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Phylogenomic analysis supports a recent change in nitrate assimilation in the White-nose Syndrome pathogen, *Pseudogymnoascus destructans*

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ABSTRACT

Cave environments are generally nutrient-poor, but can contain patches of limiting nutrients such as nitrogen in association with bats. *Pseudogymnoascus destructans*, the causative agent of White-nose Syndrome in bats, is thought to have recently emerged from cave soil saprotrophs. To determine whether changes in nitrate assimilation from a nutrient-limited to nitrate-rich environment could help explain the emergence of *P. destructans* as a pathogen, we analyzed the evolution of nitrate assimilation clusters in members of the genus. By screening the genomes of 21 members of the *Pseudogymnoascus*, we identified a very recent duplication of the high affinity nitrate transporter (NRT2) in *P. destructans*. Analyses of gene phylogeny, secondary structure, and evolutionary rates suggest the NRT2 paralog is functionally divergent, providing *P. destructans* a higher capacity for nitrate uptake. Such genomic changes may enhance the growth of *P. destructans* in bat-associated cave environments.

1. Introduction

The filamentous fungus *Pseudogymnoascus destructans* (Leotiomycetes) has been identified as the causal agent in the White-nose Syndrome (WNS) epizootic, which has killed millions of North American bats since the disease was first identified in 2007 (Gargas et al., 2009; Lorch et al., 2013; Minnis and Lindner, 2013). *Pseudogymnoascus* species are globally distributed with non-pathogenic phenotypes in multiple soil types and microenvironments (Hayes, 2012; Reynolds and Barton, 2013) including bat hibernacula (Lorch et al., 2013; Vanderwolf et al., 2013) and healthy bat fur (Johnson et al., 2013). While *Pseudogymnoascus pannorum* has caused non-fatal skin lesions in humans and domesticated animals (Gianni et al., 2003; Zelenkova, 2006; Erne et al., 2007; Christen-Zaech et al., 2008), virulence towards bats appears to have emerged only in *P. destructans* (Minnis and Lindner, 2013). In the environment, *P. destructans* appears to be a competent saprotroph, persisting and propagating in cave sediments, supporting the hypothesis of its emergence from non-pathogenic ancestors (Lorch et al., 2013; Reynolds et al., 2015). While many caves are nutrient-limited, caves have historically served as an industrially important source of nitrate through saltpeter deposits (Gale, 1912). The origin of this nitrate remains debatable, with nitrifying bacteria and archaea, groundwater, and even bat guano having been postulated to play a role (Hess, 1900; Pace, 1971; Fliermans and Schmidt, 1977; Hill, 1981; Schlottman et al., 2000). The finding that *P. destructans* can metabolize multiple sources of nitrogen, including nitrate, (Raudabaugh & Miller 2013), suggests that in the environmental the pathogen can presumably exploit nitrate present in caves and cave hibernacula.

Gene duplications are a source of evolutionary novelty (Ohno, 1970; Zhang, 2003; Hayes, 2012) and may be important in the emergence of fungal pathogens of animals. For example, the genome of the amphibian pathogen *Batrachochytrium dendrobatidis* contains multiple duplications of proteases that allow the fungus to invade host tissue (Joneson et al., 2011; Rosenblum et al., 2012, 2013). The draft genome assembly of *P. destructans* 20631-21 has similarly enabled the identification of genetic features that may

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promote its survival in cave environments and/or ability to invade bat tissue (Johnson et al., 2013; Smyth et al., 2013; O'Donoghue et al., 2015; Pannkuk et al., 2015). Recently, a subtilisin-like serine protease transcribed during host invasion (Field et al., 2015) was shown to be derived from a duplication event (O'Donoghue et al., 2015; Pannkuk et al., 2015). One prerequisite for identifying genomic features associated with the emergence of *P. destructans* ecology is a robust, well-supported *Pseudogymnoascus* phylogeny to allow for comparison of candidate gene duplication, loss and horizontal gene transfer. In single and multigene studies to date, *P. destructans* was shown to be a distinct species in *Pseudogymnoascus* (Gargas et al., 2009) with an apparent relationship to *Pseudogymnoascus roseus* and *Pseudogymnoascus verrucosus* (Minnis and Lindner, 2013), but with low support for key nodes within the genus. Additionally, environmental *P. pannorum* isolates showed this species to be polyphyletic, leaving in question how moderately pathogenic species relate to *P. destructans* (Minnis and Lindner, 2013). With the increasing number of *Pseudogymnoascus* genomes that have become publicly available (Chibucos et al., 2015; Leushkin et al., 2015), it may be possible to combine phylogenetic signals from a large number of genes (Salichos and Rokas, 2013; Solís-Lemus et al., 2015) to construct a more robust phylogeny and identify genome remodeling events within the genus.

We hypothesized that *P. destructans* might display altered nitrate metabolism, driven by evolution of the nitrate transporter. Fungi typically contain a single high-affinity nitrate transporter that functions at low nitrate concentrations (<1 mM) and is often in a three-gene nitrate assimilation cluster (Fig. 1A), but a subset of fungi maintain a second high-affinity NRT2 paralog that has been found in some cases to allow transport in higher nitrate conditions (Unkles et al., 2001; Slot et al., 2007). We investigated whether *P. destructans* might also contain genomic changes affecting nitrate assimilation compared to other *Pseudogymnoascus* spp. In this study, we: (i) generated a *Pseudogymnoascus* phylogeny using a set of 125 informative genes derived from 23 newly generated and databased whole genomes for reconstructing targeted gene family evolution; (ii) analyzed patterns of NRT2 diversification and selection in *Pseudogymnoascus*; and (iii) characterized nitrate utilization by *P. destructans* and related species.

We present evidence of a *P. destructans*-specific duplication of NRT2, informed by strong support for placement of *P. destructans* in a clade consisting of species related to *P. verrucosus*. This duplicate NRT2 shows evidence of structural divergence and a higher rate of non-synonymous substitutions. Furthermore, *P. destructans* shows expansion of nitrate uptake capacity and range compared with relatives with single NRT2 homologs. We also identified a more ancient duplication of NRT2 that is over-represented among cave-isolated *Pseudogymnoascus* spp. While it is unknown whether nitrate transport plays a role in WNS, recent shifts in *P. destructans* ecology such as these may underpin its transition pathogenesis.

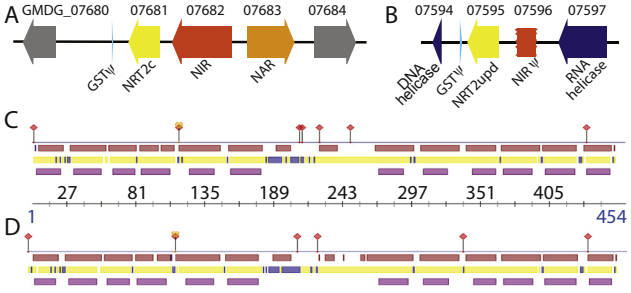

Fig. 1. Nitrate cluster structure and predicted protein structure of the *P. destructans* NRT2 paralogs. A. NRT2c cluster on scaffold 246 contains the full nitrate and nitrite reductases (NAR, NIR). B. NRT2upd on scaffold 239 is flanked by a NIR fragment and the unannotated glutathione S-transferase fragment also found on scaffold 246. The GST is complete in other *Pseudogymnoascus* species. C & D. This segment is flanked by two helicases. Numbers above the arrows indicate annotation performed by the Broad Institute (http://www.broadinstitute.org/annotation/genome/Geomyces_destructans). C. NRT2c and D. NRT2upd structural predictions. NRT2c contains four protein-binding domains in the intracellular loop. NRT2upd contains 2 protein-binding domains in the intracellular loop and one extra protein-binding domain between helices 8 & 9. Red bars: helices. Yellow bars: hidden residues. Blue bars: exposed residues. Purple bars: membrane-spanning helices. Red diamonds: protein-binding domains. Yellow circle: polynucleotide binding region.

2. Materials and methods

2.1. Genome sequencing and annotation

To improve phylogenomic sampling and reconstruct the evolution of NRT2 in *Pseudogymnoascus*, whole genome sequences for four isolates were generated: ATCC16222 *P. pannorum* (wheat field soil; Germany), 04NY16 (cave sediment; New York), BL308, and BL549 (both from *Myotis lucifugus* fur in a cave; Tennessee). Fungi were cultured on potato dextrose agar at 20 °C and genomic DNA was extracted using the Zymo Fungal/Bacterial DNA Miniprep kit (Zymo Research Corp., Irvine, CA) with RNAse treatment (4 μl of 100 mg/mL). Genomic libraries were prepared using the Indexed Truseq DNA Library Prep Kit (Illumina, San Diego, CA). Duplexed samples were then sequenced in the paired-end 2 × 300 bp format on the Illumina Mi-Seq at the Ohio State University Ohio Agricultural Research and Development Center Molecular and Cellular Imaging Center (Wooster, OH).

The quality of raw FastQ files was assessed with FastQC v. 0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), poor quality ends were removed with Trimmomatic v. 0.32 (Bolger et al., 2014), and possible sequencing errors were corrected with SOAPdenovo2 EC (Luo et al., 2012). Ten assemblies of processed reads for each genome were generated using SOAPdenovo v. 2.21 (Luo et al., 2012) with kmer sizes ranging from 35 to 115 bp, and the assembly with the highest N50 was selected for gap closing (using GapCloser v. 1.12 from the SOAPdenovo2 package) and further analyses. Genome coverage was calculated as the mean of the individual nucleotide coverage values obtained using the depth function in SAMtools v. 0.1.19 (Li et al., 2009; Li, 2011).

Gene models were predicted for each *Pseudogymnoascus* genome using two iterations of MAKER v. 2.31.8 (Cantarel et al., 2008) to obtain a protein sequence phylome. In the first iteration, *P. destructans* 20631-21 protein sequences (Broad Institute, http://www.broadinstitute.org/annotation/genome/Geomyces_destructans/MultiHome.html) were used as evidence, and genes were predicted using SNAP v. 2013-11-9 (Korf, 2004) and GeneMarkES v. 4 (Lomsadze et al., 2005; Ter-Hovhannisyan et al., 2008). The core genes predicted with CEGMA v. 2.4 (Parra et al., 2007) were used as the initial training set for SNAP. The predicted annotations from the first iteration were then used to retrain SNAP and to train Augustus v. 2.5.5 (Stanke et al., 2004, 2006, 2008; Stanke and Morgenstern, 2005) for a second MAKER iteration using all previously described models. This same workflow was used to predict comparable gene models in 15 genomes previously deposited in NCBI (as WGS) by other groups (Chibucos et al., 2015; Leushkin et al., 2015) and in the *P. destructans* 20631-21 assembly. This process yielded 20 consistently annotated *Pseudogymnoascus* genomes (Table 1).

2.2. Estimation of Pseudogymnoascus phylogeny

Three previously annotated leotiomycete genomes were selected as outgroups: *Botrytis cinerea B05.10* (Amselem et al., 2011; Staats and van Kan, 2012), *Glarea lozoyensis* ATCC 20868 (Chen et al., 2013), *Oidiodendron maius Zn* (Kohler et al., 2015). For