

Phylogenomics Reveal the Dynamic Evolution of Fungal Nitric Oxide Reductases and Their Relationship to Secondary Metabolism

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Abstract

Fungi expressing P450nor, an unconventional nitric oxide (NO) reducing cytochrome P450, are considered significant contributors to environmental nitrous oxide (N₂O) emissions. Despite extensive efforts, fungal contributions to N₂O emissions remain uncertain. For example, the majority of N₂O emitted from antibiotic-amended soil microcosms is attributed to fungal activity, yet axenic fungal cultures do not couple N-oxyanion respiration to growth and these fungi produce only minor quantities of N₂O. To assist in reconciling these conflicting observations and produce a benchmark genomic analysis of fungal denitrifiers, genes underlying denitrification were examined in >700 fungal genomes. Of 167 *p450nor*—containing genomes identified, 0, 30, and 48 also harbored the denitrification genes *narG*, *napA*, or *nirK*, respectively. Compared with *napA* and *nirK*, *p450nor* was twice as abundant and exhibited 2–5-fold more gene duplications, losses, and transfers, indicating a disconnect between *p450nor* presence and denitrification potential. Furthermore, cooccurrence of *p450nor* with genes encoding NO-detoxifying flavohemoglobins (Spearman $r=0.87$, $p=1.6e^{-10}$) confounds hypotheses regarding P450nor's primary role in NO detoxification. Instead, ancestral state reconstruction united P450nor with actinobacterial cytochrome P450s (CYP105) involved in secondary metabolism (SM) and 19 (11%) *p450nor*-containing genomic regions were predicted to be SM clusters. Another 40 (24%) genomes harbored genes nearby *p450nor* predicted to encode hallmark SM functions, providing additional contextual evidence linking *p450nor* to SM. These findings underscore the potential physiological implications of widespread *p450nor* gene transfer, support the undiscovered affiliation of *p450nor* with fungal SM, and challenge the hypothesis of *p450nor*'s primary role in denitrification.

Key words: P450nor, fungi, nitrous oxide, secondary metabolism, nitrogen cycle, horizontal gene transfer.

Introduction

Since the early 20th century, increased human reliance on fixed nitrogen (N) for agricultural practices has contributed to a 20% increase in atmospheric nitrous oxide (N₂O), a potent greenhouse gas with ozone destruction potential (Galloway et al. 2008; Canfield et al. 2010). N₂O is primarily formed by denitrifying members of the *Bacteria* (Zumft 1997), a prevailing view that has been challenged by experiments

reporting that abundant soil- and sediment-inhabiting fungi contribute up to 89% of the total N₂O emitted from these systems (Cathrine and Raghukumar 2009; Mothapo et al. 2015; Wankel et al. 2017). Notably, fungi cannot convert N₂O to inert N₂ like many denitrifying bacteria (Shoun et al. 2012), suggesting their contributions to greenhouse effects and ozone destruction could be significant. Fungi are considered to be important sources of N₂O emissions from

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agroecosystems (Mothapo et al. 2013; Chen et al. 2014), which are predicted to contribute up to two-thirds of the total N₂O emissions by 2030 (Hu et al. 2015). Studies of model fungi show that N₂O formation is due to P450nor, a heme-containing cytochrome P450, that catalyzes the two electron reduction of nitric oxide (NO) to N₂O (Nakahara et al. 1993; Shiro et al. 1995; Shimizu et al. 2002). N₂O formation by P450nor is thought to occur exclusively in fungi and the *p450nor* gene has been exploited as a distinctive biomarker in molecular assays to study fungal denitrifier diversity and abundance in the environment (Higgins et al. 2016; Li et al. 2016; Novinscak et al. 2016).

Despite these observations, the fungal contributions to N₂O emissions remain uncertain. For example, fungi do not satisfy criteria set forth to classify microorganisms as respiratory denitrifiers (Mahne and Tiedje 1995). N₂O-producing fungi in pure culture do not exhibit a balance between the inorganic N inputs and quantities of N₂O formed (Bleakley and Tiedje 1982; Shoun 1992; Tsuruta et al. 1998) and possess three to six orders of magnitude lower rates of N₂O production compared with denitrifying bacterial isolates under optimal conditions (Mothapo et al. 2015). Moreover, fungi fail to generate anoxic growth yields proportional to the quantity of inorganic N reduced in pure culture (Shoun and Tanimoto 1991; Zhou et al. 2001; Cathrine and Raghukumar 2009; Stief et al. 2014), and no significant relationship was detected between fungal denitrification activity and fungal biomass in anoxic soil incubations (Herold et al. 2012). Above all, partitioning techniques (antibiotic inhibition and isotope site preference) used to estimate fungal and bacterial contributions to N₂O emissions are biased and often lack corroborating evidence in conjunction with their application, suggesting fungal contributions to N₂O emissions are substantially inflated (Rousk et al. 2009; Ladan and Jacinthe 2016; Phillips et al. 2016; Wankel et al. 2017).

The capacity for N₂O-production conferred by *p450nor* in fungi is a uniquely eukaryotic trait, yet previous investigations have hypothesized an actinobacterial origin for *p450nor* based on sequence comparisons (Kizawa et al. 1991; Moktali et al. 2012; Shoun et al. 2012; Chen et al. 2014). Of note, *Actinobacteria* are not considered canonical denitrifying bacteria, and only a few reports of their denitrification capacity exist (Shoun et al. 1998; Kumon et al. 2002; Shapleigh 2013). Most members of the *Actinobacteria* possess a truncated denitrification pathway or lack a canonical nitric oxide reductase gene (*nor*) (with the exception of *Corynebacterium* and *Propionibacterium*) (Kumon et al. 2002; Shapleigh 2013). Hence, members of the Fungi and *Actinobacteria* share an incomplete denitrification pathway with a potentially limited capacity to perform denitrification. Consistent with the horizontal gene transfer (HGT) hypothesis are sequence similarities between fungal P450nor and actinobacterial P450s of the CYP105 family, many of which have been investigated for their contributions to secondary

metabolism (SM) (Shoun et al. 2012; Moody and Loveridge 2014). Despite these observations, the prevailing hypothesis regarding *p450nor*'s evolution and function was its acquisition from the *Actinobacteria* and subsequent evolution to fill a novel role in denitrification, specifically the reduction of NO to N₂O (Kizawa et al. 1991; Shoun et al. 2012). The hypothesis that *p450nor* was acquired from one or more members of the *Actinobacteria* and retained an ancestral function in SM surprisingly remains unexplored.

Therefore, the overarching questions this study addresses are 1) what is the breadth of denitrification genes across fungal genomes and what are their evolutionary relationships, and 2) can phylogenomic analyses reconcile the conflict in fungal contributions to N₂O formation observed in laboratory and environmental settings? Our comparative genomic and phylogenetic analyses identified a disconnect between *p450nor* and denitrification gene presence and supported a role for P450nor in SM rather than denitrification. Importantly, these results provide an explanation for the minor, nonrespiratory capacity of fungi to form N₂O, and suggests N₂O is a byproduct of active SM. These findings transform our understanding of the ecological significance and environmental consequences of *p450nor* presence/absence in fungal genomes.

Materials and Methods

Data Sets

Draft and complete fungal, algal, and bacterial genomes were accessed from the National Center for Biotechnology Information and the Joint Genome Institute on March 16, 2016 and downloaded from their respective database utilities. A list of fungal, algal, and bacterial genomes and their taxonomic and database affiliations can be found at <https://doi.org/10.6084/m9.figshare.c.3845692.v4> (figshare Data Set S1, see Data Sharing).

Gene Marker Identification

To identify gene markers within fungal genomes suitable for phylogenetic analysis, a database of 1,438 amino acid sequences of fungal single copy orthologs from the BUSCO tool v1.1b (Simão et al. 2015) were provided as queries to the genblastG search tool v1.0.138 (She et al. 2011). The genblastG tool performs amino acid alignment of protein queries against a six frame translated nucleotide subject sequence (genome) to find significant alignments and uses heuristic analysis to piece the appropriate gene models back together from high-scoring segment pairs identified using BLAST (Altschul et al. 1990). Of the BUSCO gene models queried, 238 were used for phylogenetic tree reconstruction and were annotated using PfamScan against the Pfam A database and blastp against the uniprot database with default settings (Mistry et al. 2007; Finn et al. 2010, 2015; Li et al. 2015)

(figshare Data Set S2, see Data Sharing). The genblastG tool was also used to detect gene sequences involved in denitrification (*NapA*, *NarG*, *NirK/NirS*, *NorB*, *P450nor*, and *NosZ*) from curated bacterial and fungal proteins in the FunGene repository (Fish et al. 2013) or proteins involved in NO detoxification (flavo-hemoglobins) identified in the literature (Wisecaver et al. 2016). Denitrification gene models used in downstream phylogenetic analysis were manually curated against full length fungal reference sequences to ensure that accurate gene models were predicted for each organism in which the gene was detected. After identification of these genes in fungal genomes, alignment of the fungal *NapA*, *NirK*, *NarG*, and *P450nor* amino acid sequences with blastp against the plant, archaea, bacteria, protozoa, and fungi RefSeq protein databases (Pruitt et al. 2007) was performed to identify similar sequences in each taxonomic group. Protein sequences demonstrating significant alignment ($\geq 60\%$ query coverage and $\geq 35\%$ amino acid identity) to fungal proteins were used in subsequent phylogenetic reconstructions.

Gene Prediction for Comparative Genomic Analyses

The ab initio gene predictor SNAP (Korf 2004) was used to predict gene models in fungal genomes where no such information was available (e.g., some draft genomes). In this case, one or several closely related fungal genomes containing gene models were selected based on phylogenetic affiliation to train SNAP for gene prediction. Although this methodology is limiting when closely related genomes are unavailable, gene models from close relatives were available for *p450nor*-containing genomes lacking gene predictions. All gene predictions performed with SNAP are provided in a FigShare repository (see *Data Sharing* below).

Analysis of SM Gene Clusters in Fungi

Genomic regions 50 kb up- and downstream of a *p450nor* gene in each genome were subjected to gene cluster prediction with the antiSMASH and CASSIS tools with default settings (Inglis et al. 2013; Wolf et al. 2016). Additionally, genes encoded ± 10 genes up and downstream of *p450nor* were evaluated using PfamScan searches with default settings against the profile Hidden Markov Models (pHMMs) of curated SM genes identified by antiSMASH (figshare Data Set S3, see Data Sharing) (Inglis et al. 2013). Protein sequences with significant alignment to antiSMASH pHMMs were given an "automatic" SM function status and were colored blue. In order to supplement the automated SM annotation, additional functional annotation was performed by hmmscan searches with HMMER3 (Eddy 2009) against the eggNOG database (Powell et al. 2012). These functional annotations were manually flagged as related to SM if they possessed literature entries suggesting an involvement in SM or had functions related to methyl transfer, oxidation–reduction

reactions, glycosyl transferases, fungal specific transcription factors, and other protein functions that may be important for SM outlined by antiSMASH (Inglis et al. 2013). All manual SM annotations were colored light blue to indicate potential involvement in SM. All other annotations were colored grey when no evidence connecting the function to SM could be identified.

Pairwise nucleotide alignments of *p450nor*-containing genomic regions were performed as previously described (Richter and Rosselló-Móra 2009). Briefly, the *nucmer* utility of MUMmer v3.0 (Kurtz et al. 2004) was used to align *p450nor*-containing genomic regions (~ 100 kb) against whole genomes of fungi with and without *p450nor*. The average nucleotide identity (reported as ANIm) of the alignment was calculated from the resulting delta output file. The resulting data was plotted using Matplotlib (Hunter 2007) available for the python programming language (<http://www.python.org>).

Phylogenetic Analysis

Phylogenetic reconstruction of the fungal species tree was performed using concatenated amino acid sequences from 238 single copy orthologs found in $\geq 90\%$ of all genomes (figshare Data Set S2, see Data Sharing). The genomes of *Puccinia arachidis* and *Microbotryum lychnidis-dioicae* strain p1A1 Lamole were excluded from further analysis due to an insufficient number of informative sites and inconsistent placement within the fungal tree. Alignment of amino acid sequences were performed individually on all 238 individual BUSCO gene models present within each organism using MAFFT v7.130b (Katoh et al. 2009) with linsi alignment tuning parameters ($-\text{maxiterate } 1000$ and $-\text{localpair}$ settings used). Individual alignments were concatenated using in-house python scripts, resulting in a 65, 897 column alignment. Tree reconstruction was performed using FastTree2 (Price et al. 2010) with refined tree reconstruction settings for a slower, more exhaustive search of the tree space than default settings ($-\text{bionj } -\text{slow } -\text{gamma } -\text{spr } 4 -\text{lg } -\text{mlacc } 2$ and $-\text{slownni}$ settings) (Wisecaver et al. 2016). For comparison to tree reconstruction using a concatenated alignment, individual trees from each BUSCO alignment were also constructed using FastTree2 with identical settings as above. The resultant alignments and trees were subjected to coalescent tree reconstruction using ASTRAL-II software (Mirarab and Warnow 2015). Overall, both phylogenies largely agreed except for branching patterns of some lineages (e.g., Zoopagomycota and Mucoromycota) and are available online in a Figshare repository (see *Data Sharing* below).

The predicted amino acid and intronless nucleotide sequences of fungal *napA*, *nirK*, and *p450nor* gene models were aligned using the MAFFT settings described above and manually refined in JalView and SeaView software (Galtier et al. 1996; Waterhouse et al. 2009). Maximum-likelihood

(ML) and Bayesian phylogenetic tree reconstruction was performed on both nucleotide and amino acid alignments using RAxML and MrBayes, respectively (Stamatakis 2006; Ronquist et al. 2012). For details regarding phylogenetic tree parameters, please refer to [Supplementary Material](#).

BayesTraits software was used to perform phylogenetically informed correlations between binary traits (i.e., the presence or absence of two denitrification markers) and ancestral state reconstruction (Pagel et al. 2004). Please refer to [Supplementary Material](#) for additional details on BayesTraits analyses.

Approximately unbiased (AU) tests were performed in the program CONSEL (Shimodaira and Hasegawa 2001) using default settings. The negative log likelihood values from the observed nucleotide phylogenies input into CONSEL were $-140,261$, $-37,782$, $-111,531$ for *napA*, *nirK* and *p450nor*, respectively. The observed negative log likelihood scores for amino acid phylogenies of NapA, NirK, and P450nor were $-65,158$, $-14,197$, and $-44,171$, respectively. Species-tree gene-tree reconciliation was performed using NOTUNG software v2.9 (Chen et al. 2000; Stolzer et al. 2012). Please see [Supplementary Material](#) for further details on NOTUNG parameters.

Statistical Analyses

All statistical analyses were carried out in R programming language (Team 2012) and significance of statistical tests were assessed using a *P* value cutoff ≤ 0.05 .

Data Sharing

All [Supplementary Material](#) figures, tables, and data sets, as well as gene models, alignments, and trees reported in the manuscript are made available in a Figshare data collection prepared by S.A.H. at <https://doi.org/10.6084/m9.figshare.c.3845692.v4>, last accessed September 3, 2018.

Results

Infrequent Cooccurrence among Denitrification Genes in Fungi

Bioinformatic analyses identified homologs of canonical bacterial and fungal denitrification genes (*narG*, *napA*, *norB*, *nirK*, *nosZ*, and *p450nor*) in 712 fungal genomes. Of the denitrification gene set investigated, only *narG*, *napA*, *nirK*, and *p450nor* were detected (fig. 1). Genes encoding the membrane bound respiratory nitrate reductase (*narG*) were detected in only three fungal genomes (0.42%) and were excluded from further analysis due to their low occurrence. The genes predicted to encode the periplasmic nitrate reductase (NapA) and the copper-containing nitrite reductase (NirK) were detected in 75 (10.5%) and 82 (11.5%) of the 712 fungal genomes analyzed, respectively (fig. 1 and

[supplementary table S1, Supplementary Material](#) online). In contrast, P450nor gene sequences occurred at approximately twice the frequency in 167 (23%) of the fungal genomes analyzed, supporting the claim that P450nor-mediated N₂O production may be widespread in fungi (fig. 1) (Maeda et al. 2015). A breakdown of genus- and family-level denitrification gene abundances in fungal genomes underscores the disparity in presence/absence of denitrification genes in fungi and is available in [Supplementary Material](#) (figshare Data Set S4, fig. S1).

Our analyses also revealed a low cooccurrence between *p450nor* and additional fungal denitrification pathway markers. Since *p450nor* is regarded as the sole trait encoding N₂O production in fungi, the cooccurrence of multiple denitrification gene markers would be indicative of a capacity for sequential respiratory denitrification, whereas isolated occurrences could be indicative of alternative processes such as detoxification. The three-gene set *narG/nirK/p450nor* did not cooccur in any of the fungal genomes examined, whereas cooccurrence of the gene set *napA/nirK/p450nor* was observed in 18 (10.8%) of 167 *p450nor*-containing fungal genomes. Sets of at least two cooccurring denitrification traits (i.e., *narG/p450nor*, *napA/p450nor*, and *nirK/p450nor*) were found in 0, 18 and 29% of fungal genomes, respectively. Of the *napA*-containing fungal genomes, 25 (33%) also contained a *nirK* gene, whereas 30% of the *nirK*-containing fungal genomes also harbored a *napA* gene. Evolutionary correlation was strongly supported for the gene sets *napA/nirK*, *napA/p450nor*, and *nirK/p450nor*, with average log Bayes Factor values of 31.9 ± 0.60 , 12.2 ± 0.11 , and 31.3 ± 0.04 , respectively. Hence, the genes *napA*, *nirK*, and *p450nor* occur in related fungal taxa, but cooccurrences were infrequent within the individual fungal genomes analyzed.

Evolutionary Forces Acting upon Denitrification Traits within Fungi

To identify evolutionary forces shaping the observed distribution of denitrification traits within fungi, comparisons between gene and species trees were assessed with phylogenetic tests and parsimony-informed models to quantify evolutionary events. Visual inspection of *p450nor* gene and species trees indicated potential widespread HGT of *p450nor* within fungi, examples of which included HGT of *p450nor* from the phylum Ascomycota to members of the Basidiomycota and within and among classes of ascomycetes ([supplementary fig. S2, Supplementary Material](#) online). Furthermore, the monophyly of five fungal classes containing *p450nor* (Dothideomycetes, Eurotiomycetes, Leotiomycetes, Sordariomycetes, and Tremellomycetes) were not supported by approximately unbiased (AU) tests ($P \leq 0.05$, [supplementary table S2, Supplementary Material](#) online), indicative of dynamic evolution of *p450nor* in most fungal lineages. Although cophylogeny plots are suggestive

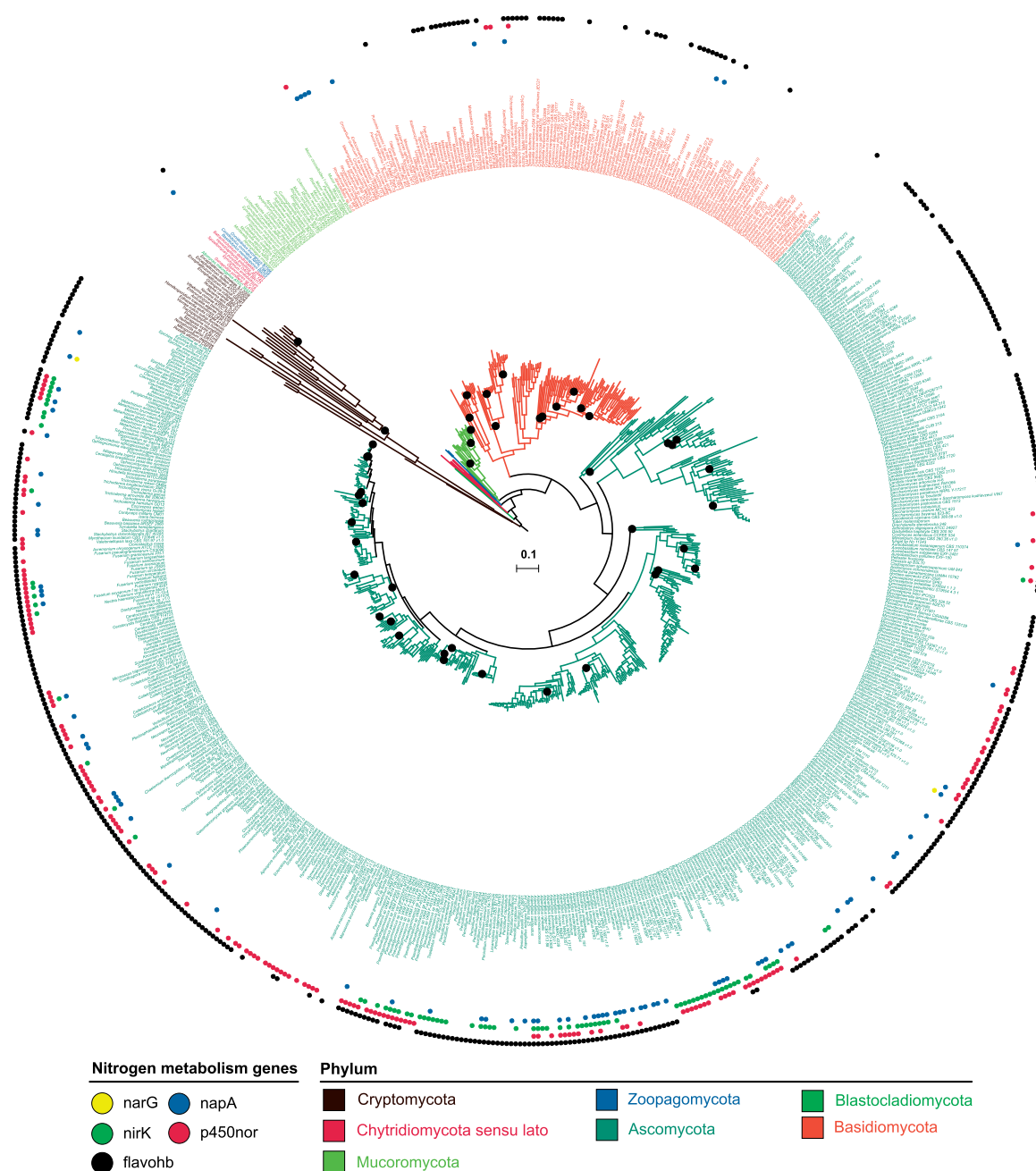


Fig. 1.—Maximum-Likelihood phylogeny of the kingdom Fungi inferred from a concatenated alignment of 238 single copy marker gene amino acid sequences (see Materials and Methods). Black circles marking branches indicate nodes with bootstrap percentages below 90%. Colored markers outside taxon names specify the presence or absence of each gene (*narG*, *napA*, *nirK*, *p450nor*, and flavohemoglobin) within a fungal genome. Flavohb, flavohemoglobin genes involved in NO detoxification. The scale bar (center of tree) represents amino acid substitutions per site.

of HGT, additional analysis using NOTUNG software was performed to model potential gene duplication (GD), gene transfer (GT), and gene loss (GL) events (Stolzer et al. 2012). Of the *napA*, *nirK*, and *p450nor* genes analyzed, the *p450nor* phylogenies had the greatest number of predicted GT events, ranging from 4 to 15 GT events despite applying stringent GT costs within NOTUNG software

(supplementary table S3, Supplementary Material online). At GT costs below 9, no temporally consistent optimal solutions were reached, suggesting that GD and GL alone are insufficient to describe the evolutionary dynamics of *p450nor* in fungi. Using the same stringent GT costs, the predicted number of GT events detected for *napA* and *nirK* were much lower, and ranged from 1 to 3 and 0 to 1 GT

events for each gene, respectively (supplementary table S3, Supplementary Material online). The reduced number of GT events detected in *napA* and *nirK* phylogenies were also apparent from cophylogeny plots of each gene (supplementary figs. S3 and S4, Supplementary Material online) compared with cophylogenetic plots for *p450nor* (supplementary fig. S2, Supplementary Material online). Although GT events detected for *napA* were lower than *p450nor* at high GT costs, GT may still represent a significant evolutionary force contributing to the observed *napA* distribution in extant fungal lineages (supplementary table S3, Supplementary Material online). For example, AU tests rejected the monophyly of three Ascomycota (Dothideomycetes, Leotiomycetes, and Sordariomycetes) and one Basidiomycota (Pucciniomycetes) lineage within the *napA* phylogeny (supplementary table S2, Supplementary Material online, $P \leq 0.05$). Specific instances of predicted HGT events for each gene are outlined in Supplementary Material (supplementary table S4 and fig. S5, Supplementary Material online).

Fungal P450nor Evolved from Actinobacterial P450s Involved in SM

Previous investigations have hypothesized an actinobacterial origin for *p450nor* based on amino acid sequence alignments (Moktali et al. 2012; Shoun et al. 2012; Chen et al. 2014), but rigorous phylogenetic tests of *p450nor*'s origins were lacking to support this hypothesis. Alignment of fungal P450nor amino acid sequences to the NCBI RefSeq protein database identified 230 bacterial sequences with significant sequence alignment ($\geq 65\%$ query coverage and $\geq 35\%$ amino acid identity) to P450nor. Of note, *p450nor* homologs were also detected within the genomes of three freshwater inhabiting green algae, *Chlorella variabilis*, *Chlamydomonas reinhardtii*, and *Monoraphidium neglectum*, expanding the known distribution of *p450nor* to photosynthetic eukaryotic microbes. Additional *p450nor* homologs were not detected in archaea, plant, protist, or other lineages housed within the RefSeq database. Of the bacterial cytochrome P450 (hereafter P450) sequences identified, approximately 6% ($n = 13$) were proteobacterial in origin, whereas the remaining sequences belonged to members of the bacterial phylum *Actinobacteria* (supplementary fig. S6, Supplementary Material online). Ancestral character state reconstruction of select P450 families on a subset of these sequences supported the monophyly of *p450nor* and bacterial P450 gene sequences of the P450 family CYP105 (fig. 2) (Nelson 2009). The same relationships were preserved when phylogenetic reconstruction was performed using the complete set of 408 P450 amino acid sequences (supplementary fig. S7, Supplementary Material online). Importantly, NO-utilizing P450 sequences from the CYP107 family belonging to members of the *Streptomyces* formed a larger monophyletic clade containing P450nor and other CYP105 sequences (supplementary

fig. S7, Supplementary Material online). The CYP107 family includes *txtE* genes encoding nitrating enzymes that use NO as a substrate for the production of secondary metabolites and have no known role in respiratory denitrification or detoxification (Barry et al. 2012; Dodani et al. 2014). Thus, P450nor and TxtE are related (Barry et al. 2012; Dodani et al. 2014), yet TxtE is involved in SM and is the only other P450 observed to directly utilize NO as a substrate.

Sequences of the bacterial CYP105 family of P450s include diverse actinobacterial genera such as *Streptomyces* ($n = 159$), *Amycolatopsis* ($n = 12$), *Saccharothrix* ($n = 5$), *Streptacidiphilus* ($n = 4$), *Frankia* ($n = 4$), *Kutzneria* ($n = 4$), *Nocardia* ($n = 3$), and members from 17 additional actinobacterial genera ($n = 39$). The proteobacterial sequences were affiliated with members of the genera *Burkholderia* ($n = 5$), *Paracoccus* ($n = 3$), *Bradyrhizobium* ($n = 3$), *Pseudomonas* ($n = 1$), and *Halomonas* ($n = 1$). Bacterial P450 gene and species tree comparisons of 60% identity clustered P450 amino acid sequences ($n = 57$) and cognate 16S rRNA genes ($n = 55$) supported HGT of one or more actinobacterial P450 genes to members of the *Alpha*-, *Beta*-, and *Gamma*proteobacteria (supplementary fig. S6, Supplementary Material online). Furthermore, ancestral character state reconstruction overwhelmingly supported *Actinobacteria* as the root state (root probability = 0.99 ± 0.06) of the bacterial CYP105 family P450 phylogeny. When forcing the root state of the P450 phylogeny to be *Proteobacteria* (simple model) and comparing to the complex model where the root is allowed to vary, the simpler model with a proteobacterial root was not supported (average log Bayes Factor = 0.03 ± 0.18). Therefore, *p450nor* likely evolved from one or more CYP105 family P450 genes found in members of the *Actinobacteria*. This finding underscores *p450nor*'s distinct origin compared with the fungal denitrification traits *napA* and *nirK*, which exhibit a proteobacterial ancestry consistent with the majority of bacterial denitrifiers (supplementary fig. S8, Supplementary Material online).

Widespread Cooccurrence of *p450nor* and NO-Detoxifying Flavohemoglobins

Poor conversion of inorganic N-oxides to N_2O by fungal isolates supports the hypothesis that P450nor is involved in NO detoxification (Morozkina and Kurakov 2007; Shoun et al. 2012). Fungi also possess NO-detoxifying flavohemoglobins responsible for detoxification of NO to NO_3^- under oxic conditions or NO to N_2O under anoxic conditions (Poole and Hughes 2000; Morozkina and Kurakov 2007; Wisecaver et al. 2016). Flavohemoglobins were detected in 450 (63%) fungal genomes investigated and were widespread within ascomycete and basidiomycete fungi. Within *p450nor*-containing genomes, 125 (75%) also possessed a flavohemoglobin gene (fig. 1 and supplementary table S1, Supplementary Material online). Furthermore, the number of genomes in

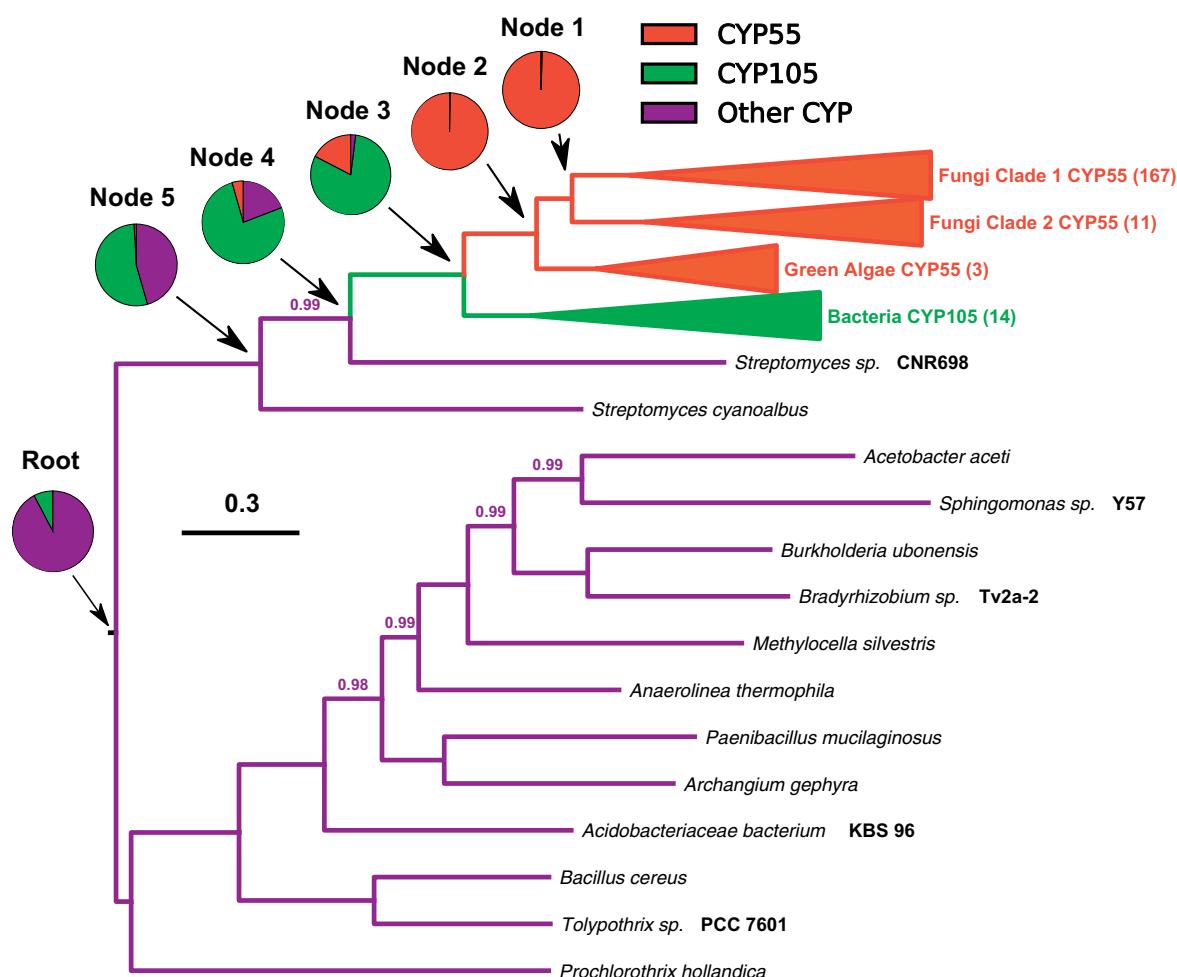


Fig. 2.—Midpoint-rooted Bayesian phylogeny of select families of cytochrome P450 amino acid sequences from fungi, algae, and bacteria. Ancestral state reconstruction was performed using CYP55 (orange), CYP105 (green), and other CYPs (purple) to uncover the shared ancestry of algal and fungal N_2O -producing cytochrome P450s with their most recent common bacterial ancestor. The scale bar indicates substitutions per site and posterior probability values <1 are displayed above branches of the Bayesian MCMC analysis. Numbers in parentheses next to collapsed clades indicate the number of sequences in the clade. Values in pie charts are average probabilities of each character state across one representative Bayesian MCMC analysis.

fungal families containing *p450nor* and NO-detoxifying flavohemoglobin genes were significantly correlated (Spearman $r = 0.87$, $p = 1.6e^{-10}$), suggesting a competitive or compensatory functioning of P450nor with flavohemoglobins in fungi where they coexist.

Evidence of a Role for *p450nor* in Secondary Metabolism

p450nor is actinobacterial in origin, yet *Actinobacteria* are not considered canonical denitrifiers and evidence for their role in denitrification was lacking when *p450nor* was initially identified (Kaspar 1982; Kizawa et al. 1991). Subsequent investigations did not posit a role for *p450nor* in SM despite the affiliation of *p450nor* and CYP105 P450s with documented roles in SM (O'Keefe and Harder 1991; Moody and Loveridge 2014). To assess genomic evidence linking *p450nor* to SM,

we queried genes encoded within genomic regions approximately 50 kb on either side of *p450nor* for functions related to SM. The biosynthetic gene cluster (BGC) prediction tool antiSMASH detected putative BGCs containing *p450nor* in 19 (11%) of the 167 *p450nor*-containing genomes analyzed (figshare Data Set S3). The number of open reading frames in a predicted SM cluster ranged from 34 to 97, spanning 21,086 to 55,473 nucleotides in length. Inspection of protein-coding genes surrounding *p450nor* using curated antiSMASH pHMMs resulted in the identification of hallmark SM features (e.g., polyketide synthases (PKS), nonribosomal peptide synthases (NRPS), terpene cyclases, dimethylallyl tryptophan synthases) in an additional 40 (24%) of the 167 *p450nor*-containing genomes analyzed (figshare Data Set S3, see Materials and Methods for details). The distribution of automatic and manually curated protein-coding genes

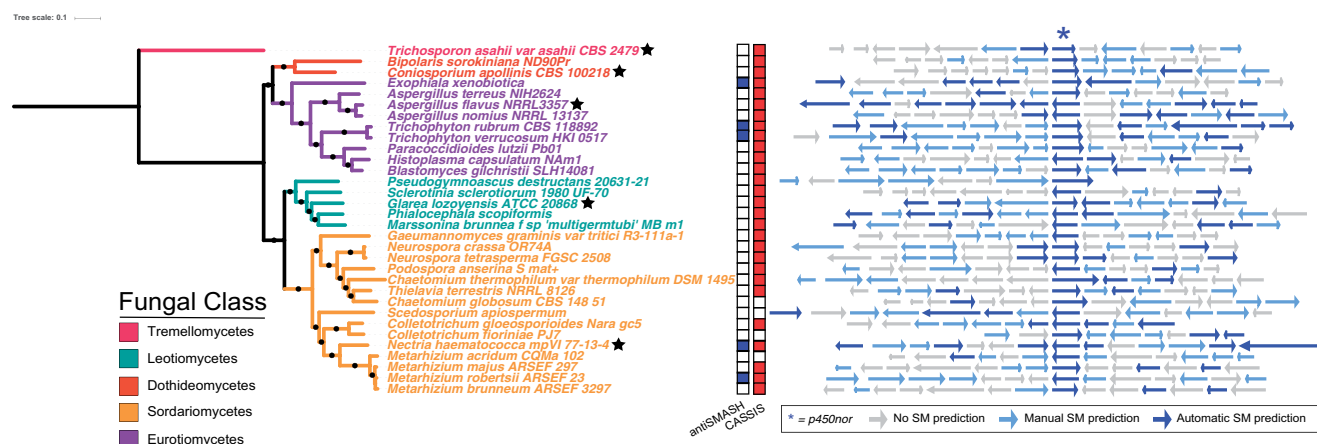


Fig. 3.—SM gene cluster predictions for a subset of 32 (167 total) *p450nor*-containing fungi. The boxes to the right of the rooted Maximum-Likelihood phylogeny indicate whether the *p450nor*-containing genomic region was predicted by antiSMASH (blue squares) or CASSIS (red squares) to be an SM gene cluster. White squares indicate no prediction. Colored arrows indicate protein-coding genes surrounding *p450nor* that were automatically predicted (dark blue arrow), manually predicted (light blue arrow) or not predicted (grey arrow) to be involved in SM (see Materials and Methods for details). The black stars next to species names are individuals chosen for in depth presentation of the genes surrounding *p450nor* (supplementary fig. S9, Supplementary Material online).

surrounding a subset of 32 *p450nor*-containing fungi suggests that *p450nor*-containing BGCs are structurally and functionally diverse (fig. 3). An additional BGC prediction tool, CASSIS, which detects BGCs based on shared transcription factor binding sites upstream and downstream of a user specified anchor gene (*p450nor*) (Wolf et al. 2016), predicted as many as 105 (63%) *p450nor*-containing gene regions to be BGCs (figshare Data Set S5). Furthermore, CASSIS analysis corroborated 74% of the 19 BGCs predicted by antiSMASH (figshare Data Set S5). A detailed accounting of antiSMASH and CASSIS predictions, gene annotations, and gene organization surrounding *p450nor* in all 167 *p450nor*-containing genomes is available in the Supplementary Material (supplementary fig. S9 and figshare Data Set S5). A diversity of secondary metabolite biosynthesis pathways were predicted to be encoded by *p450nor*-containing BGCs, including nonribosomal peptides ($n = 7$), polyketides ($n = 5$), terpenes ($n = 2$), hybrid terpene-polyketide-indoles ($n = 2$), indoles ($n = 1$), or currently unclassifiable compounds ($n = 2$). Phylogenetic reconstruction of C-type and ketosynthase domains encoded by NRPS and PKS genes surrounding *p450nor* enabled the prediction of potential secondary metabolites encoded by fungal *p450nor*-containing BGCs (fig. 4). Of the 94 fungal genera harboring *p450nor*, 21 (22%) genera contained species with and without a copy of *p450nor* (supplementary table S5, Supplementary Material online). Nucleotide alignments of *p450nor*-containing genomic regions (81.3 ± 27.8 kb in length) against other fungal genomes revealed a disproportionately high nucleotide identity and alignment length between genomes with and without *p450nor* from the same genus (fig. 5A–C). For example, genomic regions surrounding *p450nor* in *Exophiala xenobiotica* are highly conserved in other *Exophiala* species without *p450nor* (fig. 5D), and

additional examples of large, high identity regions between closely related fungal genomes with and without *p450nor* are abundant (fig. 5A–C and figshare Data Set S6).

Discussion

Hypotheses Regarding the Biological Role of *p450nor*

The leading hypotheses regarding the biological role of fungal *p450nor* are respiratory denitrification (Shoun et al. 2012), hybrid respiration of N-oxides and O₂ (Takaya et al. 2003), NO detoxification (Morozkina and Kurakov 2007), and based on the work presented here, secondary metabolism. The respiratory denitrification hypothesis is misleading since evidence is lacking to classify fungi as respiratory denitrifiers (Shoun 1992; Mahne and Tiedje 1995; Tsuruta et al. 1998; Guengerich and Munro 2013; Mothapo et al. 2015). Furthermore, unaccounted for methodological biases inherent to partitioning techniques, especially antibiotic inhibition, raises substantial concerns over the validity of fungal N₂O production in situ (Rousk et al. 2009; Mothapo et al. 2015; Ladan and Jacinthe 2016; Phillips et al. 2016). For example, site preference measurements of the intramolecular distribution of ¹⁵N within the linear N₂O molecule (i.e., N₂O isotopocules) of cultured microorganisms have been increasingly applied to partition microbial sources of N₂O in situ (Toyoda et al. 2017; Wankel et al. 2017). Although promising, the limitations of N₂O isotopocule measurements used in isolation are becoming apparent (Decock and Six 2013; Yang et al. 2014; Phillips et al. 2016). Of primary concern is the significant overlap in, and difficulty discretizing, site preference measurements of distinct processes or diverse microbial assemblages (Baggs 2008; Butterbach-Bahl et al. 2013; Yang et al. 2014).

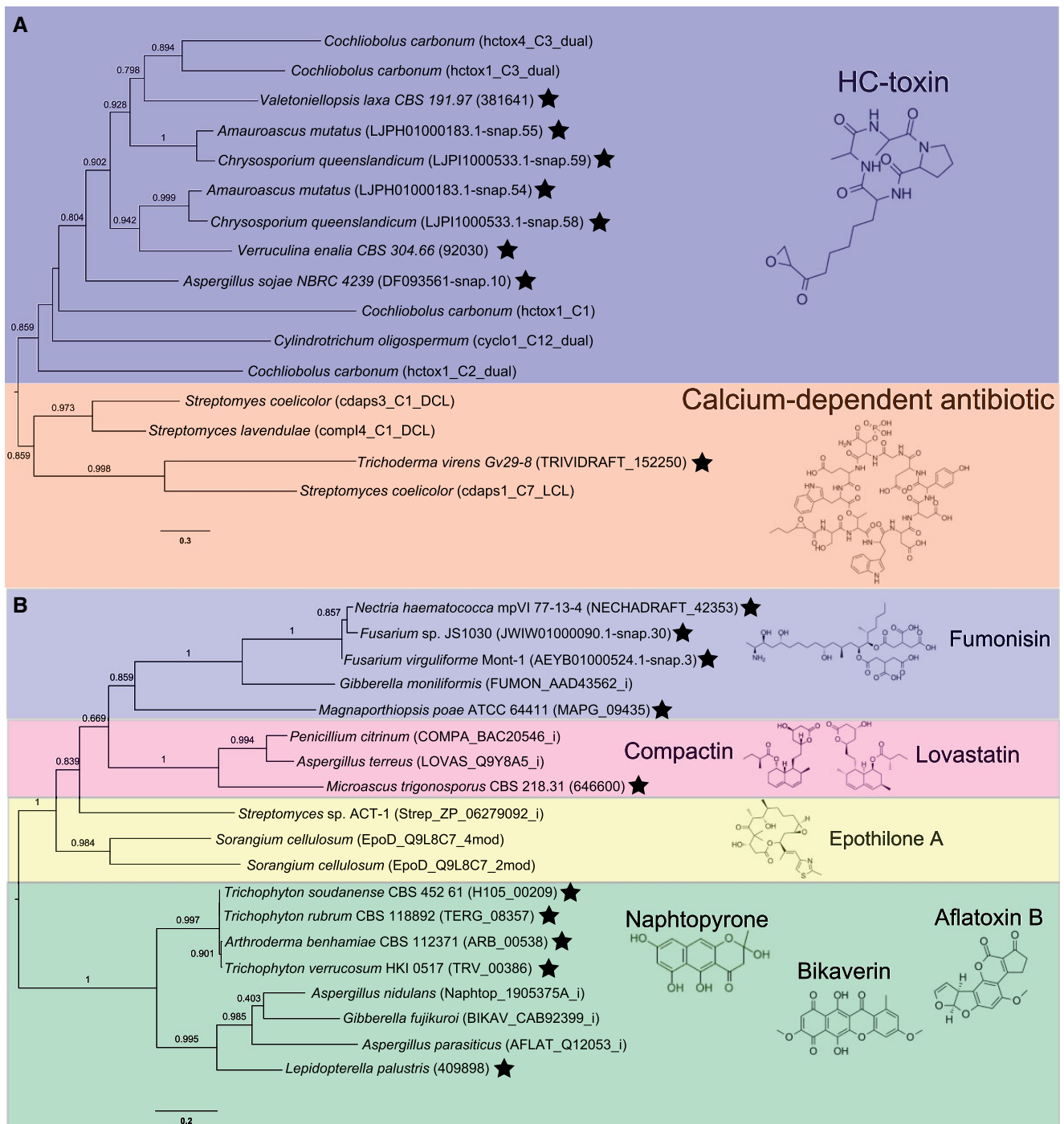


Fig. 4.—Maximum-Likelihood phylogenetic trees of nonribosomal peptide synthase and polyketide synthase domains encoded within *p450nor*-containing genomic regions. Each phylogeny displays relationships of C-type condensation (C-type) (A) or ketosynthase (KS) domains (B) detected in nonribosomal peptide and polyketide synthase amino acid sequences, respectively, encoded within *p450nor*-containing genomic regions. A black star next to taxa indicates C-type or KS domains identified in fungal genomes nearby *p450nor*. The NCBI or JGI accession numbers are shown in parentheses next to taxa with black stars. Taxa without black stars are reference amino acid sequences of C-type and KS domains curated by the NAPDOS database, and their NAPDOS accession numbers are indicated in parentheses. Proteins from species without accession numbers were predicted ab initio using SNAP (Korf 2004) (see Materials and Methods for details). Chemical structures and names of secondary metabolites produced by NAPDOS reference sequences are indicated and highlighted distinct colors for clarity. Scale bars indicate amino acid substitutions per site. Values along branches indicate bootstrap support for the adjacent node.

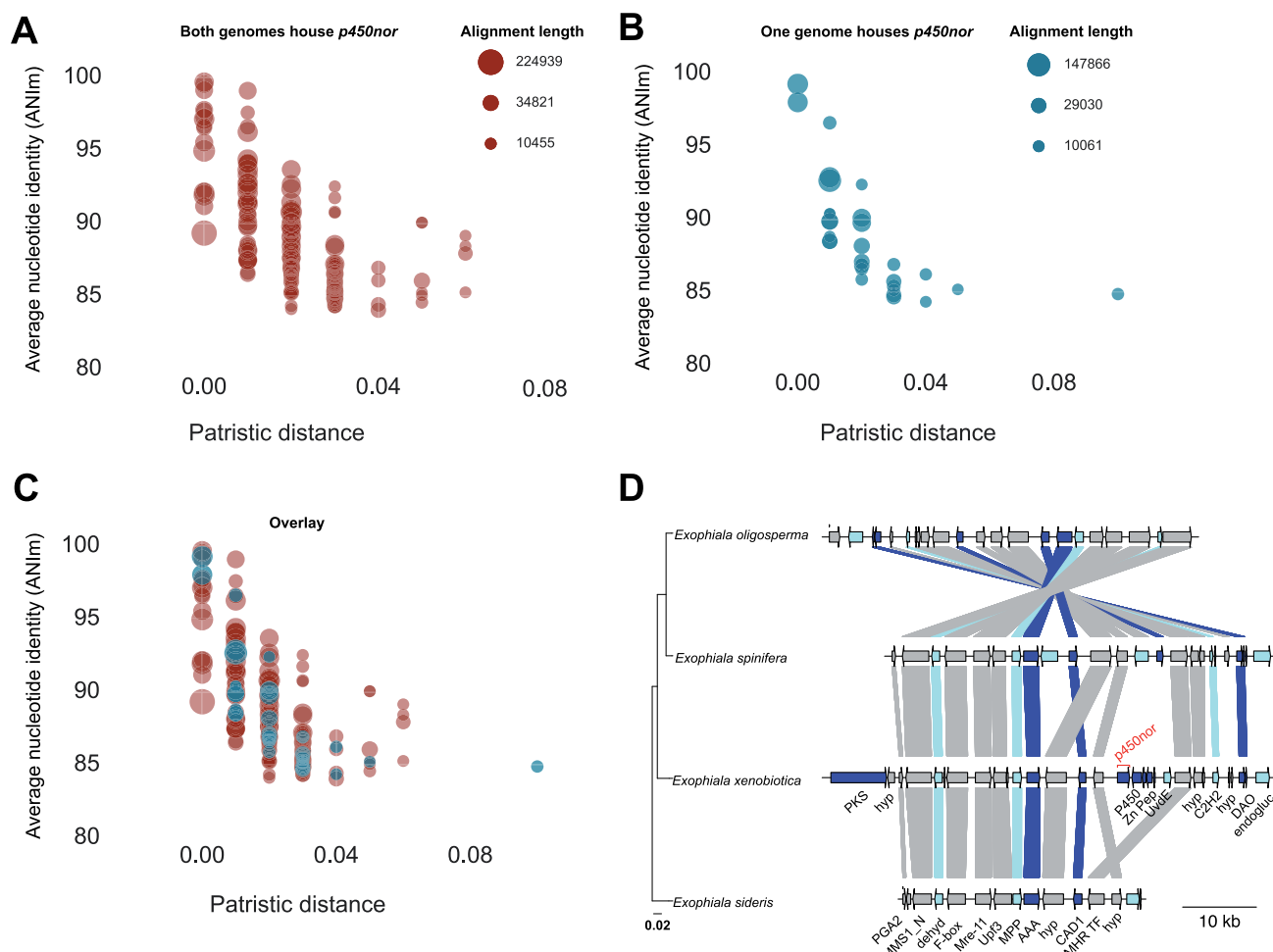


Fig. 5.—Within genera alignments of *p450nor*-containing genomic regions ($N = 136$) from species with and without *p450nor*. Alignment of two species with *p450nor*-containing genomic regions (red circles) (A) or between two species where only one member possesses *p450nor* (teal circles) (B). The overlay of the two plots in (C) indicates conservation of genomic architecture regardless of *p450nor* presence or absence. The size of the circles is proportional to the square root of the aligned length of the genomic regions. The gene synteny plot (D) highlights conservation of genomic architecture using a *p450nor*-containing genomic region from *Exophiala xenobiotica* aligned to three additional closely-related *Exophiala* species that do not possess *p450nor*. The arrows represent gene models within the gene region displayed. Both arrows and connecting lines above and below arrows are colored according to figure 3 (see Materials and Methods for details). Lines connecting arrows between species indicate the genes are homologous. The scale bars (left to right) in (D) indicate substitutions per site and genome size in kilobases, respectively. The location of *p450nor* in *E. xenobiotica* is indicated in red font. The labels in black font describes the putative functions of proteins encoded by each gene. PKS, polyketide synthase; hyp, hypothetical protein; PGA2, protein trafficking protein; MMS1_N, MMS1-like protein; dehyd, dehydrogenase; F-box, F-box domain containing protein; Mre-11, double strand break repair protein Mre-11; Upf3, nonsense mediated mRNA decay protein 3; MPP, metallophosphatase; AAA, ATPase; CAD1, cinnamyl alcohol dehydrogenase; MHR TF, middle homology region transcription factor; P450, cytochrome P450 reductase; Zn Pep, zinc peptidase superfamily protein; UvdE, UV-endonuclease; C2H2, zinc finger C2H2 type; DAO, D-amino acid oxidase; endogluc, endoglucanase.

In addition to isotopocule measurements, antibiotic inhibition has been frequently used to assess the contributions of fungi and bacteria to N_2O formation (Mothapo et al. 2015). For example, streptomycin, a widely applied bactericide used to inhibit bacterial N_2O emissions, only inhibits a fraction of the active bacteria present in soil (Rousk et al. 2009; Ladan and Jacinthe 2016). The ineffectiveness of antibiotics to partition microbial respiration has been previously demonstrated (Rousk et al. 2009), yet antibiotics continue to be used to

support the prevalence of fungal respiratory denitrification in situ. Therefore, the respiratory denitrification hypothesis is predicated on biased approaches often used in isolation that are unable to correctly assess fungal contributions to denitrification.

Another hypothesis for the retention or acquisition of denitrification traits in fungi is their role in hybrid respiration and survival under hypoxic conditions. Hybrid respiration involves the flow of electrons to two terminal electron acceptors

(e.g., O_2 and NO_3^-) simultaneously when O_2 is limiting (Takaya et al. 2003). Presumably, electron flow via terminal N-oxide reductases conserves additional energy under O_2 -limiting conditions (Chen and Strous 2013; Simon and Klotz 2013). Hybrid respiration has been investigated in *Fusarium oxysporum* strain MT-811 (Takaya et al. 2003), but the effect of denitrification trait presence/absence on fungal survival under hypoxic/anoxic conditions has yet to be determined. The only proteins encoded in the fungal genomes examined and linked to proton motive force generation were NarG and NirK (Takaya et al. 2003; Chen and Strous 2013; Simon and Klotz 2013), the former of which was only detected in three genomes, suggesting a limited role for NO_3^- in fungal hybrid respiration. NirK contributes indirectly to proton translocation via the activity of the cytochrome bc1 complex (Takaya et al. 2003), but investigations examining the contributions of NirK to proton motive force generation in fungi exposed to hypoxic conditions are lacking to support this hypothesis. The gene encoding the catalytic subunit of the periplasmic nitrate reductase, NapA, was detected at much greater frequency than NarG (10.5% of genomes examined), but NapA is not involved in proton translocation and energy conservation (Simon and Klotz 2013). Finally, P450nor is a soluble cytochrome P450 with no known role in energy conservation in fungi (Shoun et al. 2012). Hence, it remains to be determined whether denitrification trait acquisition/retention is driven by episodic O_2 deficiency experienced by fungi in the environment.

Another alternative function suggested for P450nor is NO detoxification, which was initially postulated in experiments using the fungus *Fusarium oxysporum* strain 11n1 (Kurakov et al. 2000). This hypothesis was supported by low growth yields and a poor mass balance between the N-oxyanion inputs and N_2O formed by the fungus (Shoun 1992; Tsuruta et al. 1998; Rohe et al. 2014). Although plausible, the NO detoxification hypothesis is confounded by extensive cooccurrence between *p450nor* and genes encoding canonical NO-detoxifying flavohemoglobins, which also produce N_2O under anoxic conditions (Zumft 2005; Wisecaver et al. 2016) (fig. 1 and [supplementary table S1, Supplementary Material](#) online). The binding affinity of NO by P450nor is reported between 0.1 and 0.6 mM (Nakahara et al. 1993; Shiro et al. 1995) and is orders of magnitude higher than the 0.1 to 0.25 μ M NO binding affinity reported for flavohemoglobins (Gardner et al. 2000), suggesting flavohemoglobins would outcompete P450nor for NO binding at physiological NO concentrations. Thus, the higher affinity of flavohemoglobins for NO and their greater distribution in fungi ([supplementary table S1, Supplementary Material](#) online) would suggest a limited role for P450nor in NO detoxification.

The SM hypothesis has traction considering that P450nor is derived from CYP105 P450s (fig. 2), all of which share a functional role in SM (O'Keefe and Harder 1991;

Yasutake et al. 2007; Moody and Loveridge 2014). Thus, the adaptation of P450nor to a novel niche in NO reduction and denitrification is unlikely. A more parsimonious hypothesis is that P450nor has maintained a role in SM as observed for related actinobacterial enzymes. The monophyly of P450nor with the SM enzyme TxtE, the only other NO-utilizing P450, provides additional *a priori* support for P450nor's role in SM ([supplementary fig. S7, Supplementary Material](#) online). P450nor's role in SM is further corroborated by SM prediction tools where a sizeable proportion (35%) of gene regions surrounding *p450nor* contained genes predicted to encode hallmark SM functions, and as many as 105 (63%) *p450nor*-containing genomic regions were automatically predicted to be involved in SM (fig. 3). Although phylogenomic evidence supports a role for P450nor in the biosynthesis of secondary metabolites, direct physiological evidence should be a target for future research efforts. For example, transcriptomic approaches may provide corroborating evidence of P450nor's role in SM, but experimental conditions used in investigations of *p450nor*-containing fungi are not optimized for *p450nor* gene expression (i.e., growth with low O_2 and N-oxyanions present) (Delmas et al. 2012; Ehrlich and Mack 2014; Pullan et al. 2014). Further, transcriptomic analysis of fungal SM clusters requires knowledge of the specific growth conditions for their induction under laboratory conditions (Inglis et al. 2013). Emerging technologies enabling the expression of full length BGCs and metabolite identification should enable robust experimentation to test the SM hypothesis (Clevenger et al. 2017).

Predicting P450nor's Role in Secondary Metabolism

A variety of metabolites containing nitro functional groups have been detected in fungal genera known to harbor denitrifying representatives (Chen et al. 2016), yet mechanistic explanations for nitration reactions in fungi remain elusive. The addition of a nitro functional group to a metabolite represents a potential mechanism for enhancing its toxicity or functional specificity (Kovacic and Somanathan 2014). The hypothesis of a role for P450nor in nitration, or possibly nitrosylation, of fungal metabolites is attractive given P450nor's affiliation with the nitrating enzyme TxtE (Barry et al. 2012) ([supplementary fig. S7, Supplementary Material](#) online). The inclusion of *p450nor* within BGCs may be adaptive in fungal lineages in which this gene was acquired due to the augmenting effects nitro or nitroso groups impart on their substrates. Support for this hypothesis stems from the widespread distribution of *p450nor* within secondary metabolite producing members of the Ascomycota (Keller 2015; Macheleidt et al. 2016), and previous reports of HGT between members of *Actinobacteria* and fungi in enhancing fungal SM (Janssen et al. 2002). Furthermore, the high nucleotide identity shared between *p450nor*-containing genomic regions from closely related fungal species suggests *p450nor* gain or loss may

have important consequences for the secondary metabolites potentially produced by the enzymatic machinery encoded in *p450nor*-containing BGCs (fig. 5D). The variability in *p450nor* gene presence/absence among closely related fungal species suggests *p450nor* is not essential for SM (supplementary table S5, Supplementary Material online). Indeed, genomic regions between closely related species with and without *p450nor* contain canonical SM elements (fig. 5D). Although *p450nor* may not be essential for SM generally, it may provide a selective advantage in specific contexts (e.g., plant pathogenesis) or environments (e.g., N-oxide rich [agricultural] soils). Specifically, the introduction of a nitro/nitroso group catalyzed by P450nor would result in a secondary metabolite with altered functionality with benefits to the host. Future research should explore the essential or ancillary role *p450nor* plays in fungal SM.

Additional unknowns related to P450nor's role in SM is the identification of putative substrates and the sources of NO required to fuel the hypothesized nitration or nitrosylation reactions. To date, P450nor is solely reported to bind the electron donors NADH or NADPH and the electron acceptor NO (Shoun et al. 2012). However, N₂O formation by P450nor is O₂ dependent (Zhou et al. 2001; Mothapo et al. 2013), suggesting O₂ may be an additional substrate as observed for TxtE (Barry et al. 2012). TxtE and NovI, both P450s affiliated with P450nor, bind to and transform L-tryptophan and L-tyrosine to produce the secondary metabolites thaxtomin A and novobiocin, respectively (Chen and Walsh 2001; Barry et al. 2012). It is conceivable that P450nor might also bind O₂ and aromatic amino acids, but direct experimental evidence is required to confirm this hypothesis. A potential source of NO in fungi could result from nitrite reductase activity of the copper containing nitrite reductase, NirK. The NO synthase (TxtD) from *Streptomyces turgidiscabies* produces NO to fuel TxtE nitration of L-tryptophan (Barry et al. 2012), but *txtD* homologs were not detected in the fungal genomes examined. Although evidence of NO synthases in fungi exist, knowledge regarding their distribution is limited (Ninnemann and Maier 1996; Samalova et al. 2013). Given the functional redundancy between NO synthases and NirK, it is conceivable that one of NirK's functions in fungi is to generate NO for use by P450nor in SM.

Causes and Consequences of *p450nor* Evolution in Fungi

A limited understanding of *p450nor* evolution represented an impediment to our knowledge of fungal N₂O formation. For example, closely related fungi vary in their ability to produce N₂O (Shoun 1992; Tsuruta et al. 1998; Maeda et al. 2015; Higgins et al. 2016), and the evolutionary forces (e.g., HGT, gene gain/loss, and incomplete lineage sorting) contributing to this observation were unexplored. For *p450nor*, many HGT events were observed between distantly related fungal lineages using gene and species tree comparisons (supplementary

fig. S2, Supplementary Material online). Although HGT events are challenging to precisely quantify given the level of uncertainty in deeply branching nodes of the functional gene trees reported here, a signal of potentially double digit HGT events were observed using gene tree-species tree reconciliation (supplementary table S3, Supplementary Material online). Genetic elements encoding *pogo* family transposases ($N = 9$), retrotransposons ($N = 4$), and reverse transcriptases ($N = 1$) were in some cases detected adjacent to *p450nor* and may act as vehicles for dissemination of *p450nor* within fungi and between fungal chromosomes (figshare Data Set S3).

N₂O production was previously coined a widespread trait in fungi (Maeda et al. 2015), yet genomic analysis suggests fortuitous N₂O formation by fungi is largely restricted to members of the Ascomycota. N₂O production has been reported for fungal isolates assigned to the recently revised phylum Mucoromycota (Mothapo et al. 2015; Spatafora et al. 2016), yet no evidence of genes underlying denitrification were detected in available genomes from members of this phylum (fig. 1). Denitrification markers were also absent from ascomycete yeast genomes (i.e., *Candida*, *Yarrowia*), though a number of N₂O-producing ascomycete yeasts have been reported (Tsuruta et al. 1998). Even within the Basidiomycota, N₂O formation is restricted to a few taxa within the Tremellomycetes and Agaricomycetes (Mothapo et al. 2015), and at least for members of the Tremellomycetes, was likely the result of HGT from one or more members of the Ascomycota (supplementary fig. S2, Supplementary Material online). The finding that genomes from fungi (e.g., ascomycete yeasts) previously observed to produce N₂O did not possess denitrification traits was unexpected and suggests that experimental artifacts or other mechanisms, such as the NO-detoxifying activity of flavohemoglobins, may also contribute to N₂O formation in fungi. In addition to fungi, species of green algae have been reported to produce small quantities of N₂O, the production of which could, at least in part, be attributed to the presence of *p450nor* within this lineage (Weathers 1984; Guieysse et al. 2013; Plouviez et al. 2017). However, like fungi, respiratory denitrification by green algae is unlikely (Weathers 1984; Plouviez et al. 2017); thus, the SM hypothesis appears to be an attractive explanation for the presence of *p450nor* in green algae as well.

p450nor genes within fungi also have implications for fungal pathogenesis (Mothapo et al. 2015). At least for some bacteria (e.g., *Neisseria*, *Brucella*, and *Mycobacterium*), the presence of denitrification genes has been demonstrated to enhance virulence or detoxification of N-oxides produced by the host (Philippot 2005). Although the impact of denitrification gene acquisition on fungal pathogenesis is not well established, there is growing evidence for P450nor involvement in fungal virulence (Shoun et al. 2012; Mothapo et al. 2015). For example, *p450nor* gene expression is linked to *Fusarium* wilt in banana and cotton plants, yet mechanistic explanations of

P450nor's function during plant infection are lacking (McFadden et al. 2006; Sutherland et al. 2013). Notably, more than half of all *p450nor*-containing fungal species are known plant pathogens (Mothapo et al. 2015), and the involvement of *p450nor* in SM is consistent with and would support the plant pathogenic life history strategies of many *p450nor*-containing fungi.

The diversity of denitrifying microorganisms and the modularity of the pathway has led to the view of denitrification as a community function (Philippot 2002; Jones et al. 2011; Graf et al. 2014). Therefore, limited cooccurrence and correlated evolution between *napA*, *nirK*, and *p450nor* might suggest mutualistic interactions occur between fungal or bacterial species performing denitrification. However, gene cooccurrences and evolutionary correlations should be interpreted with caution as additional factors (e.g., shared ecological niche, selection pressures) related to fungal life history strategies may explain their distribution equally well. For example, N₂O-producing fungi are frequently detected in, and cultivated from, highly disturbed, N-amended agricultural soils (Chen et al. 2014; Mothapo et al. 2015; Higgins et al. 2016) and detoxification or N-oxide utilization traits may merely cooccur more frequently due to selection imposed by environments with regular N inputs. Fungi also contain genes homologous to bacterial denitrifiers, but their presence does not guarantee a role in respiratory denitrification. For example, the presence of genes homologous to the bacterial NO reductase (*norB*) is not sufficient evidence for respiratory denitrification potential in pathogenic bacteria (Mahne and Tiedje 1995; Philippot 2005). The same is true of the abundant *napA* gene homologs detected in fungal genomes, which would suggest a robust capacity of fungi to perform dissimilatory nitrate reduction. Yet this is not the case, and many fungi only produce N₂O when NO₂⁻ is present (Shoun 1992; Tsuruta et al. 1998; Mothapo et al. 2015). Hence, the role of the *napA* gene in fungal biology is intriguing since they exist in >10% of the 712 fungal genomes examined.

In summary, fungi often produce little or no gaseous N from reduction of N-oxyanions and do not grow proportionally to the quantity of N-oxyanions consumed; thus, fungi cannot be classified as respiratory denitrifiers (Mahne and Tiedje 1995). Given the limited accounting of methodological bias in the study of N₂O production by fungi (Rousk et al. 2009; Ladan and Jacinthe 2016; Phillips et al. 2016), alternative explanations for the biological function of *p450nor* in fungi are likely and raises concerns over the validity of these techniques in estimating fungal contributions to N₂O emissions. NO detoxification by P450nor is plausible, but their cooccurrence with flavohemoglobins and weak binding affinity for NO suggests insufficient evidence exists to support the NO detoxification hypothesis. The added benefit of hybrid respiration using N-oxyanions in concert with O₂ is also a possible explanation for the acquisition/retention of

denitrification traits in fungi, but evidence of growth or cell maintenance conferred by these traits under hypoxic conditions is currently lacking. The analyses presented here support the affiliation of *p450nor* with nondenitrifying actinobacterial sequences involved in SM and provide multiple lines of evidence for their inclusion in candidate BGCs. Nevertheless, a premier goal of future research should be direct experimentation to support or refute a role for P450nor in SM. Recent developments in technologies geared at characterizing fungal SM products and identification of novel fungal BGCs should facilitate these experiments. Considering that many putatively denitrifying fungi are also plant disease causing secondary metabolite producers and agricultural pests, our results strongly endorse a biological role for *p450nor* in SM rather than denitrification.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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