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Detection and Diversity of Fungal Nitric Oxide Reductase Genes (*p450nor*) in Agricultural Soils

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ABSTRACT

Members of the Fungi convert nitrate (NO_3^-) and nitrite (NO_2^-) to gaseous nitrous oxide (N_2O) (denitrification), but the fungal contributions to N loss from soil remain uncertain. Cultivation-based methodologies that include antibiotics to selectively assess fungal activities have limitations, and complementary molecular approaches to assign denitrification potential to fungi are desirable. Microcosms established with soils from two representative U.S. Midwest agricultural regions produced N_2O from added NO_3^- or NO_2^- in the presence of antibiotics to inhibit bacteria. Cultivation efforts yielded 214 fungal isolates belonging to at least 15 distinct morphological groups, 151 of which produced N_2O from NO_2^-. Novel PCR primers targeting the *p450nor* gene, which encodes the nitric oxide (NO) reductase responsible for N_2O production in fungi, yielded 26 novel *p450nor* amplicons from DNA of 37 isolates and 23 amplicons from environmental DNA obtained from two agricultural soils. The sequences shared 54 to 98% amino acid identity with reference P450nor sequences within the phylum *Ascomycota* and expand the known fungal P450nor sequence diversity. *p450nor* was detected in all fungal isolates that produced N_2O from NO_2^-, whereas *nirK* (encoding the NO-forming NO_2^- reductase) was amplified in only 13 to 74% of the N_2O-forming isolates using two separate *nirK* primer sets. Collectively, our findings demonstrate the value of *p450nor*-targeted PCR to complement existing approaches to assess the fungal contributions to denitrification and N_2O formation.

IMPORTANCE

A comprehensive understanding of the microbiota controlling soil N loss and greenhouse gas (N_2O) emissions is crucial for sustainable agricultural practices and addressing climate change concerns. We report the design and application of a novel PCR primer set targeting fungal *p450nor*, a biomarker for fungal N_2O production, and demonstrate the utility of the new approach to assess fungal denitrification potential in fungal isolates and agricultural soils. These new PCR primers may find application in a variety of biomes to assess the fungal contributions to N loss and N_2O emissions.

Denitrification is a key process responsible for loss of fixed nitrogen (N) in soils and sediments and is mediated by both abiotic and microbial processes (1, 2). Of the microorganisms involved in denitrification, members of the *Bacteria* are well studied and considered key contributors; however, some saprotrophic fungi also conserve energy from the reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to nitrous oxide (N_2O), the main end product of fungal denitrification (3–5). Fungi have been implicated in N turnover for over 30 years (1, 3, 6), but the ecological importance of fungal contributions to denitrification remain uncertain. Potential roles of fungi in soil N loss and greenhouse gas (i.e., N_2O) emission are underexplored, and monitoring tools (e.g., quantitative or endpoint PCR) to specifically address the presence and abundance of denitrifying fungi are largely lacking. To date, the majority of the known denitrifying fungal isolates reside in the phylum Ascomycota (7–9). Although some reports suggest that fungi within the Basidiomycota and Zygomycota are denitrifiers (8–10), only a few isolates are available, and it remains to be confirmed if members of other phyla harbor *nar*, *nap*, *nir*, and *nor* gene clusters implicated in steps of the denitrification pathway. Previous investigations of fungal denitrification have relied on laboratory cultivation and substrate-induced respiration inhibition (SIRIN) tests (3, 7, 10). The SIRIN technique applies NO_3^- or NO_2^- in concert with broad-spectrum antibiotics (e.g., streptomycin) or fungicides (e.g., cycloheximide) to partition the bacterial and fungal contributions to N_2O or dinitrogen (N_2) formation. These efforts suggested that fungi play a large but unrecognized role in N turnover and N_2O flux (9, 11–13). Although the SIRIN methodology has provided valuable insight into the fungal contribution to denitrification, the application of SI-

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RIN to soils can be problematic as incomplete inhibition can bias the observations (14–16). Hence, alternative approaches (e.g., PCR) that selectively target fungal genes involved in denitrification are desirable to advance understanding of the fungal diversity contributing to N cycling in environments such as soils, aquifers, and deep-ocean sediments, where denitrifying fungi have been cultivated (7, 13, 17, 18).

Denitrifying fungi possess a unique protein of the CYP55 family of P450 cytochromes, cytochrome P450 nitric oxide reductase (P450nor). P450nor lacks mono-oxygenase activity and a conserved N-terminal membrane anchor domain found in other eukaryotic P450 cytochromes (19). It directly binds the electron donor NAD(P)H but otherwise is structurally conserved and shares similarity with other cytochrome P450 proteins (19). P450nor is responsible for the reduction of nitric oxide to N_2O, the dominant end product, although N_2 formation has been observed (5, 7, 20). Denitrifying bacteria and archaea are also capable of reducing nitric oxide to N_2O but possess a distinct gene locus (*norB*) that encodes two isozymes, cNor and qNor, each relying on a distinct electron donor (*c*-type cytochrome or ubiquinol pool, respectively) (21, 22). Furthermore, only bacterial and archaeal denitrifiers possess the *nosZ* gene, which encodes the enzyme responsible for reduction of the greenhouse gas N_2O to inert N_2. Thus, the *p450nor* gene could serve as a diagnostic marker for fungal denitrification potential, but the lack of molecular tools targeting *p450nor* currently limits efforts to assess the contribution of fungi to N cycling. In the present study, a translated alignment of available *p450nor* sequences identified key conserved amino acid residues within P450nor and enabled the design of *p450nor*-specific degenerate primers. We demonstrated the broad utility of these new primers by performing PCR amplification of *p450nor* genes from fungal isolates and environmental DNA obtained from two agricultural soils with distinct physicochemical properties. Metagenomic data sets derived from the same agricultural soils did not reveal the presence of fungal denitrification genes within either soil. This finding emphasizes the value of cultivation-based efforts and the *p450nor*-targeted PCR assays for assessments of fungal contributions to denitrification in complex environmental systems such as soils.

MATERIALS AND METHODS

Medium preparation. A modified Czapek liquid medium containing nitrate (2 mM) or nitrite (1 mM) (23) was utilized for fungal isolation and cultivation unless otherwise noted. Briefly, ultrapure water (≥18.0 MΩ · cm) (Milli-Q system; Millipore, Billerica, MA) was combined with (in g liter^{-1}) KCl (0.5), K_2SO_4 (0.35), magnesium glycerophosphate (0.5), and $FeCl_2$ (0.01), and the pH was adjusted to 6.8 by adding sodium hydroxide (0.5 M) before the medium was autoclaved.

Fungal enrichment and isolation. Triplicate soil cores were collected in November 2012 from agricultural sites in Havana, IL (latitude 40.296, longitude −89.994), and Urbana, IL (latitude 40.075, longitude −88.242), using a soil corer with a 30-cm depth range. The soil cores were placed in coolers and transported to the University of Illinois, Urbana-Champaign, for processing. Soil aliquots (5 to 10 g) from the 0- to 5-, 5- to 20-, and 20- to 30-cm depth ranges were shipped overnight on ice to the University of Tennessee. Soil samples were pooled and homogenized using an aseptic technique, and 5 g of soil was added to 30 ml of oxic Czapek medium (pH 6.8) in 60-ml glass serum bottles fitted with black butyl rubber stoppers and incubated at room temperature. Microcosms ($n = 1$ per treatment) were amended with either 2 mM sodium nitrate (NO_3^-) or 1 mM sodium nitrite (NO_2^-) as the electron acceptor and either sodium acetate, sodium formate, sodium pyruvate (3 mM each), or an autoclaved 1.5% (wt/vol) milled corn and soybean plant suspension as an exogenous substrate. All the serum bottles were amended with 30 μg ml^{-1} chloramphenicol (Fisher Scientific, USA) and 100 μg ml^{-1} streptomycin sulfate (Fisher Scientific, USA) from ethanolic or aqueous stocks, respectively, to inhibit bacterial growth. After 1 month of monitoring N_2O production, the soil microcosms were shaken vigorously by hand, and 0.5 ml of homogenized suspension was transferred, using a sterile 1-ml plastic syringe fitted with a 19-gauge needle (Becton Dickinson, Franklin Lakes, NJ), to fresh anoxic medium containing the same carbon and nitrogen sources as the parent microcosms. The transfer cultures ($n = 2$ per treatment) received 50 μg ml^{-1} each of kanamycin sulfate (Fisher Scientific, USA) and ampicillin sodium salt (Fisher Scientific, USA) from sterile aqueous stocks to inhibit bacteria that might have possessed natural resistance to the antibiotics added to the initial enrichments. The cultures were then amended with 0.6 ml of filter-sterilized air (2% [vol/vol] headspace volume), and the NO_3^-, NO_2^-, and N_2O concentrations were monitored. Following a 2-week incubation period, 2 ml of culture samples was transferred to 25 ml liquid (45°C) agar medium containing the same substrates as the parent cultures. Each tube was gently mixed, and its contents were poured into individual sterile plastic plates. The plates were incubated in the dark at room temperature and visually inspected each day for fungal growth. After filamentous colonies were observed, approximately 1-cm^2 agar sections containing fungal mycelia were transferred to fresh agar plates. Subsequent transfers (at least three) were of 1-cm^2 agar sections of the fungal colony margins to quarter-strength R2A agar (Fisher Scientific, USA) plates containing 100 μg ml^{-1} streptomycin sulfate. Contamination was monitored by microscopic observation and avoided by routine transfer of agar sections containing hyphae of consistent appearance to fresh R2A agar plates containing streptomycin or ampicillin and kanamycin. *Aspergillus terreus* strain NRRL 255 and *Aspergillus flavus* strain NRRL 3357 were obtained from the USDA Agricultural Research Service culture collection (the NRRL Collection). Both fungi were grown on quarter-strength R2A agar (Becton Dickinson, Franklin Lakes, NJ) plates amended with 50 μg ml^{-1} streptomycin for tissue collection and DNA extraction (see below).

Analytical procedures. NO_3^- and NO_2^- were measured by ion chromatography with a Dionex 3000 instrument (Dionex, Sunnyvale, CA) as described previously (24, 25). N_2O was monitored via 1-ml headspace injections into a MicroGC 3000A (Agilent Technologies, Palo Alto, CA) equipped with a polystyrene-divinylbenzene PlotQ column and a thermal conductivity detector. All nitrogen species were quantified using a dilution series of standards prepared in medium. The instrument limits of detection for NO_2^-, NO_3^-, and N_2O were 7, 4, and 3 μmol, as determined by preparing replicate standards ($n = 20$) and following established procedures (26).

Denitrification activity of fungal isolates. Fungal isolates were tested for denitrifying potential by transferring individual 1-cm^2 agar sections to 1.5-ml plastic microcentrifuge tubes containing 1 ml of sterile water, followed by brief manual homogenization with sterile steel forceps. Then, 250 μl of the water-agar-mycelium mixture was inoculated into 26-ml Balch tubes containing 15 ml of oxic Czapek medium amended with 5 mM sodium acetate, 2 mM NO_2^-, and 100 μg ml^{-1} streptomycin. The headspace of each Balch tube was monitored over a 12-day period for N_2O production.

DNA extraction. Fungal biomass (~0.3 g [wet weight]) was placed in a 2-ml screw-cap plastic tube containing approximately 200 mg of 0.5-mm silica and zirconium beads (Fisher Scientific, USA), and DNA was extracted following an established procedure (27) (see the supplemental material for details). Soil DNA extraction from soil samples (0.5 g of homogenized soil from 0- to 30-cm depth from Havana and Urbana and a separate 0- to 5-cm-depth Havana sample) was performed using the FastDNA spin kit for soil (MP Biomedicals, Santa Ana, CA) following the manufacturer's protocol. The soil DNA concentration and purity were analyzed with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA) using the

TABLE 1 Characteristics of the *p450nor* gene-targeted primers designed and used in this study

Primer[a]	Primer sequence[b] (5′→3′)	Melting temp range (°C)[c]	GC (%)[c]	Fold degeneracy[d]
*p450nor*394F	SCIACITTYGTIGAYATGGA	45.6–55.9	35–60	8 (256)
*p450nor*809R	ATCATGTTIACBAIIGTIGCIT	45.5–56.7	27–55	3 (3,072)
*p450nor*1008R	GMSGCRATKATNCCYTC	42.2–54.3	41–71	128

[a] The numeric positions of the primers are based on the *A. terreus* strain NIH2624 *p450nor* gene sequence.
[b] S = G or C; Y = C or T; B = C, G, or T; M = A or C; R = A or G; K = G or T; N = A, G, C, or T; I = inosine.
[c] Ranges were calculated using OligoCalc (70).
[d] The numbers in parentheses indicate fold degeneracy if the IUPAC symbol "N" had been used instead of an inosine nucleoside.

dsDNA BR Assay kit (Life Technologies) following the manufacturer's recommendations.

Primer design. To design primers targeting *p450nor* gene sequences, primary literature resources and the National Center for Biotechnology Information (NCBI) database were queried to identify available *p450nor* gene and protein sequences (28). Multiple-sequence alignment was used to identify conserved residues, followed by selection of additional sequences by querying public sequence databases (GenBank and UniProt) using BLAST (29). Overall, 38 *p450nor* reference sequences were obtained and aligned using the program T-Coffee (30), followed by manual inspection. PAL2NAL (31) was used to generate a codon-aware nucleotide alignment, and conserved sites were selected for forward and reverse primer binding (Table 1; see the supplemental material for additional details about primer site selection). The *p450nor* sequences of *A. terreus* strain NIH2624 and *A. flavus* strain NRRL 3357 were among the 38 sequences used for primer design (see Data Set S1 in the supplemental material), and *p450nor* sequences derived from *A. terreus* strain NRRL 255 and *A. flavus* strain NRRL 3357 served as positive controls in all *p450nor*-targeted PCR assays (32, 33). Genomic DNA from the bacterium *Dehalogenimonas lykanthroporepellens* strain BLDC-9 (ATCC BAA-1523) was used as a negative control in PCR assays.

PCR conditions. PCR assays using the primer pair *p450nor*394F and *p450nor*809R (Table 1) consisted of 9.125 μl DNase-free water (Life Technologies, Carlsbad, CA), 2.5 μl of 10 mM magnesium chloride solution (Applied Biosystems, CA, USA), 2.5 μl of *Ex Taq* 10× buffer (Mg^{2+} free) (Clontech Laboratories, Inc., Mountain View, CA), 2.5 μl of 2.5 mM each deoxynucleoside triphosphate (dNTP) (Clontech), 0.5 μl each 100 μM primers, 6.25 μl of 200-ng/μl bovine serum albumin (BSA) solution (Fisher Scientific, USA), 0.125 μl of 5-U/μl TaKaRa *Ex Taq* DNA Polymerase (Clontech), and 1 μl DNA template (approximately 10 to 100 ng/μl) in a total reaction volume of 25 μl.

PCR conditions for *p450nor* amplification from soil DNA samples required a seminested approach for consistent results, with initial PCR using primers *p450nor*394F and *p450nor*1008R, followed by a second round of PCR using primers *p450nor*394F and *p450nor*809R. The initial PCR mixture contained the same ingredients described above, except that 0.8-μl volumes (each) of 25 μM primers *p450nor*394F and *p450nor*1008R were utilized in the initial amplification reaction. Then, 1 μl of the amplicon solution was used as the template DNA in a second round of PCR with primers *p450nor*394F and *p450nor*809R employing PCR conditions identical to those for fungal genomic DNA amplification.

Amplification of fungal *nirK* genes from isolate DNA were performed as previously described by using primers nirKfF/nirKfR and fnirK2F/fnirK1R (34, 35) with the following modifications. The durations of the annealing and elongation steps were increased by 15 s (34) or decreased by 15 s (35), respectively, to allow simultaneous amplification of similar-size amplicons (~450 to 500 bp). A total of 35 amplification cycles were performed for both the nirKfF/nirKfR and the fnirK2F/fnirK1R primer sets.

Amplification of the fungal internal transcribed spacers 1 and 2 was performed using primers ITS4 (36) and EF3RCNL (37). Amplification and sequencing of the nuclear small-subunit ribosomal (18S rRNA) gene from fungal isolates utilized 0.5 μl each of 10 μM primers PNS1, NS1, NS3, NS4, NS7, NS8, SR6, and NS19b (36, 38, 39) and the PCR conditions described above for the *p450nor*394F and *p450nor*1008R primers.

All DNA amplifications were carried out on a Veriti thermal cycler (Life Technologies, Carlsbad, CA) at 95°C for 2 min and 10 s, followed by 35 cycles each of 95°C for 30 s, 47°C for 45 s, and 72°C for 1 min (1.5 min for 18S rRNA genes), with a final extension step at 72°C for 7 min. For amplification of *p450nor* from soil DNA templates, as well as amplification of isolate ITS regions or isolate 18S rRNA genes, the annealing temperature was increased to 50, 53, or 55°C, respectively. Annealing temperatures for amplifications with *nirK*-targeted primer pairs nirKfF/nirKfR and fnirK2F/fnirK1R were 54 and 50°C, respectively (34, 35). For visualization of amplicons, 5 μl of the PCR assay mixture was combined with 1 μl of a loading dye solution (25 mg each bromophenol blue and xylene cyanol and 4 g sucrose dissolved in 10 ml of ultrapure water) and loaded onto a 1% (wt/vol) agarose gel, and electrophoresis was conducted at 90 V for 1 h at an initial amperage of 200 mA.

Cloning and sequencing. Cloning of the ITS region and *p450nor* amplicons from DNA obtained from isolates and agricultural soil was performed using the TOPO TA cloning kit (Life Technologies, Carlsbad, CA) following the manufacturer's protocols. Of the 60 amplicons generated from soil DNA, 23 were selected for sequencing based on restriction digest analysis (HaeIII digestion using NEBuffer 4 solution [New England BioLabs, Ipswitch, MA]). Briefly, PCR amplicons (5 μl) were incubated at 37°C for 12 h in a 12-μl reaction volume (5.3 μl PCR grade water, 1.2 μl 10× NEBuffer 4 solution, 0.5 μl HaeIII), and fragment patterns were analyzed via gel electrophoresis. The cloned amplicons were Sanger sequenced using an ABI Big-Dye v3.1 cycle-sequencing mixture on an ABI 3130 analyzer (Applied Biosystems) at the University of Tennessee Molecular Biology Resource Facility. Electropherograms of sequenced amplicons were manually inspected using Geneious software (Biomatters Ltd., Auckland, New Zealand) following automatic trimming with a 0.03 error probability limit, and overlapping regions were assembled *de novo* after vector sequence removal using the UniVec database within Geneious.

Fungal taxonomy. The script FungalITSPipeline.pl (40) was used to extract ITS1 and ITS2 regions from the cloned ITS sequences of the fungal isolates and to determine taxonomic affiliations within the International Nucleotide Sequence Database Collaboration (INSDC) (41). The 18S rRNA gene sequences were annotated using the SILVA database and the SINA alignment tool (42, 43).

Metagenomic analysis. Metagenomic reads previously generated from Havana (SRR1152189) and Urbana (SRR1153387) soils (44) were retrieved from NCBI's Sequence Read Archive and unpacked with the SRA Toolkit (45). SolexaQA++ (46) was used to trim reads with a 0.01 error probability limit (Phred score, 20), followed by discarding reads shorter than 50 nucleotides. This procedure resulted in totals (including paired and single reads) of 236,437,515 and 291,298,696 high-quality sequences from Havana and Urbana soils, respectively. Fungal presence in the metagenomes was established by nucleotide alignment (BLASTn) against a custom ITS database provided by the FHiTINGS ITS analysis software, followed by analysis of the results with the same software (47). BLASTn alignment of the metagenomic reads against the cloned ITS sequences from the enriched fungal isolates was performed to uncover the presence/absence and relative abundances of isolated fungi within the metagenomes. A similar analysis was performed with cloned *p450nor* sequences, except a translated nucleotide alignment (BLASTx) was used.

P450nor sequence analyses. The 38 reference P450nor sequences were combined with newly obtained *p450nor* sequences from isolates and

soil DNA. Following the removal of identical sequences, all the sequences were aligned with the UniRef90 database with BLASTp (29, 48). Significant alignments (≥70% alignment length and ≥30% sequence identity) with eukaryotic sequences were retained for each query sequence, and hits with ≥90% identity to P450nor query sequences or uncertain NCBI taxonomy were removed. MAFFT v7.130b (49) was used with default parameters to initially align all the sequences, followed by manual editing with Jalview and SEA VIEW to retain the region amplified by the primer set *p450nor*394F and *p450nor*809R (50–52). The resulting alignment consisted of 108 taxa with an average length of 153 (minimum, 128; maximum, 158) amino acids. The alignment was analyzed with ProtTest v3.4 (53) to select an optimized evolutionary model for the aligned sequences prior to tree building. Phylogenetic reconstruction was performed using the FastTree 2 v2.1.7 SSE3 software package (54). Approximate maximum-likelihood tree reconstruction with FastTree 2 was performed using the Whelan and Goldman (55) amino acid substitution model with gamma-distributed rate heterogeneity (discrete distribution with 4 rate categories) and the subtree-pruning-regrafting algorithm. Support for internal nodes was assessed using 5,000 replications of the Shimodaira-Hasegawa test performed within FastTree 2. An additional alignment was generated using MAFFT that contained full-length sequences from a subset ($n = 16$) of all the sequences used in phylogenetic reconstruction. This alignment was submitted to the ESPript 3.0 Web server (http://espript.ibcp.fr; 56) to visualize key conserved residues in a diversity of P450nor sequences.

Nucleotide sequence accession numbers. The *p450nor*, ITS, and 18S rRNA gene sequences are available under GenBank accession numbers KM889490 to KM889526 and KT714145 to KT714154, KM889527 to KM889556, and KT714155 to KT714191, respectively.

RESULTS

Fungal isolation. Fungal cultivation techniques applied to Havana and Urbana soil-derived transfer cultures produced 214 fungal isolates. Growth experiments in medium containing NO_2^- demonstrated that about two-thirds of the isolates (151 out of 214) produced N_2O within a 12-day incubation period (data not shown). Based on the gross morphology of mature fungal colonies, the 214 isolates from both the Havana and Urbana soils were divided into 15 groups, and 30 distinct isolates (including at least one representative isolate from each morphological group) were selected for further analysis. Since each morphological grouping may not align with the underlying isolate genotype, ITS region analysis was performed, which revealed 10 phylogenetically distinct groups, four of which were composed of singleton ITS sequences (see Data Set S2 in the supplemental material). Five additional isolates were later selected for *p450nor* analysis based on morphology consistent with that of N_2O-producing isolates in previously assigned groupings, but only 18S rRNA gene identification was performed on these isolates (see Data Set S2 in the supplemental material).

Fungal-denitrifier morphology and taxonomy. The majority of the representative fungal isolates were assigned to the genera *Fusarium* (teleomorph *Giberella*; 19 in total) or *Trichoderma* (5 in total) based on ITS and 18S rRNA gene sequence analysis. Microscopic observations of mature fungal colonies corroborated these results and revealed characteristic features of both the *Fusarium* (e.g., banana-shaped septate ascospores) and *Trichoderma* (e.g., presence of green conidia aggregated into fascicles) isolates (see Fig. S1 in the supplemental material) (57). Taxonomic prediction based on 18S rRNA gene sequence classification was successful for all but two fungal isolates. ITS sequence analysis identified these two isolates as putative *Trichoderma* sp. and *Fusarium* sp. Morphotyping (e.g., appearance of aerial hyphae with a white, feathery appearance on agar plates) and 18S rRNA gene sequence analysis assigned two additional isolates to the order Mortierellales. Other fungi isolated in this study included members of the genera *Verticillium* (1 isolate), *Humicola* (2 isolates), *Chaetomium* (3 isolates), *Podospora* (1 isolate), *Lecythophora* (1 isolate), and *Guehomyces* (1 isolate) (see Data Set S3 in the supplemental material), all of which belong to fungal lineages known to harbor denitrifying representatives (33).

p450nor and *nirK* PCR amplification. Application of primers *p450nor*394F and *p450nor*809R to isolate genomic DNA consistently resulted in a DNA band between 650 and 850 bp in size, and sequencing confirmed the amplification of *p450nor* gene fragments in 23 out of 23 (100%) N_2O-producing fungal isolates (Fig. 1; see Data Set S2 in the supplemental material). Several nonspecific amplicons 100 to 200 bp below the predicted *p450nor* amplicon size of 536 to 890 bp were consistently observed (see Data Set S4 in the supplemental material), requiring gel extraction and cloning of the amplicons prior to sequencing. Sequence similarity among nontarget amplicons indicated that they originated from a putative ATPase gene and a noncoding genomic region. Nine isolates displayed a *p450nor* amplicon, but N_2O was not detected in liquid culture under the conditions tested (Table 2). *nirK* amplification was detected in 17 out of 23 (74%) N_2O-producing fungal isolates using primer set nirKfF/nirKfR. Amplification of both a *p450nor* and a *nirK* gene was observed in 20 out of 37 (54%) isolates. There were two instances in which *nirK* was amplified but *p450nor* could not be detected (isolate identifiers [ID] 50 and 141), and in 12 cases, *p450nor* was amplified but *nirK* was not detected (Table 2). Using primer set fnirK2F/fnirK1R, *nirK* was detected in only 3 out of 23 (13%) N_2O-producing fungal isolates (Table 2).

The nested-PCR amplification of soil DNA resulted in the recovery of a total of 23 environmental *p450nor* clone sequences. Pairwise nucleotide and amino acid identities of the 23 environmental *p450nor* sequences ranged from 65.8 to 100% and 63.8 to 100%, respectively. Phylogenetic analysis of the environmental *p450nor* sequences revealed the presence of five distinct soil clades (e.g., Urbana clone groups I and II and Havana clone groups I, II, and III) (Fig. 2). The clone groups from both soils were affiliated with sequences of representative Sordariomycetes denitrifiers (e.g., *Fusarium* and *Trichoderma*) and *p450nor* sequences derived from N_2O-producing fungal isolates recovered from these soils (isolate ID 213 and 179) (Fig. 2). Overall, the environmental clone sequences displayed 59 to 100% amino acid identity with P450nor sequences from fungal isolates derived from the same agricultural soils. The alignment of the amplified region of the *p450nor* genes (approximately 160 amino acids) and P450nor tree reconstruction supported the monophyly of the cloned *p450nor* sequences with previously reported *p450nor* sequences from confirmed fungal denitrifiers (Fig. 2). The *p450nor* sequences recovered from isolate DNA and soil DNA clustered with *p450nor* sequences from members of the Leotiomycetes (one sequence), Eurotiomycetes (one sequence), and Sordariomycetes (47 sequences), three diverse fungal lineages within the phylum Ascomycota that are known to harbor denitrifying representatives (33).

Soil denitrification activity. Previous investigations have demonstrated that the majority of denitrifying fungal isolates lack the capacity to reduce NO_3^-. Therefore, replicate microcosms received NO_3^- or NO_2^- to assess denitrification activity. N_2O

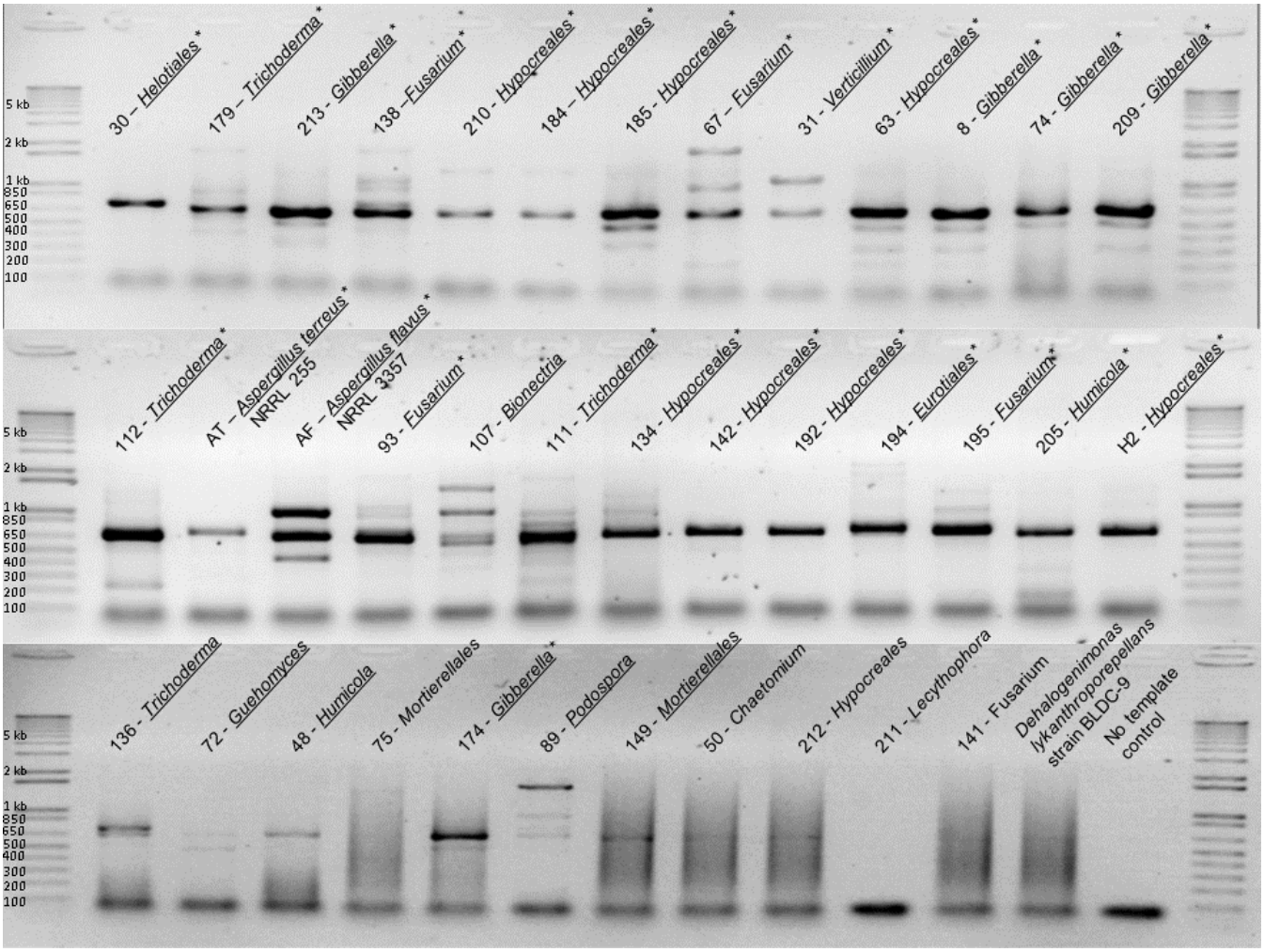

FIG 1 PCR amplification of *p450nor* genes from 37 fungal isolates. The names of isolates with positive *p450nor* bands between 650 and 850 bp are underlined. Fungal isolates whose *p450nor* genes have been sequenced are marked with asterisks. All the isolates have unique identifiers (shown before the taxonomic names) and possess their lowest taxonomic designation (e.g., genus) using the 18S rRNA gene classification from the SILVA database. *A. terreus* strain NRRL 255 and *A. flavus* strain NRRL 3357 were used as positive controls for *p450nor* amplification. Genomic DNA from the bacterium *Dehalogenimonas lykanthroporepellens* strain BLDC-9 was used as a negative control.

production and visible fungal biomass (i.e., hyphae) were observed in all microcosms (16 total) that received antibiotics to inhibit bacterial growth (see Fig. S3 in the supplemental material); however, N_2O formation in Havana soil microcosms amended with NO_3^- was low (see Fig. S3, top left, in the supplemental material). Havana transfer cultures amended with NO_2^- mirrored patterns observed in the NO_2^--amended soil microcosms, but N_2O formation was variable in the transfer cultures that received NO_3^- (see Fig. S4 in the supplemental material). N_2O concentrations declined in Urbana microcosms after 2 weeks of incubation, whereas N_2O production continued in Havana microcosms (see Fig. S3 in the supplemental material). A decline in the NO_3^- concentration in Urbana transfer cultures was observed, but concomitant formation of N_2O did not occur (see Fig. S4 in the supplemental material).

Metagenomic analyses. BLAST analysis of the Havana and Urbana metagenomic sequences identified 5,213 and 3,373 unique reads out of 236 and 291 million reads, respectively, that were alignable with fungal ITS database sequences. Fewer than 200 reads from each metagenome aligned with ITS sequences amplified from the representative fungal isolates obtained from the same soil samples. No metagenomic reads aligned with amplified and sequenced *p450nor* (this study) or previously reported *p450nor* sequences.

DISCUSSION

A new means to assess fungal denitrification potential. The *p450nor* sequences identified in this study formed a monophyletic group with reference *p450nor* sequences and demonstrated the specificity of the degenerate primers for *p450nor* genes in isolate and soil DNA (Fig. 2). New sequences sharing 54 to 98% amino acid identity to reference *p450nor* sequences were recovered, suggesting the primers allow the recovery of novel sequence diversity within the predicted 536- to 890-bp sequence region spanned by the conserved primer binding sites (see Data Set S4 in the supplemental material). Phylogenetic analysis of the *p450nor* environmental clones permitted the identification of distinct groups (Havana clone groups I, II, and III and Urbana clone group I) (Fig. 2).

TABLE 2 Select characteristics and gene contents of fungal isolates related to their capacity to perform denitrification

Isolate ID	Isolate source[a]	Taxonomic affiliation[b]	N_2O production[c]	Targeted PCR		
				p450nor[d]	*nirK* primer set 1[e]	*nirK* primer set 2[f]
30	H	*Helotiales*	+	+	+	−
179	U	*Trichoderma*	+	+	+	−
213	U	*Gibberella*	+	+	+	−
138	H	*Fusarium*	+	+	+	+
210	U	*Hypocreales*	+	+	−	−
184	U	*Hypocreales*	+	+	−	−
185	U	*Hypocreales*	+	+	+	+
67	U	*Fusarium*	+	+	+	−
31	U	*Verticillium*	+	+	+	−
63	H	*Hypocreales*	+	+	+	−
8	H	*Gibberella*	+	+	+	−
74	H	*Gibberella*	+	+	+	−
209	U	*Gibberella*	+	+	+	−
112	H	*Trichoderma*	−	+	−	−
93	H	*Fusarium*	+	+	+	+
107	H	*Bionectria*	+	+	−	−
111	U	*Trichoderma*	+	+	+	−
134	H	*Hypocreales*	−	+	−	−
142	H	*Hypocreales*	−	+	+	−
192	H	*Hypocreales*	−	+	+	−
194	H	*Eurotiales*	+	+	+	−
195	U	*Fusarium*	−	+	−	−
205	H	*Humicola*	−	+	−	+
H2	H	*Hypocreales*	+	+	−	−
136	H	*Trichoderma*	−	+	+	−
72	U	*Guehomyces*	+	+	−	−
48	U	*Humicola*	−	+	−	−
75	U	*Mortierellales*	−	−	−	−
174	U	*Gibberella*	+	+	+	−
89	H	*Podospora*	−	+	−	−
149	H	*Mortierellales*	+	+	+	−
50	U	*Chaetomium*	−	−	+	+
212	H	*Hypocreales*	−	−	−	−
211	H	*Lecythophora*	−	−	−	−
141	H	*Fusarium*	−	−	+	−
AT[g]	USDA	*Aspergillus*	+	+	−	−
AF[h]	USDA	*Aspergillus*	+	+	+	−

[a] H and U, Havana and Urbana soils, respectively; USDA, Agricultural Research Service culture collection.
[b] Lowest taxonomic rank based on 18S rRNA gene classification (see Materials and Methods for more information). In some cases, the ITS sequence was used for classification when 18S rRNA gene classification was unsuccessful.
[c] +, detected; −, not detected. N_2O production was determined in defined mineral salts medium using nitrite as an electron acceptor (see Materials and Methods).
[d] +, detected; −, not detected. *p450nor* was classified as detected when a visible band was confirmed between 536 and 890 bp by electrophoresis on a 1% (wt/vol) agarose gel (see Materials and Methods).
[e] +, detected; −, not detected. *nirK* amplification was with primer set nirKfF/nirKfR (34). *nirK* positive detection was indicated by a visible band at 480 bp by electrophoresis on a 1% (wt/vol) agarose gel (see Materials and Methods).
[f] +, detected; −, not detected. *nirK* amplification was with primer set fnirK2F/fnirK1R (35). *nirK* was classified as detected when a visible band was confirmed at 468 bp by electrophoresis on a 1% (wt/vol) agarose gel (see Materials and Methods).
[g] AT, *Aspergillus terreus* strain NRRL 255.
[h] AF, *Aspergillus flavus* strain NRRL 335.

These data suggest that geochemical differences between Havana and Urbana soils could be driving fungal-denitrifier diversity, but additional efforts are needed to determine to what extent geochemical conditions determine fungal *p450nor* diversity.

Recent investigations into fungal denitrification have relied on PCR primers targeting the fungal *nirK* gene to demonstrate the NO_2^--reducing potential of the fungal community (34, 58). Although a significant advance in our ability to target fungal denitrifiers, *nirK* primer sets targeting the NO_2^--to-NO step do not directly reveal the N_2O-producing potential of the fungal community. The application of the fungal *nirK* gene-targeted primer set fnirK2F/fnirK1R (35) yielded only five positive results, whereas primer set nirKfF/nirKfR (34) revealed that many (17 out of 23) of the N_2O-producing fungal isolates possessed *nirK*. Apparently, the latter primer set targets a greater diversity of fungal *nirK* genes but still failed to amplify *nirK* genes from 26% of the NO_2^--reducing and N_2O-producing fungal isolates. Interestingly, in two nondenitrifying fungal isolates, *nirK* was amplified but *p450nor* was not detected. On the other hand, in 12 isolates, *p450nor* amplification occurred but *nirK* was not detected (Table 2). Of these

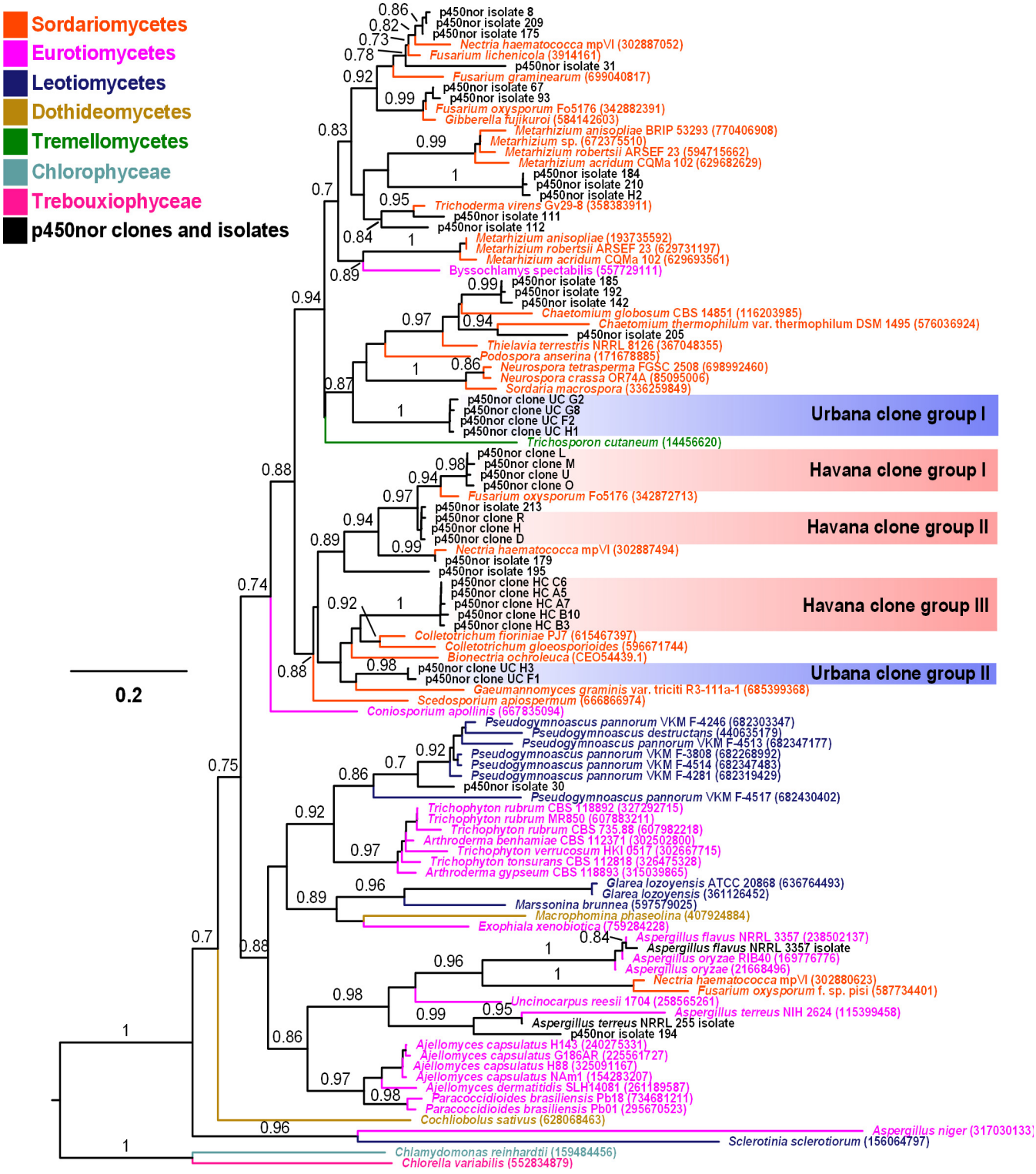

FIG 2 Rooted maximum-likelihood tree of fungal P450nor and related P450 amino acid sequences. Translated *p450nor* sequences amplified from isolate and clone sequences generated in this study are in black letters. Values along branches represent support (out of 5,000 resamples) for the tree topology at the nearest node, determined using the Shimodaira-Hasegawa test implemented in FastTree2. Only values ≥0.70 are reported. The scale bar indicates amino acid substitutions per site. GenInfo Identifier (GI) numbers of proteins from the NCBI database are in parentheses. HC, Havana clone; UC, Urbana clone. Clones with single letters were generated from a distinct Havana soil sample compared to the HC clones. Note that for *Bionectria ochroleuca*, an EMBL protein accession number is provided in parentheses, since a GI number was not available.

12 organisms, five were shown to reduce NO_2^- to N_2O. These observations suggest that the characterization of fungal *nirK* sequence space is incomplete, and additional sequence information from denitrifying fungal isolates is desirable. To date, *p450nor* is the sole gene known to encode the N_2O-producing phenotype in fungi (3) and thus represents an ideal target for assessing N_2O formation potential. Due to substantial heterogeneity in available *p450nor* sequences, primer design is challenging (33). Consequently, the design of PCR primers that amplify all *p450nor* sequences while excluding all other sequences of the p450 gene family will probably not be possible. Still, the new *p450nor* primers presented here represent a major advance for assessing fungal N_2O production potential.

The variable presence/absence of introns within *p450nor* genes can lead to amplicon size variations and create additional difficulties in identifying *p450nor* amplicons. To assist in identifying a range of expected amplicon sizes, we analyzed the intron structure in *p450nor* sequences from 47 *p450nor*-containing fungal genomes (see Fig. S5 and Data Set S4 in the supplemental material). Overall, predicted *p450nor* amplicons contained between one and four introns and varied in size between 536 and 890 bp. Although introns or other genetic variations may prevent *p450nor* amplification, their presence within a primer binding region was observed in only a single case (*Glarea lozoyensis* strain ATCC 20868) (see Fig. S5 in the supplemental material). The observed intron in *G. lozoyensis* is more likely due to low transcript coverage at the *p450nor* locus, since the forward primer site (present in exons of all other fungi examined) was detected within the predicted *p450nor* gene sequence. Primer sequence degeneracy contributes to nonspecific amplification, and thus, cloning and sequencing were required to confirm *p450nor* amplification. A general limitation of degenerate PCR primers is diminished binding efficiency leading to uneven amplification of target sequences (Table 1). In spite of these obstacles, all the sequenced amplicons fell in the expected 536- to 890-bp size range and were identified as *p450nor*. Importantly, primers *p450nor*394F/*p450nor*809R amplified *p450nor* from all N_2O-producing isolates, indicating the utility of this approach to target a broad diversity of fungal denitrifiers. In nine instances, *p450nor* was detected but N_2O was not produced by fungal isolates in liquid culture. Amplicon sequencing ruled out false positives, suggesting that the chosen cultivation conditions were not conducive to N_2O production in these isolates.

The seminested-PCR methodology provides additional confidence in assaying for the presence/absence of *p450nor* in soils where fungi may not be abundant at the time of sampling or fungal DNA is underrepresented in soil DNA extracts. Application of the seminested-PCR approach to a dilution series of genomic DNAs extracted from *A. terreus* strain NRRL 255, *A. flavus* strain NRRL 3357, and *Fusarium graminearum* indicated a 10- to 100-fold increase in sensitivity over the direct (i.e., nonnested) approach. Experiments with dilutions of genomic DNA suggested that the seminested-PCR approach yielded positive results when at least 3,000 to 24,000 *p450nor* gene copies were present per assay (data not shown). Thus, the nonnested-PCR approach may yield false-negative results (inability to detect *p450nor* when present), and failure to detect *p450nor* amplicons in isolate or environmental DNA samples should be interpreted accordingly.

Diversity of the denitrifying fungi. It is widely accepted that the majority of soil bacteria cannot be cultivated by conventional methods (59). Although this issue may not be as extensive for fungi, only approximately 2 to 7% of the estimated 1.5 to 5.1 million fungal species have been cultivated (60, 61). Furthermore, only a few hundred of the 100,000 documented fungal species have been tested for the ability to produce N_2O from N oxyanions. Our knowledge of fungi capable of denitrification is constantly expanding, and fungal representatives spanning eight orders and three phyla have now been documented to produce N_2O (33, 58), including a diversity of organisms examined here. Therefore, it is very likely that a far greater diversity of fungi contributing to this process exists in nature than is currently acknowledged.

The enrichment and isolation techniques utilized in the present study resulted in the cultivation of fungal taxa largely representative of currently classified fungal denitrifiers. For example, several members of the genera *Fusarium* and *Trichoderma*, both known to harbor species capable of N_2O production, were cultured (8, 17, 18). Also identified were two *Mortierella* isolates (Mortierellomycotina, formally Zygomycota), a genus that includes members known to produce N_2O (33, 58). Only one of the two *Mortierella* isolates produced N_2O in liquid culture after 12 days of incubation, indicating that not all members of the genus may share this phenotype. Several other fungal isolates possessed ITS regions highly similar (>99% similarity) to those of known denitrifiers, but not all produced N_2O under the cultivation conditions tested (see Data Set S3 in the supplemental material). Similarly, a recent study demonstrated that only 50 of 113 species in the order *Hypocreales*, many belonging to the same genus (i.e., *Fusarium*), tested positive for N_2O production, suggesting the denitrifying phenotype varies even among closely related fungi (58).

N_2O formation in soil microcosms and enrichments. The Havana and Urbana site microcosms amended with NO_2^- consistently produced N_2O, but N_2O formation from NO_3^- was limited to Urbana soil microcosms. Possible explanations for this observation include a lack of fungal denitrifiers possessing nitrate reductase (*nar* and *nap*) genes, inhibition of fungal nitrate reductase activity by antibiotics intended to inhibit bacterial denitrifiers (62), and the rapid reduction of NO_3^- to ammonium in Havana soils (63). Of note, the N_2O concentration declined or N_2O was not detected in Urbana microcosms (see Fig. S1 and S2 in the supplemental material), likely because bacterial denitrifiers were not completely inhibited by the addition of antibiotics and consumed the available N_2O. In support of this explanation, inoculation of solid medium with suspensions from Urbana soil microcosms resulted in the formation of colonies of presumably antibiotic-resistant bacteria. Microscopic observations corroborated bacterial growth on antibiotic-treated solid medium. Incomplete inhibition of all or specific microbial processes is a known weakness of SIRIN analyses and may explain the observed variation in N_2O production between sites (13, 17, 64, 65). The observed variability in NO_3^- reduction is consistent with other studies describing a diversity of fungi that are incapable of reducing NO_3^- to N_2O (7, 10). Interestingly, most sequenced genomes of fungal denitrifiers possess a putative *napA* gene encoding periplasmic nitrate reductase (3), but a detailed understanding of the variation in fungal NO_3^- reduction capacity has yet to be obtained. Overall, the observed production of N_2O in microcosms established with Havana and Urbana soils is in line with the magnitude and variability observed in other studies, in which antibiotics were employed to probe the fungal contribution to N_2O production (8, 13, 18).

Low sequence depth limits detection of fungal sequences in metagenomes. *p450nor* was not detected in Havana and Urbana metagenomes, but fungal presence was established by the presence of fungal ITS sequences (~5,200 and 3,400 reads, respectively) and alignment of >200 million metagenomic reads to the fungal-isolate ITS regions (~200 reads in both metagenomes). The newly designed primer sets amplified *p450nor* genes using soil DNA extracted from the same soil samples used for metagenomics analysis, emphasizing their utility to screen for fungal denitrification potential. In most documented cases, a single *p450nor* gene copy was found per fungal genome, although exceptions have been noted (e.g., three *p450nor* gene copies were detected in *Nectria haematococca* [Fig. 2]). Not surprisingly, the detection of single-copy protein-encoding genes in metagenomics data sets is more challenging than detecting high-copy-number ribosomal operons (which includes the fungal ITS region) (66). Hence, current metagenomics approaches may not be optimal for detection of sequences from organisms that contribute small amounts of DNA (e.g., fungi) or are present in low abundance, and increased sequencing depth of environmental DNA samples is needed to uncover the functional potential of fungi in soils (67–69).

Conclusions. A curated set of 38 *p450nor* sequences enabled the design of degenerate PCR primers, which were demonstrated to effectively uncover fungal N_2O production potential in denitrifying isolates and soil DNA samples. Hence, the new *p450nor*-targeted primers complement existing primer sets to assess fungal NO_2^- reduction potential (34, 58). Given the potential biases associated with DNA extraction and direct-sequencing methodologies (e.g., metagenomics analysis), approaches that rely on the targeted amplification of low-abundance, process-specific genes are crucial for unraveling the functional potential of microorganisms in soils.

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