 fungi and bacteria to N₂O production. *Microb Ecol* 69: 180-191
- 476 Crenshaw C, Lauber C, Sinsabaugh R, Staveland L (2008) Fungal control of nitrous oxide
 477 production in semiarid grassland. *Biogeochemistry* 87: 17-27
- 478 Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity
 479 and speed of chimera detection. *Bioinformatics* 27: 2194-2200
- 480 Finn RD, Clements J, Eddy SR (2011) HMMER web server: interactive sequence similarity
 481 searching. *Nucleic Acids Res* gkr367 doi: 10.1093/nar/gkr367
- 482 Fierer N, Jackson JA, Vilgalys R, Jackson RB (2005) Assessment of soil microbial community
 483 structure by use of taxon-specific quantitative PCR assays. *Applied and Environmental*
 484 *Microbiology* 71: 4117-4120
- 485 Geiser DM, del Mar Jiménez-Gasco M, Kang S, Makalowska I, Veeraraghavan N, Ward T J et al
 486 (2004) "FUSARIUM-ID v 10: A DNA sequence database for identifying *Fusarium*" in
 487 *Molecular Diversity and PCR-detection of Toxigenic Fusarium Species and Ochratoxigenic*
 488 *Fungi Springer* 473-479
- 489 Gulvik CA, Effler TC, Wilhelm SW, Buchan A (2012) De-MetaST-BLAST: a tool for the
 490 validation of degenerate primer sets and data mining of publicly available metagenomes.
 491 *PLoS ONE* 7: e50362

- 492 Herold MB, Baggs EM, Daniell TJ (2012) Fungal and bacterial denitrification are differently
493 affected by long-term pH amendment and cultivation of arable soil. *Soil Biol Biochem* 54: 25-
494 35
- 495 Henry S, Baudoin E, López-Gutiérrez JC, Martin-Laurent F, Brauman A, Philippot L (2004)
496 Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. *Journal of*
497 *Microbiological Methods* 59: 327-335
- 498 Jirout J (2015) Nitrous oxide productivity of soil fungi along a gradient of cattle impact. *Fungal*
499 *Ecol* 17: 155-163
- 500 Jirout J, Šimek M, Elhottová D (2013) Fungal contribution to nitrous oxide emissions from cattle
501 impacted soils. *Chemosphere* 90: 565-572
- 502 Jones CM, Stres B, Rosenquist M, Hallin S (2008) Phylogenetic analysis of nitrite nitric oxide
503 and nitrous oxide respiratory enzymes reveal a complex evolutionary history for
504 denitrification. *Mol Biol Evol* 25: 1955-1966
- 505 Kim SW, Fushinobu S, Zhou S, Wakagi T, Shoun H (2009) Eukaryotic *nirK* genes encoding
506 copper-containing nitrite reductase: originating from the protomitochondrion? *Appl Environ*
507 *Microb* 75: 2652-2658
- 508 Kobayashi M, Shoun H (1995) The copper-containing dissimilatory nitrite reductase involved in
509 the denitrifying system of the fungus *Fusarium oxysporum*. *J Biol Chem* 270: 4146-4151
- 510 Kwok S, Kellogg D, McKinney N, Spasic D, Goda L, Levenson C et al (1990) Effects of primer-
511 template mismatches on the polymerase chain reaction: human immunodeficiency virus type
512 1 model studies. *Nucleic Acids Res* 18: 999-1005

- 513 Laughlin RJ, Rütting T, Müller C, Watson C J, Stevens R J (2009) Effect of acetate on soil
 514 respiration N₂O emissions and gross N transformations related to fungi and bacteria in a
 515 grassland soil. *Appl Soil Ecol* 42: 25-30
- 516 Laughlin RJ, Stevens RJ (2002) Evidence for fungal dominance of denitrification and
 517 codenitrification in a grassland soil. *Soil Sci Soc Am J* 66: 1540-1548
- 518 Long A, Song B, Fridley K, Silva A (2015) Detection and diversity of copper containing nitrite
 519 reductase genes (*nirK*) in prokaryotic and fungal communities of agricultural soils. *FEMS*
 520 *Microbiol Ecol* 91: 1-9
- 521 Maeda K, Spor A, Edel-Hermann V, Heraud C, Breuil M C, Bizouard F et al (2015) N₂O
 522 production a widespread trait in fungi. *Scientific Reports* 5: 9697
- 523 McWilliam H, Li W, Uludag M, Squizzato S, Park Y M, Buso N et al (2013) Analysis tool web
 524 services from the EMBL-EBI. *Nucleic Acids Res* 41: W597-W600
- 525 Morales SE, Cosart T, Holben WE (2010) Bacterial gene abundances as indicators of greenhouse
 526 gas emission in soils. *ISME J* 4: 799-808
- 527 Mothapo NV, Chen H, Cubeta MA, Shi W (2013) Nitrous oxide producing activity of diverse
 528 fungi from distinct agroecosystems. *Soil Biol Biochem* 66: 94-101
- 529 Mothapo NV, Chen H, Cubeta MA, Grossman J M, Fuller F, Shi W (2015) Phylogenetic
 530 taxonomic and functional diversity of fungal denitrifiers and associated N₂O production
 531 efficacy. *Soil Biol Biochem* 83: 160-175
- 532 Nakanishi Y, Zhou S, Kim S-W, Fushinobu S, Maruyama J, Kitamoto K et al (2010) A
 533 eukaryotic copper-containing nitrite reductase derived from a NirK homolog gene of
 534 *Aspergillus oryzae*. *Biosci Biotech Biochem* 74: 984-991

- 535 O'Donnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a
 536 monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol Phylogenet Evol* 7:
 537 103-116
- 538 O'Donnell, K Rooney AP, Proctor RH, Brown DW, McCormick SP, Ward TJ et al (2013)
 539 Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade
 540 comprising all agriculturally and medically important fusaria. *Fungal Genet Biol* 52: 20-31
- 541 Philippot L, Andert J, Jones CM, Bru D, Hallin S (2011) Importance of denitrifiers lacking the
 542 genes encoding the nitrous oxide reductase for N₂O emissions from soil. *Glob Change Biol*
 543 17: 1497-1504
- 544 Philippot L, Čuhel J, Saby N, Cheneby D, Chronakova A, Bru D et al (2009) Mapping
 545 field-scale spatial patterns of size and activity of the denitrifier community. *Environ Microb*
 546 11: 1518-1526
- 547 Rütting T, Huygens D, Boeckx P, Staelens J, Klemmedtsson L (2013) Increased fungal dominance
 548 in N₂O emission hotspots along a natural pH gradient in organic forest soil *Biol Fertil Soils*
 549 49: 715-721
- 550 Schloss PD, Westcott SL, Ryabin T, Hall J R, Hartmann M, Hollister EB et al (2009)
 551 Introducing mothur: open-source platform-independent community-supported software for
 552 describing and comparing microbial communities. *Appl Environ Microb* 75: 7537-7541
- 553 Seo DC, DeLaune RD (2010) Fungal and bacterial mediated denitrification in wetlands:
 554 influence of sediment redox condition. *Water Res* 44: 2441-2450
- 555 Shade A, Peter H, Allison SD, Baho DL, Berga M, Burgmann H et al (2012) Fundamentals of
 556 microbial community resistance and resilience. *Front Microb* 3

- 557 Shoun H, Fushinobu S, Jiang L, Kim SW, Wakagi T (2012) Fungal denitrification and nitric
558 oxide reductase cytochrome P450nor. *Philos T Roy Soc B* 367: 1186-1194
- 559 Smith S, Vigilant L, Morin PA (2002) The effects of sequence length and oligonucleotide
560 mismatches on 5' exonuclease assay efficiency. *Nucleic Acids Res* 30: e111-e111
- 561 Thompson JR, Marcelino LA, Polz MF (2002) Heteroduplexes in mixed-template amplifications:
562 formation consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Res* 30:
563 2083-2088
- 564 Wei W, Isobe K, Nishizawa T, Zhu L, Shiratori Y, Ohte N et al (2015a) Higher diversity and
565 abundance of denitrifying microorganisms in environments than considered previously. *ISME*
566 *J* 2015a doi:101038/ismej20159
- 567 Wei W, Isobe K, Shiratori Y et al (2015b) Development of PCR primers targeting fungal nirK to
568 study fungal denitrification in the environment. *Soil Biol Biochem* 81:282-286
- 569 Wei W, Isobe K, Shiratori Y, Nishizawa T, Ohte N, Ise Y et al (2013) N₂O emission from
570 cropland field soil through fungal denitrification after surface applications of organic
571 fertilizer. *Soil Biol Biochem* 69: 157-167
- 572 Yanai Y, Toyota K, Morishita T, Takakai F, Hatano R, Limin SH et al (2007) Fungal N₂O
573 production in an arable peat soil in Central Kalimantan Indonesia. *Soil Sci Plant Nutr* 53: 806-
574 811
- 575 Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012) Primer-BLAST: a
576 tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:
577 134
- 578 Zumft WG (1997) Cell biology and molecular basis of denitrification. *Microb Mol Biol Rev* 61:
579 533-616

Figure Captions

Fig. 1. Positions of degenerate primers on the nitrite reductase gene (*nirK*) of *Aspergillus oryzae* (GU144509.1). Of total 1592 base pairs (bp), the first 168 bp will be transcribed into the 5' UTR (untranslated region) of mRNA, and last 65 bp will be transcribed into the 3' UTR of mRNA. The forward and reverse primers of this study and those developed by Maeda et al. (2015), Wei et al. (2015b), and Long et al. (2015) are within the range from 169 to 1527 of CDS (coding DNA sequence).

Fig. 2. Maximum likelihood phylogenetic tree of *nirK* of fungal isolates as well as clones from soil DNA amplified with the primer pairs FnirK_F2/R2 and FnirK_F3/R2. Fungal and bacterial species in bold represent reference strains from public databases (see Supplementary Table S1 for identification/accession number). Fungal species in italics represent fungal cultures isolated from our previous work (Mothapo et al., 2013). When an operational taxonomic unit (OTU) has more than one clone, the number is given in parentheses. Bootstrap values (> 65%) from 1000 replicates are shown adjacent to the nodes. Branch lengths represent sequence differences, indicated by a scale bar.

Fig. 3. Maximum likelihood phylogenetic tree of *nirK* of clones from soil DNA amplified with the primer pairs FnirK_F1/R1 and FnirK_F1/R2. Fungal and bacterial species in bold represent reference strains from public databases (see Supplementary Table S1 for identification/accession number). When an operational taxonomic unit (OTU) has more than one clone, the number is given in parentheses. Bootstrap values (> 65%) from 1000 replicates are shown adjacent to the nodes. Branch lengths represent sequence differences, indicated by a scale bar.

Fig. 4. Maximum likelihood phylogenetic tree of *nirK* of clones from soil DNA amplified with the primer pair FnirK_F3/R2 and the primers developed by Wei et al. (2015b) and Maeda et al.

(2015), i.e., Chen, Wei, and Maeda-OTU, respectively. Fungal and bacterial species in bold represent reference strains from public databases (see Supplementary Table S1 for identification/accession number). When an operational taxonomic unit (OTU) has more than one clone, the number is given in parentheses. For a compressed subtree having more than one OTU, total number of clones is given in parentheses. Bootstrap values (> 65%) from 1000 replicates are shown adjacent to the nodes. Branch lengths represent sequence differences, indicated by a scale bar.

Fig. 5. Amino acid sequence alignment of NirK of eukaryotic and prokaryotic microorganisms and amino acid oligomers corresponding to forward and reverse primer pairs used in this study and others; solid and dash arrows indicating the ligand positions of type I and type II copper domains with *Aspergillus oryzae* as the template, and LL and TL meaning the linker loop and tower loop positions with *Aspergillus oryzae* as the template.

Fig. 1

619 **Fig. 2**

622 **Fig. 3**

623
624
625

626 Fig. 4

Actinosynnema mirum (C6WP79_ACTMD)
Cellvibrio gilvus (F8A0Z0_CELGA)
Oligotropha carboxidovorans (B6JIX6_OLICO)
Phenylobacterium zucineum (B4RF40_PHEZH)
Gemmatimonadetes bacterium (WOR9Z9_9BACT)
Sphaerobacter therophilus (D1C654_SPHTD)
Ocenimonas sp. (H2FXF5_OCESG)
Kangiella korensis (C7R6X8_KANKD)
Neisseria meningitidis (Q9JYE1)
Moraxella catarrhalis (D5V9Q4_MORCR)
Galdieria sulphuraria (M2W536_GALSU)
Naegleria gruberi (D2VM78_NAEGR)
Aspergillus oryzae (ACZ26344.1)
Penicillium rubens (B6HS74_PENCW)
Fusarium oxysporum (ABU88100.1)
Fusarium lichenicola (ADB82651.1)
Coccidioides posadasii (E9DHQ8_COCPS)
Ajellomyces capsulatus (F0U738_AJEC8)
Paracoccidioides brasiliensis (C1FZP8_PARB0)
Microsporium gypseum (E4V080_ARTGP)
Trichophyton rubrum (F2SUL0_TRIRC)
Metarhizium acridum (E9E2M2_METAQ)
Chaetomium globosum (Q2GPV9_CHAGB)
Myceliophthora thermophila (G209K5_THIHA)

VRQVMWTYNGT**YAGPV**LRLGKVGDVFEIELHNDGS--VDHGVD**F**HAGALAP
 VTQRLWTFGGQ**APG**PVLHGRVGDTFVTVLVNDGS--IGHSID**F**HAGALAP
 TTFGYWTFNGK**VP**GPMLRVRVGD**T**VEVHLKNAENS**GM**VHVS**D**FHAATGPG
 ATFKYWTFNSKL**PG**PMVRVRVGD**T**VEVTLHNPADS**SM**NMHNV**D**FHAVTGPG
 VDYP**I**WTFGGSV**PG**KFIRVREGD**I**VELHLKVD**PK**AT**MP**HNID**L**HAVTGPG
 VAYKFWT**F**NGTV**PG**PMIRVREGD**T**VELTLKNSLD**ST**APHNID**L**HAVTGPG
 VEYMFWT**F**GGSV**PG**QFLRVREGD**E**IEFHL**SN**HPDS**K**MPHNID**L**HAVTGPG
 VEYVYWS**F**GGTV**PG**SFIRVKEG**D**IEFHL**SN**HPSS**K**MPHNID**L**HAVTGPG
 VEYRWTFDGD**V**PGRMIRVREGD**T**VEVEFS**NN**PS**ST**VPHNV**D**FHAATGQG
 VEYQFWT**F**GGQ**V**PGQ**M**IRVREGD**T**IEVQFS**SN**HPDS**K**MPHN**V**D**F**HAA**T**GPG
 YTYEYWT**F**QGS**V**PG**PM**IRARVGD**I**MQV**KL**VNRDQ**S****GM**MHN**L**D**F**HAV**L**GPG
 YKYPYWT**F**NGT**V**PG**PF**IRARVGD**V**MQV**NY**LNLDE**T****GM**AHN**I**D**F**HC**V**TGPG
 YKYEKWTFNNS**V**PG**PF**IRARVGD**I**VNL**KIT**NHDES**GM**PHN**ID**CHA**FL**GPG
 HKYEQWN**F**NG**I**VPG**PF**IRARVGD**V**VELS**LT**NRK**AG**PNHN**ID**CHA**FI**GPG
 YKYEQWTFNGT**V**PG**PF**IRAKVGD**V**VELS**LT**NKD**PAG**PNHN**ID**CHA**FT**GPG
 YKYEQWTFNGT**V**PG**PF**IRAKVGD**V**VELS**LT**NKDT**AG**PNHN**ID**CHA**FT**GPG
 YKYDQWTFNDS**V**PG**PF**IRARAGD**V**IEL**TT**NKD**PS**GNPHN**ID**CHA**FI**GPG
 YKYEQWTFNNQ**V**PA**PF**IRARVGD**V**IEL**SL**TNKD**VT**GNPHN**ID**CHA**FT**GPG
 YKYEQWTFNNK**V**PG**PF**IRARVGD**V**IEL**TL**TNRD**VS**GNPHN**ID**SHA**FT**GPG
 YKYEQWTFNDT**V**PG**PF**IRARVGD**I**VEL**TL**TNRD**VT**GNPHN**ID**CHA**FT**GPG
 YKYEQWTFNGN**V**PG**PF**IRARVGD**V**VEL**TL**TNHD**VT**GNPHN**ID**CHA**FT**GPG
 YKYEAWTFNGT**T**PG**PF**IRARVGD**V**VELS**LT**NRKD**VT**GNPHN**ID**SHA**FAG**GPG
 YKYEQWTFNDT**V**PG**PF**IRARVGD**V**VDME**FT**NKD**IT**GNPHN**ID**SHA**FT**GPG
 YQYEQWTFNGT**V**PA**PF**IRARVGD**V**VD**LT**LTNRD**AT**GNPHN**ID**CHA**FT**GPG

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Phenylobacterium zucineum (B4RF40_PHEZH)
Gemmatimonadetes bacterium (W0R9Z9_9BACT)
Sphaerobacter therophilus (D1C654_SPHTD)
Ocenimonas sp. (H2FXF5_OCESG)
Kangiella korensis (C7R6X8_KANKD)
Neisseria meningitidis (Q9JYE1)
Moraxella catarrhalis (D5V9Q4_MORCR)
Galdieria sulphuraria (M2W536_GALSU)
Naegleria gruberi (D2VM78_NAERG)
Aspergillus oryzae (ACZ26344.1)
Penicillium rubens (B6HS74_PENCW)
Fusarium oxysporum (ABU88100.1)
Fusarium lichenicola (ADB82651.1)
Coccidioides posadasii (E9DHQ8_COCPS)
Ajellomyces capsulatus (F0U738_AJEC8)
Paracoccidioides brasiliensis (C1FZP8_PARBD)
Microsporium gypseum (E4V080_ARTGP)
Trichophyton rubrum (F2SUL0_TRIRC)
Metarhizium acridum (E9E2M2_METAQ)
Chaetomium globosum (Q2GPMV9_CHAGB)
Myceliophthora thermophila (G2Q9K5_THIHA)

251/2 260 265

 D-GPMRPIEPGESLTYRFTATKAGIWMYHCSTMPMMLH**I**ANGMYGAVIVD
 D-DVMRTIAPQGELTYRFTATRSGVWMYHCSTMPMSV**H**IANGMLGAVVIE
 GGAASTQANPGETKTVKFKALVPGLYVYHCATPMVA**H**IANGMYGLLIVE
 GGAAATLANPGETKTF SFKALQPGLYIYHCAAPMVA**Q**H**I**ANGMYGAILVE
 GGAKASLTIPGHESVFTFTAMNAGLFVYHCATSPVPM**H**MANGYMGILVE
 GGAAATTVSPGKQATIQFLAMHPGVVYVYHCATPPVAM**H**IANGMYGLIVVE
 GGAAASFTAPGETSTVFENKALNPGLYVYHCATPVG**M**H**I**ANGMYGLLIVE
 GGATSSFTAPGHTSTFAFKALNPGLYVYHCATAPV**G**M**H**IANGMYGLLIVE
 GGAAATFTAPGRTSTFSFKALQPGLYIYHCAVAPV**G**M**H**IANGMYGLLIVE
 GGAEASFTAPGHTSTFSFKALQPGLYVYHCAVAPV**G**M**H**IANGMYGLLIVE
 GGAPLLNCGDGETKVAQFRLLTYPGLIYHCAVDPVSV**H**ISNGMFGLLIVE
 GGAEMLLAEKDEEKTVGFQFKLLTSGLFYIYHCAAGVPV**S**HISNGMYGLMLVE
 GGSALTTVNEGETKTARFRLQNPGLIYIYHCAVGPV**G**V**H**IANGMYGLLYVQ
 GGSALTTAEEGQTKTARFRLHYPLGLIYHCAAAAPVPV**H**IANGMYGLMYVE
 GGAAVTTAAEGETKVGRFKLLYPGLIYVYHCAAAAPVPV**H**IANGMYGLMYVQ
 GGAAVTTAEENESKTARFKLLYPGLIYVYHCAAAAPVPV**H**IANGMYGLMYVQ
 GGAPLTTVGFNESKTARFQLLYPGLIYVYHCAAAAPVPV**H**IANGMYGMIYVQ
 GGASLTTAEENQNKTGRFKLLYPGLFIYHCAAAAPVPV**H**IANGMYGLIYVQ
 GGAALTTAEENQTKTGRFKLMYPGLFFYHCAAAAPVPV**H**IANGMYGLIYVQ
 GGAALTTAEENESKTGRFKLLHPLGLIYVYHCAAAAPVPV**H**IANGMYGLIYVQ
 GGAALTTAEESKSTGLFKLLHPLGLIYVYHCAAAAPVPV**H**IANGMYGLIYVQ
 GGSALTTAEENETKTGRFKLLCPGLIYVYHCAAAAPVPV**H**MANGYGLMYVQ
 GGAALTTTEENETKSARFKLLTPGLIYHCAAAAPVPV**H**IANGMYGLIYVQ
 GGSALTTAEETGKAAARFLLRPGLIYHCAAAAPVPV**H**IANGMYGLMYVQ

$$\begin{array}{r} \text{FnirK_F2} \\ \text{Wei_B_F} \\ \text{Long_F} \\ \text{Wei F F} \end{array}$$

1

		LL	TL
Actinosynnema mirum (C6WP79_ACTMD)	PPD-----LPVDRREYVLVQSELYLGGDQ-----AKMRA		
Cellvibrio gilvus (F8A0Z0_CELGA)	PDG-----LAQADRQYVVVQHELYLGEQGG-----VDAAKVAG		
Oligotropha carboxidovorans (B6JIX6_OLICO)	PEG-----GLPPVDRREYVVMQGEIYTAQPFQ-----HGS�DFDVEKLLN		
Phenyllobacterium zucineum (B4RF40_PHEZH)	PEE-----GLPKVDRREYVVMQGEIYTEEDHDA-----QGELTESIGKLLD		
Gemmatimonadetes bacterium (W0R9Z9_9BACT)	PKG-----GLPKVDRREYVVMQSEFYTKGKFGE-----HGLQTLDMDKGVD		
Sphaerobacter therophilus (D1C654_SPHTD)	PEG-----GLPQVDRREYVVMQGDFYLSGERGE-----KGLHGFDMEQMLA		
Ocenimonas sp. (H2FXF5_OCESG)	PKE-----GLPPVDRREYVVMQGDFYTKGAYGE-----OGLQSFDMDKAIR		
Kangiella koreensis (C7R6X8_KANKD)	PKE-----GLPKVDRREYVVMQGDFYTKGKYGD-----PGLQPFDMQKAID		
Neisseria meningitidis (Q9JYE1)	PKE-----GLPKVDRREYVVMQGDFYTKGKKA-----OGLQPFDMDKAVA		
Moraxella catarrhalis (D5V9Q4_MORCR)	PKE-----GLPKVDRREYVVMQGDFYTKGKYGE-----OGLQPFDMDKAIR		
Galdieria sulphuraria (M2W536_GALSU)	PDK-----GLQAVDRREYVVMQAEVYTEDNPITVDS---RCLPQSIDKLM		
Naegleria gruberi (D2VM78_NAEGR)	PEE-----GLPKVDRREYVVMQSEFYCEPSDD-----PKLMEHSYANGLD		
Aspergillus oryzae (ACZ26344.1)	PEQ-----DLPPVDRREYVVMQSEFYHEPPEPDNDGQMSSTVEFSWPHALR		
Penicillium rubens (B6HS74_PENCW)	PAT-----GDLPPADRREYVVMQSEFYHEPPEIEDNGKPSIIVEFSYPNALR		
Fusarium oxysporum (ABU88100.1)	PEG-----NDLPPVDRREYVVMQSEFYHEPPEVDDGRRSEIVEFSYPNGLR		
Fusarium lichenicola (ADB82651.1)	PEG-----GDLPPVDRREYVVMQSEFYHEPPEVDDGRRSEIVEFSYPNGLR		
Coccidioides posadasii (E9DHQ8_COCPS)	QAE-----GDLPPVDRREYVVMQSEFYHEPPEVEDDGRPSIIVEFSYPNALR		
Ajellomyces capsulatus (F0U738_AJEC8)	PEE-----DLPKVDREYVVMQSEFYHEPPEIEENGKRSSLVEFSYPNALR		
Paracoccidioides brasiliensis (C1FZP8_PARBD)	PEK-----DLPKVDREYVVMQSEFYHEPPEIEENGKRSSLVEFSYPNALR		
Microsporium gypseum (E4V080_ARTGP)	PED-----GDLPPVDRREYVVMQSEFYHEPPEVLENGKRSSLVEFSYPNALD		
Trichophyton rubrum (F2SUL0_TRIRC)	PED-----GDLPPVDRREYVVMQSEFYHEPPEVLENGKRSSLVEFSYPNALE		
Metarhizium acridum (E9E2M2_METAQ)	PEE-----QDLPPVDRREYVVMQSEIYHEPPEGLDDGRLNVVEFSYPNGLR		
Chaetomium globosum (Q2GPV9_CHAGB)	PAE-----GDLPPVDRREYVVMQSEFYHEPPEIMDDGRPSKTVEFSYPNGLA		
Myceliophthora thermophila (G2Q9K5_THIHA)	PAVPGGGEGLPADRREYVVMQSEFYHEPPEVGGDGRPSRTVEFSYPNGLA		

FnirK_F3/R1

2
3
4
5

Actinosynnema mirum (C6WP79_ACTMD)	ENPDAMAFNGYAAQ-YAHRPLTAKAGERVRFWLLDVGPNRSGAFHVVGQAQ
Cellvibrio gilvus (F8A0Z0_CELGA)	QVPDLVLVNGYANQ-YRDRPLTAAVGERVRLWLDAGPNRPSAFHVVGGO
Oligotropha carboxidovorans (B6JIX6_OLICO)	ERPEYFVFNQSVGALSGLHPLQAKVGETVRFYFVGVPNFTSSFHLIGE
Phenyllobacterium zucineum (B4RF40_PHEZH)	EDAEFYVFNQAFKALTGEKQLEAKVGETVRIYFVGVPNFTSSFHVIGEI
Gemmatimonadetes bacterium (W0R9Z9_9BACT)	ERPTYVVFNGAVGALTGDGALQANVGQVRIFFGDAGPNLTSSFHVIGEV
Sphaerobacter therophilus (D1C654_SPHTD)	ENPDYVVFNGSVGALTGDNALKAKTGETVRIFFVGVPNLTSSFHVIGEV
Ocenimonas sp. (H2FXF5_OCESG)	EQADYVVFNGAVGALNGDKALTANVGETVRLYVNGGPNLTSSFHVIGEI
Kangiella koreensis (C7R6X8_KANKD)	EHADYVVFNGAVGSLTGDKSLKANVGETVRLYVNGGPNLTSSFHVIGEI
Neisseria meningitidis (Q9JYE1)	EQPEYVVFNGHVGAIAGDNALKAKAGETVRMYVNGGPNLTSSFHVIGEI
Moraxella catarrhalis (D5V9Q4_MORCR)	EDAEYVVFNGSVGALTGENALKAKVGETVRLFVNGGPNLTSSFHVIGEI
Galdieria sulphuraria (M2W536_GALSU)	ERNPYVVFNGRQSGHLENNPFKAKVGDVRFYVGNAGPALVSSFHIIGTI
Naegleria gruberi (D2VM78_NAEGR)	EKPTYVVFNGREGSLI-DTPLLANTGERIRIFYGNAGPNLTSSFHVIGAI
Aspergillus oryzae (ACZ26344.1)	EADVVVFNGSEAAIT-EKPLKATLDDTVRIFFNGGPNLTSSFHVIGTC
Penicillium rubens (B6HS74_PENCW)	EEPSVVVFNGHEAALTRDKPLKASVGKVRIFFGNAGPNLTSSFHVIGSA
Fusarium oxysporum (ABU88100.1)	EEPQVVFNGSESALTRDHLKAVGDDVRIFFGNAGPNLTSSFHIIGTH
Fusarium lichenicola (ADB82651.1)	EEPQVVFNGSESALTRDPLKANVGDNVRIFFGNAGPNLTSSFHIIGTH
Coccidioides posadasii (E9DHQ8_COCPS)	EEPNNVFNGSESALTRDKPLTANINETVRLFFGNAGPNLTSSFHVIGSN
Ajellomyces capsulatus (F0U738_AJEC8)	EDPDVVVFNGSETALTMHPLKATAGETIRVFFGNAGPNLTSSPHIIGST
Paracoccidioides brasiliensis (C1FZP8_PARBD)	EDPDVIVFNGSETALTDNPLKAKTGETIRVFFGNAGPNLTSSPHIIGST
Microsporium gypseum (E4V080_ARTGP)	ENPNLVVFNGSESALTRDQPLKAKTGESVRIFFGNAGPNLTSSFHIIGTT
Trichophyton rubrum (F2SUL0_TRIRC)	ENPSLVVFNGSESALTRDQPLKAKSGETVRIFFGNAGPNLTSSFHVIGTT
Metarhizium acridum (E9E2M2_METAQ)	EEPNVVFNGRESALTRDAPLKARVGETVRIFFGNAGPNLTSSFHVIGSH
Chaetomium globosum (Q2GPV9_CHAGB)	EEPNVVFNGSESALTRDKPLKAKTGETVRIFFGNAGPNLTSSFHVIGSN
Myceliophthora thermophila (G2Q9K5_THIHA)	EEPAVVVFNGRESAATRDALARTGENVRLFFGNAGPNLTSSLHVIGSC

Maeda-R FnirK_R2

6

7

Actinosynnema mirum (C6WP79_ACTMD)	FDRVYAEGAYRLQPD---DPGGSQVLPLAPAEAGGFVETVFPEAGHYPFVS
Cellvibrio gilvus (F8A0Z0_CELGA)	FDTVFREGDYTLRAGGSTGTGGAQVLALQPAEGGFVELTFPQAGTYPFVT
Oligotropha carboxidovorans (B6JIX6_OLICO)	FDKVYNLGGVLSLEPL-----HGIQTVTVPAGGAVITEAKLDVPNGYTLVD
Phenyllobacterium zucineum (B4RF40_PHEZH)	FDNVYNLGSLSLTPPV-----KDVQTVTVAPGGATVVDFKLEVPGNYVLVD
Gemmatimonadetes bacterium (W0R9Z9_9BACT)	FDNVYADGGTEV-TQ-----HGVQTTAVPAGGSVIVEFVGEKPGDLVLVD
Sphaerobacter therophilus (D1C654_SPHTD)	FDRVAPEGAAEW-V-----SHVQTTMVPAGGATIVEFQVEVPNGYTLVD
Ocenimonas sp. (H2FXF5_OCESG)	FDKVNVEGGTAI-N-----ENVQTTLPVAGGAAMAEFRLDVPNGFIMVD
Kangiella koreensis (C7R6X8_KANKD)	FDKVYVEGGNLV-N-----ENIQTTLPVAGGSAIVEFKVEVPNGNLILVD
Neisseria meningitidis (Q9JYE1)	FDKVYVEGGKLI-N-----ENVQSTIVPAGGSAIVEFKVDIPGSYTLVD
Moraxella catarrhalis (D5V9Q4_MORCR)	FDKVHFEKGKGE-N-----HNIQTTLPVAGGAAITEFKVDVPNGYVLVD
Galdieria sulphuraria (M2W536_GALSU)	FDKVWKGDSLNLNPQ-----LGLQTMIPSGSSAVVELDAVVPNGFTLVD
Naegleria gruberi (D2VM78_NAEGR)	FDKVYREGDLVSPPE-----RNVQVTQVPPGGATMIEFAIVPGTYSFVD
Aspergillus oryzae (ACZ26344.1)	FNKVYRSDVLSPPG-----QCQVTVSVPPGGSTIVDMKMVPNGTYTIVD
Penicillium rubens (B6HS74_PENCW)	FEKVYRSDVLSPPG-----QFVQTVSVPPGGSTIVDMKMVPNGTYTIVD
Fusarium oxysporum (ABU88100.1)	FKNVYRDGGVTSNPS-----KGIQTVSVPCGGSTIVDLKMAVPNGTYTIVD
Fusarium lichenicola (ADB82651.1)	FKNVYRDGGVINPFS-----QGIQTVSVSPGGSTIVDLKMAVPNGTYTIVD
Coccidioides posadasii (E9DHQ8_COCPS)	FKNVYREGDVISPPG-----QCIQTVSVPPGSATIVDMEMSVPGTYALVD
Ajellomyces capsulatus (F0U738_AJEC8)	FHNLYRDGDIILSPPA-----RNVQTTTVPVPPGGATIFDMKAVVPNGTYTIVD
Paracoccidioides brasiliensis (C1FZP8_PARBD)	FHKLYRDGDVLSPPA-----QNVQTTATVPVPPGAASIFDSKMVPNGTYTIVD
Microsporium gypseum (E4V080_ARTGP)	FCKLYRDGDVLSPPA-----NWXSTASVPPGGSTIVDLKMAVPNGTYTIVD
Trichophyton rubrum (F2SUL0_TRIRC)	FSKLYRDGDVLSPPA-----SWVPTTSVPPGGSTIVDLKLVVPNGTYTIVD
Metarhizium acridum (E9E2M2_METAQ)	FLNCYRDGDVLSPPA-----RFVGTTSVPPGGSTIVDMKMVPNGSYTIVD
Chaetomium globosum (Q2GPV9_CHAGB)	FHSVYRDGDVLSPPG-----RFVSTVTVPPGGATVVDMMKMVPNGTYTIVD
Myceliophthora thermophila (G2Q9K5_THIHA)	YRRAYRDGDVLPSPA-----RFVSTVSVPPGGATILDMKMVPNGTYTIVD

Long_R

8

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10

11

412

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Actinosynnema mirum (C6WP79_ACTMD)	HAMVD-AERGARGIVEVTG-----
Cellvibrio gilvus (F8A0Z0_CELGA)	HVMSD-AEKGAMGRVRVTP-----
Oligotropha carboxidovorans (B6JIX6_OLICO)	HALSR-MERGLVGILHVEGAA-NPDIYDGVIEPGH-----
Phenyllobacterium zucineum (B4RF40_PHEZH)	HALSR-VERGGAGILHVTGPE-NPEIFKAEANPNQDHG-----
Gemmatimonadetes bacterium (W0R9Z9_9BACT)	HAIFRAFNKGALGILKVTGPD-NPKVFASIKGLV-STGE-----
Sphaerobacter therophilus (D1C654_SPHTD)	HSLGR-LAKGAAGVLQVEGPD-NPEVYTVIEHGDQSGGH-----
Ocenimonas sp. (H2FXF5_OCESG)	HSIFRAFNKGALGMMQVSGPE-DHIVYSGKVADNVYLPEGSQVMPNGH
Kangiella koreensis (C7R6X8_KANKD)	HSIFRAFNKGALGMLAVSGKE-DKAIYSGKIDRIYRPEGSQVSDKKV
Neisseria meningitidis (Q9JYE1)	HSIFRAFNKGALGQLKVEGAE-NPEIMTQKLSDTAYAGNGAAPASAPAA
Moraxella catarrhalis (D5V9Q4_MORCR)	HAIFRAFNKGALGILKVEGEE-NHEIYSHKQTDVYLPAGAPQAIDTQEA
Galdieria sulphuraria (M2W536_GALSU)	HSITR-IEKGCVAFLQVVGETKDPYLYCSEEGSPSPCGCKIHP-----
Naegleria gruberi (D2VM78_NAEGR)	HAIFR-IEKGAVGFLKIAGAP-RPDIYHGMDRPRKRCPCGCKLHQ-----
Aspergillus oryzae (ACZ26344.1)	HAIFR-LEKGAGFLNVSGEP-RPMLYYSTLPPQPCGCKLHP-----
Penicillium rubens (B6HS74_PENCW)	HAIFR-LEKGGVGYLNVGTGEP-RPDIYHIQKPKLA-SGLYVSDRPFLLWFK
Fusarium oxysporum (ABU88100.1)	HSIFR-LDKGAVGFLNVSGPQ-NPGVYQSSQPPRCPVCGCKLHS-----
Fusarium lichenicola (ADB82651.1)	HAIFR-LDKGAVGFLNVSGPP-NPAVYQSPQPPRCPVCGCKLHA-----
Coccidioides posadasii (E9DHQ8_COCPS)	HAIFR-MDKGAVGYLNVSGEQ-RPDIYGSTQPPAPCVGCKLHP-----
Ajellomyces capsulatus (F0U738_AJEC8)	HAIFR-LDKGAVGFINVGTGNP-RPDVYQSNEHAEPVCGCKLHP-----
Paracoccidioides brasiliensis (C1FZP8_PARBD)	HAIFR-LDKGCVGFINVSGKP-RPDIYQSAEHPESVCGCKLHP-----
Microsporium gypseum (E4V080_ARTGP)	HAIFR-LDKGAVGYLNVSGKQ-RPDIYQSTLPPAPCVGCKLHP-----
Trichophyton rubrum (F2SUL0_TRIRC)	HAIFR-LDKGAVGYLNVSGKQ-RPDVYQSTLPPAPCVGCKLHP-----
Metarhizium acridum (E9E2M2_METAQ)	HAIFR-IDKGAVGFINVSGPQ-RPDIYVGSLLPQPCVCGCKLHA-----
Chaetomium globosum (Q2GPV9_CHAGB)	HAIFR-LDKGAVGFLNVSGKP-REDVFGSSQPPAPCVGCKLHA-----
Myceliophthora thermophila (G2Q9K5_THIHA)	HAIFR-LDKGAVGFLNVSGKP-RPDIYGSQEPAPCVGCKLHS-----

Wei_B_R

Wei_F_R

Primers in this study

Primers in Maeda et al. (2015)

Primers in Wei et al. (2015b)

Primers in Long et al. (2015)

Primers in Wei et al. (2015a)

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Table 1 Degenerate primers targeting Cu-containing nitrite reductase genes (*nirK*) of N₂O-producing fungi and the percentage of > 3-bp mismatches between primers and sequences in database (DB) I, II, III and IV[§]

Primer	Oligonucleotide (5' – 3')	% of > 3-bp mismatches [¶]			
		I	II	III	IV
FnirK_F1	GTCCCHGGMCCHTTCAT	0	0	38	81
FnirK_F2	CATYGCCAAYGGYATGTA	0	0	0	38
FnirK_F3	GCARAGCGAGTTYTACCAYG	0	0	0	88
FnirK_R1	CRTGGTARAACCTCGCTYTGC	0	0	0	88
FnirK_R2	TVCCGATDAYRTGGAAYGARC	0	0	17	63

I: no-single mismatches between primers and sequences

II: ~ 90% non-mismatches and 10% 1-bp mismatches between primers and sequences

III: of total ≤ 3 -bp mismatches between primers and sequences, majority are ≤ 2 -bp mismatches, in which almost all the mismatches are in the 5' half of primers and mismatches are rarely consecutive.

Primer pairs to be tested[¶]

FnirK_F1 and FnirK_R1, bp $\approx$ 331, $T_a = 58$ °C

FnirK_F1 and FnirK_R2, bp $\approx$ 544, $T_a = 56$ °C

FnirK_F2 and FnirK_R2, bp $\approx$ 314, $T_a = 55$ °C

FnirK_F3 and FnirK_R2, bp $\approx$ 233, $T_a = 56$ °C

5.8S and ITS1f, bp = 300, $T_a = 55$ °C, positive control

nirKfF and nirKfR, bp = 480, $T_a = 56$ °C, positive control

nirK876 and nirK1040, bp = 165, $T_a = 58$ °C, positive control

[§]DB I includes 3 fungal *nirK* CDS in NCBI-Nucleotide; DB II includes 34 *nirK* from fungal whole genome shotgun contigs that are homologs to fungal *nirK* in DB I by NCBI-Blast; DB III includes 24 *nirK* sequences from ENA with corresponding protein homologs to fungal NirK in DB I by HMMER; and DB IV includes non-fungal *nirK* of a protozoan, a eukaryotic alga, and 12 bacteria from ENA and were most homologous to fungal NirK by HMMER.

[¶] T_a represents annealing temperature.

28 **Table 2** Primer specificity tested against a number of fungal and bacterial denitrifier cultures[§]

Microbial cultures	FnirK [¶]				nirKf
	F1/R1	F1/R2	F2/R2	F3/ R2	F/R
Fungi					
<i>Actinomucor elegans</i> Con-1417	—	—	+	+	+
<i>Aspergillus fischerianus</i> Suc-5625	+	+	+	+	+
<i>A. oryzae</i> For-7014	+	+	+	+	+
<i>A. sydowii</i> Org-4511	—	—	+	+	+
<i>A. terreus</i> Org-4110	—	—	+	+	+
<i>A. versicolor</i> Rot-3308	I	I	—	—	I
<i>Fusarium incarnatum</i> Con-1501	—	—	+	+	—
<i>F. neocosmosporiellum</i> Con-0506	+	+	+	+	+
<i>F. oxysporum</i> Con-1802	+	+	+	+	+
<i>F. oxysporum</i> Rot-3203	+	+	+	+	+
<i>F. oxysporum</i> Org-4004	+	+	+	+	+
<i>F. oxysporum</i> For-6805	+	+	+	+	+
<i>Gibberella fujikuroi</i> Con-1929	+	+	+	+	+
<i>G. fujikuroi</i> Rot-3131	+	+	+	+	+
<i>G. fujikuroi</i> Org-3532	+	+	+	+	+
<i>G. fujikuroi</i> Org-3733	+	+	+	+	+
<i>Hypocrea intricata</i> Con-0816	I	I	—	—	—
<i>H. lixii</i> Rot-2620	I	I	—	—	I
<i>Ophiocordyceps heteropoda</i> Rot-2319	—	—	—	+	+
<i>Penicillium brasilianum</i> Suc-5826	—	—	+	+	+
<i>P. citrinum</i> Rot-2721	I	I	—	—	I
<i>P. pinophilum</i> Con-1718	—	—	+	+	—
<i>P. verruculosum</i> Org-4622	—	—	+	+	—
<i>Phoma macrostoma</i> Suc-5524	I	I	—	—	—
<i>Purpureocillium lilacinum</i> For-7627	—	—	—	+	+
<i>P. lilacinum</i> Rot-2930	—	—	—	+	+
<i>Trichoderma asperellum</i> Suc-6412	—	—	—	+	+
<i>T. asperellum</i> For-7535	+	+	+	+	+
<i>T. hamatum</i> Suc-5023	—	—	+	+	+
<i>T. piluliferum</i> For-6713	I	I	+	+	+
Bacteria					
<i>Actinoplanes missouriensis</i> ATCC 31681	—	—	—	—	—
<i>Alcaligenes faecalis</i> ATCC 8750	—	—	—	—	—
<i>Ochrobactrum anthropi</i> ATCC BAA-749	—	—	—	—	—
<i>Paracoccus pantotrophus</i> ATCC 35512	—	—	—	—	—
<i>Pseudoalteromonas haloplanktis</i> ATCC 14393	—	—	—	—	—
<i>Pseudomonas stutzeri</i> ATCC 53819	—	—	—	—	—

29 [§]Symbol “+” denotes the presence of a single band of desired size and also sequences of amplicons
30 between different primers are of high similarity (i.e., ≥ 97% identities) in aligned regions; “—” means the
31 absence of desired band; “I” means that amplifications are incongruent between products amplified by
32 different primer sets.

33 [¶]FnrK represents the primers designed in this study, and nirKf is the primer pair used by Wei et al.
34 (2015b).

Table 3 Comparisons of clone sequences amplified by the primers FnirK_F3/R2, denoted as Chen's primers, with clone sequences amplified by the primers developed by Wei et al. (2015b) and Maeda et al. (2015).

	No. of clones	OTU	% of OTU [§]		% of clones	
			Fungi	Bacteria	Fungi	Bacteria
Chen's primers	87	25	84.0	16.0	73.6	26.4
Wei's primers	102	21	76.1	23.9	92.2	7.8
Maeda's primers	65	40	0	100	0	100

[§]Percentage of OTUs belonged to fungal or bacterial *nirK*, and percentage of clones belonged to fungal or bacterial *nirK*.