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Short communication

Opening up the N_2O-producing fungal community in an agricultural soil with a cytochrome *p450nor*-based primer tool

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ABSTRACT

Fungi play an important role in soil N_2O emissions; yet little is known on the community ecology of N_2O-producing fungi in soil. We explored denitrifying fungi using a cytochrome *p450nor*-based primer tool. Clone library and sequencing analysis revealed that soil harbored diverse denitrifying fungi in Ascomycota and Basidiomycota. Ascomycotal fungi were widely distributed across orders, Eurotiales, Hypocreales, and Sordariales. Denitrifying fungi were also expanded to include *Cylindrocarpon*, *Neurospora*, *Thielavia*, and *Trichosporon* that were undetected in our previous culture-based work. These results indicate that the *p450nor*-based primer tool can provide a more comprehensive characterization of denitrifying fungal community in soil environment.

1. Introduction

Eukaryotic fungi play an important role in soil N_2O emissions (Chen et al., 2014; Crenshaw et al., 2008; Laughlin et al., 2009; Laughlin and Stevens, 2002; Yanai et al., 2007). Laboratory soil studies with antibiotic application showed that fungi made significant contributions to soil N_2O emissions, although denitrifying fungi in culture produced N_2O orders of magnitude lower than their counterparts, bacteria (Mothapo et al., 2015). Recently, process-level investigations have provided rudimentary insights into the regulatory roles of soil pH, Eh, moisture and organic matter quality on fungal denitrification (Chen et al., 2015b, 2015a; Herold et al., 2012; Seo and DeLaune, 2010). While both denitrifying fungi and bacteria preferred anoxic and neutral or slightly alkaline conditions in producing N_2O, fungi contributed more to soil N_2O production under sub-anoxic and acidic conditions. Fungi also contributed more to soil N_2O production following the addition of complex substrates. Still, our knowledge on denitrifying fungal community in soil environment is rather limited.

Fungal denitrification normally ends at N_2O due to the absence of N_2O reductase (Shoun et al., 2012). Thus, the essential enzymes for catalyzing denitrification by fungi are nitrite reductase (NirK) and nitric oxide reductase cytochrome P450nor (Shoun and Tanimoto, 1991). Primers targeting fungal *nirK* genes have been developed and used to examine denitrifying fungi in soil environment (Chen et al., 2016; Long et al., 2015; Maeda et al., 2015; Wei et al., 2015). However, these primers can co-amplify a significant portion of denitrifying bacteria because fungal and bacterial NirKs share the same ancestry (Chen et al., 2016). In contrast, P450nor belongs to the cytochrome P450 family and is far different from bacterial nitric oxide reductases, e.g., cytochrome bc-type NORs and qNOR that uses the quinol as the electron donor. This unique feature of P450nor makes *p450nor* gene a suitable marker for detecting denitrifying fungi in soil environment. In this study, we aimed to use *p450nor*-based primers to explore denitrifying fungal community of an agricultural soil that was found to have fungal denitrification (Chen et al., 2014). While *p450nor*-based primer sets had recently been developed, validated using denitrifying fungal cultures, and succeeded in detecting a number of denitrifying fungi of Ascomycota, they did not detect any sequences closely related to Basidiomycota *p450nor* (Higgins et al., 2016; Novinscak et al., 2016). Therefore, we developed two new primer sets and tested if they could broaden the detection of diverse denitrifying fungi in soil environment.

2. Materials and methods

P450nor amino acid sequences were retrieved from the databases of National Center for Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI) for fungi, including *Ajellomyces dermatitidis*, *Aspergillus oryzae*, *Colletotrichum fioriniae*, *Coniosporium apollinis*, *Cylindrocarpon tonkinense*, *Fusarium oxysporum*, *Marssonina brunnea*, *Metarhizium anisopliae*, *Neurospora crassa*, *Paracoccidiodes brasiliensis*, *Trichoderma harzianum*, *Trichosporon asahii*, and *Uncinocarpus reesii*. Sequence alignment using Clustal Omega revealed that P450nor amino acid sequences varied markedly, and there were only two conserved regions by the definition here as ≥five consecutive identical amino

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Table 1
Primer specificity tested against a number of fungal and bacterial cultures.[a]

	Fnor1	Fnor2
Fungi		
Actinomucor elegans Con-1417	+	−
Aspergillus oryzae For-7014	+	−
A. sydowii Org-4511	+	+
A. terreus Org-4110	−	+
Fusarium neocosmosporiellum Con-0506	+	+
F. oxysporum Con-1802	+	+
F. oxysporum Rot-3203	+	+
F. oxysporum Org-4004	+	+
F. oxysporum For-6805	+	+
Gibberella fujikuroi Con-1929	+	+
G. fujikuroi Rot-3131	+	+
G. fujikuroi Org-3532	+	+
G. fujikuroi Org-3733	+	+
Hypocrea lixii Rot-2620	−	+
Purpureocillium lilacinum Rot-2930	−	+
Bacteria		
Alcaligenes faecalis ATCC 8750	−	−
Paracoccus pantotrophus ATCC 35512	−	−
Pseudoalteromonas haloplanktis ATCC 14393	−	−
Pseudomonas stutzeri ATCC 53819	−	−

[a] Symbols "+" and "−" denote the presence or absence of a single band of desired size, respectively.

acids. One conserved region and its extension (P/ATFVDMP/AP, "/" for separating different amino acids in the same position) were used for designing forward primers; the other conserved region and its extension (AGNATL/M/VVNM) were used for designing reverse primers. To broaden the taxonomic coverage of denitrifying fungi in soil environment, two degenerate primer sets were developed, (1) Fnor1F (5′-CCVACITTYGTBGAYATGGA-3′) and Fnor1R (5′-TBACB AYRGTIGCRTTICC-3′) and (2) Fnor2F (5′-TTYGTBGAYATGGAYSCICC-3′) and Fnor2R (5′-TCATGTTBACCATRGTIGCRT-3′). The PCR products were expected to be ~650 bp (e.g., 642 bp for *F. oxysporum* and 666 bp for *A. oryzae*).

Fifteen denitrifying fungi, which were isolated from agricultural soils at the Center for Environmental Farming Systems (CEFS) (Mothapo et al., 2013) were used as the positive controls, and four denitrifying bacterial strains of ATCC (American Type Culture Collection) were used as negative controls (Table 1). Soil from the organic farming system at the CEFS that was shown to have significant fungal denitrification (Chen et al., 2014) was collected in August 2015. Fungal and total soil DNA were extracted using a FastDNA™ SPIN Kit (MP Bio, Solon, OH).

The PCR reaction was made based on our previous method (Chen et al., 2016). Bands of desired size were excised and purified using the PureLink Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA). The gel-purified PCR products amplified from soil DNA were further ligated into pCR2.1 vector and cloned by One Shot TOP10F' *E. coli* using TA Cloning kit (Invitrogen, Carlsbad, CA). All PCR products were sequenced by Sanger sequencing (Eton Bioscience RTP, NC). The sequences of amplicons from fungal and soil DNA were deposited to the NCBI database (KX384966-KX385009).

3. Results and discussion

The two primer sets did not amplify denitrifying bacteria, but only fungi, indicating good specificity (Table 1). Of total 15 positive controls representing 6 genera and 9 species, Fnor1 and Fnor2 were able to amplify 12 and 13 strains, respectively (Table 1). While both together amplified all the 15 fungal strains, they only shared 10, suggesting dissimilar taxonomic coverage between the two.

Both primers amplified soil DNA with a single band of desired size, but operational taxonomic units (OTUs) at 97% similarity that were inferred from clone libraries differed noticeably between Fnor1 and Fnor2 (Fig. 1). Fnor1 detected 22 OTUs of 608–672 bp, whereas Fnor2 only revealed 11 OTUs of 602–669 bp. Further, only 5 OTUs were amplified by both primes, and 17 OTUs were detected solely by Fnor1 and 6 OTUs solely by Fnor2. This soil analysis corroborated with the analysis on cultural strains and further emphasized the need of using both primers to broaden taxonomic coverage of denitrifying fungi in soil environment. Unlike the *nirK* primers that detected a significant portion of denitrifying bacteria from this soil (Chen et al., 2016), no denitrifying bacteria were found out of > 200 clones by both *p450nor* primer sets by blast analysis. Using AUGUSTUS, a web server for gene finding in eukaryotes (Stanke et al., 2006), OTU sequences were translated into 161–165 amino acid sequences, which had 50–99% identity to P450nor in the NCBI database.

Fungal OTUs were mainly clustered with references in phylum Ascomycota across the orders Eurotiales, Hypocreales and Sordariales (Fig. 1). And soil fungi amplified by Fnor1 and Fnor2 were closely related to species of *Aspergillus*, *Cylindrocarpon*, *Fusarium*, *Neurospora*, *Thielavia*, and *Trichosporon*. Agreed with the phylogeny derived from fungal *nirK* sequences (Chen et al., 2016), *p450nor* of denitrifying fungi in the order Hypocreales were also distantly related. One interesting observation was that a number of denitrifying fungal OTUs in the order Sordariales were detected, including those related to *Neurospora* and *Thielavia*. OTUs related to *Trichosporon asahii* in the phylum Basidiomycota were also detected, which has not been detected from the previous studies (Higgins et al., 2016; Novinscak et al., 2016). However, our previous culture-based investigation did not detect any denitrifying fungi in the order Sordariales nor in the phylum Basidiomycota (Mothapo et al., 2013). The inconsistency between culture–dependent and –independent approaches highlighted the need of using the primer tool to expand knowledge on the diversity and composition of denitrifying fungi at a given environment.

In summary, the two primer sets, Fnor1 and Fnor2, targeting *p450nor* were proven suitable for detecting diverse denitrifying fungi from the soil environment and more specifically targeted on denitrifying fungi than the fungal *nirK* primer tool. This primer tool expanded denitrifying fungi to include *Cylindrocarpon*, *Neurospora*, *Thielavia*, and *Trichosporon* that were undetected in our previous culture-based work. This study further emphasized that a combined use of primer sets could improve the detection of diverse denitrifying fungi from soil environment and therefore may help better to understand the composition and structure of denitrifying fungal community.