Molecular characterization of a heterothallic mating system in *Pseudogymnoascus destructans*, the fungus causing white-nose syndrome of bats

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Keywords: Geomyces, sexual reproduction, mating type, white-nose syndrome

ABSTRACT

White-nose syndrome (WNS) of bats has devastated bat populations in eastern North America since its discovery in 2006. WNS, caused by the fungus *Pseudogymnoascus destructans*, has spread quickly in North America and become one of the most severe wildlife epidemics of our time. While *P. destructans* is spreading rapidly in North America, nothing is known about the sexual capacity of this fungus. To gain insight into the genes involved in sexual reproduction, we characterized the mating-type locus (*MAT*) of two *Pseudogymnoascus* spp. that are closely related to *P. destructans* and homothallic (self-fertile). As with other homothallic Ascomycota, the *MAT* locus of these two species encodes a conserved α-box protein (*MAT1-1-1*) as well as two high mobility group (HMG) box proteins (*MAT1-1-3* and *MAT1-2-1*). Comparisons to the *MAT* locus of the North American isolate of *P. destructans* (the ex-type isolate) revealed that this isolate of *P. destructans* was missing a clear homolog of the conserved HMG box protein (*MAT1-2-1*). These data prompted the discovery and molecular characterization of a heterothallic mating system in isolates of *P. destructans* from the Czech Republic. Both mating types of *P. destructans* were found to coexist within hibernacula, suggesting the presence of mating populations in Europe. Although populations of *P. destructans* in North America are thought to be clonal and of one mating type, the potential for sexual recombination indicates that continued vigilance is needed regarding introductions of additional isolates of this pathogen.

INTRODUCTION

Since its discovery in 2006 in New York, USA, white-nose syndrome (WNS) of hibernating bats has spread to more than 25 states and 5 Canadian provinces while killing more than 5.5 million bats (Froschauer and Coleman 2012; www.whitenosesyndrome.org). The causative agent of WNS is a psychrophilic fungus named *Pseudogymnoascus destructans* (≡*Geomyces destructans*) (Gargas et al. 2009; Lorch et al. 2011; Warnecke et al. 2012; Minnis and Lindner 2013) that has been hypothesized to be an introduced pathogen, possibly from Europe, where the pathogen has been consistently detected (Martínková et al. 2010; Puechmaille et al. 2010; Puechmaille et al. 2011; Wibbelt et al. 2010) and can cause WNS (Pikula et al. 2012), although no mass mortality has been observed in Europe. The fungus is spreading rapidly in North America (Lorch et al. 2013); isolates collected thus far appear to have been derived from a single clonal introduction in the northeastern United States (Rajkumar et al. 2011; Ren et al. 2012). This has recently been supported by whole genome sequencing of 26 North American isolates of *P. destructans* (Chibucos et al. 2013; K. Drees and J. Foster, unpublished data). Taken together, these data suggest that *P. destructans* is spreading in North America exclusively through asexual reproduction, given that conidia are commonly observed in clinical specimens and in culture (Meteyer et al. 2009; Chaturvedi et al. 2010). While it is known that *P. destructans* reproduces asexually, its capacity for sexual reproduction is unknown.
Because fungi and humans are related members of the opisthokonts, sexual reproduction in fungi has been a topic of intense research interest (reviewed in (Heitman et al. 2013; Dyer and O’Gorman 2012; Ni et al. 2011; Nielsen and Heitman 2007). While sex involving two mating partners, male and female, is obligatory in some eukaryotes (e.g. humans), mating in fungi can involve multiple mating types, but there are no male and female genders and thus no sex chromosomes (e.g. human X & Y). Fungal mating types are determined by a single genetic locus termed the mating-type locus (MAT locus), which consists of highly divergent non-homologous genes that are termed idiomorphs (Heitman et al. 2013). Generally, the MAT idiomorphs encode for two key transcriptional regulators: where the MAT1-1 mating type is controlled by the MAT1-1-1 α-box transcription factor and the MAT1-2 mating type is controlled by the MAT1-2-1 high mobility group (HMG) transcription factor (Ni et al. 2011). Whereas some fungal species have a heterothallic (outcrossing) mating system (e.g., Neurospora crassa (Metzenberg and Glass 1990) and Aspergillus fumigatus (O’Gorman et al. 2009) involving each individual having either the MAT1-1 or the MAT1-2 idiomorph, others can reproduce homothally; the individual carries both idiomorphs and thus a single strain is capable of mating with itself, i.e. it is self-fertile (e.g., Aspergillus nidulans (Paoletti et al. 2007), Sclerotinia sclerotiorum (Amselem et al. 2011), Sordaria macrospora (Klix et al. 2010)). The specific gene organization of the MAT locus can be variable amongst fungal species, although the canonical MAT1-1-1 and MAT1-2-1 are always present (Figure 1). In several species, additional proteins are encoded in the MAT locus: for example Neurospora crassa (Ferreira et al. 1996) and Sordaria macrospora (Klix et al. 2010) contain an additional HMG-box gene (MAT1-1-3) (Figure 1).

While the infectious particles of many fungal pathogens are asexual spores, sexual spores can be infectious, as in the human pathogenic fungus Cryptococcus neoformans (Giles et al. 2009; Velagapudi et al. 2009). This is also true of many fungal pathogens of plants, such as Venturia inaequalis (apple scab) (MacHardy et al. 2001) and S. sclerotiorum (white mold) (Amselem et al. 2011). In many pathogenic fungi, sexual spores also function as important overwintering or survival structures, allowing the fungus to persist for long periods of time in the absence of a host. Moreover, sexual reproduction in pathogenic fungi is of interest as it is the basis for genetic variability, which has the potential to create additional virulent genotypes. Mating populations of P. destructans in North America could potentially exacerbate WNS, so information regarding the sexual capabilities of this fungus is needed to help inform management and to develop effective mitigation strategies, especially in relation to long-distance (inter-continental) movement of P. destructans.

Based on a recent phylogenetic study of the genus Pseudogymnoascus (Minnis and Lindner 2013), we selected two unnamed homothallic (self-fertile) species that produce sexual structures (gymnothecia) in culture and are relatively closely related to P. destructans as exemplars for understanding the mating-type locus in Pseudogymnoascus. We cloned and sequenced the mating-type (MAT) locus from these two homothallic species and discovered that these species share a nearly identical gene structure at the MAT locus (see results section). Comparison of the homothallic Pseudogymnoascus MAT locus to the P. destructans genome reference strain suggested that the bat pathogen was likely heterothallic. We screened isolates of P. destructans from central Europe and...
discovered the opposite mating type (MAT1-2). Differential expression of *P. destructans* genes involved in mating was also examined in cultures of each mating type individually as well as in mixed culture.

**MATERIALS AND METHODS:**

Fungal strains used in this study are listed in Table 1. New strains of *P. destructans* were isolated from muzzles and wings of WNS-suspected bats (*Myotis myotis*) using sterile cotton or plastic swabs and cultured on yeast extract glucose chloramphenicol agar or Sabouraud dextrose agar at 10°C. For routine laboratory experiments, isolates were maintained on a combination of glucose minimal medium (GMM) (Shimizu and Keller 2001) and Champe’s medium (Champe and el-Zayat 1989). All isolates have been preserved in the culture collection of the Center for Forest Mycology Research (CFMRR). The Culture Collection of Fungi (CCF) and the Collection of Microscopic Fungi (CMF) Czech Republic, also maintain cultures as indicated by their acronyms in Table 1. All primers are listed in Table S1. *Pfu*Ultra II polymerase (Stratagene) was used for all PCR reactions according to the manufacturer’s recommendations. Standard molecular biology techniques were used as previously described (Sambrook and Russell 2001). BLAST searches were conducted using the draft genome sequence of the North American isolate 20631-21 of *P. destructans* (Geomyces destructans Sequencing Project, Broad Institute of Harvard and MIT - http://www.broadinstitute.org/).

**DNA extraction from fungi** - Fungal cultures were grown in liquid stationary culture for three weeks in Champe’s medium (Champe and el-Zayat 1989), mycelium was collected, lyophilized overnight, ground to a fine powder, mixed with 700 ul of LETS Buffer (100 mM lithium chloride, 20 mM EDTA, 10 mM Tris-HCL, pH 8.0, and 0.5% SDS), extracted with an equal volume of phenol:chloroform:isoamyl alcohol (Ambion), and the aqueous phase was collected after centrifugation for 10 min at 12,000 x g at 4°C. DNA was precipitated by adding 1.0 ml of 95% ethanol and centrifuged for 10 min (12,000 x g, 4°C). The DNA pellet was washed with 70% ethanol and subsequently resuspended in 10 mM Tris-HCl (pH 8.0) containing 20 units of RNAsaeA (5 Prime).

**Cloning of MAT locus in homothallic *Pseudogymnnoascus* species** - Primers designed at conserved internal locations of *P. destructans* MAT1-1-1 (α-box) were used to amplify a PCR fragment of the MAT1-1-1 gene from the homothallic *Pseudogymnnoascus* species (WSF 3629 and 23342-1-I1). A ~ 900 bp fragment was obtained for isolate WSF 3629 and a ~ 400 bp fragment was obtained for 23342-1-I1. The PCR fragments were subsequently cloned using pCR®-Blunt II-TOPO® (Life Technologies) and sequenced. Sequencing of the region flanking the MAT1-1-I gene was achieved by using a modified version of thermal asymmetrical interlaced PCR (TAIL-PCR) called fusion primer and nested integrated PCR (FPNI-PCR) (Wang et al. 2011). Briefly, degenerate fusion primers (FP1 – FP9) were pooled in batches of three and used in combination with gene specific primers (GSP) followed by two consecutive nested PCR reactions. The largest PCR product from the final nested reaction was gel purified, cloned into pCR®-Blunt II-TOPO®, and subsequently sequenced. Five successive rounds of FPNI-PCR were conducted for isolate 23342-1-I1 and four rounds for WSF 3629, which was sufficient to identify the conserved flanking gene sla2. The remaining portion of the MAT locus for each isolate was PCR amplified by using a primer anchored in the conserved flanking gene apn2 and a GSP primer from the FPNI-PCR walking, was cloned into pCR®-Blunt II-TOPO®, and sequenced. Gene prediction was done using a combination of FGENESH (Solovyev et al. 2006) and AUGUSTUS 2.7 (Stanke et al. 2004) using the pre-trained hidden-Markov models for *Botrytis cinerea*.

**Identification of *P. destructans* MAT1-2 locus** - Twenty-three isolates of *P. destructans* from central Europe were screened via Southern blot using a 900-bp PCR fragment of MAT1-1-I as a radio-labeled P-32 probe according to standard procedures (Sambrook and Russell 2001). Isolates missing this fragment were suspected of having the other mating type. The MAT1-2 locus of *P. destructans* was cloned and sequenced from isolates CCF3942 and CCF4124 by PCR amplifying the entire region between apn2 and sla2 and putting it into the pCR®-Blunt II-TOPO® vector. The previous Southern blot was stripped and re-probed with a 1.1 kb radio-labeled P-32 probe corresponding to the MAT1-2-I sequence.

**RNA extraction and semi-quantitative RT-PCR** - Conidia were harvested in sterile water supplemented with 0.01% Tween-80 from 8-week-old cultures of *P. destructans* grown on GMM medium at 15°C. Conidia from a MAT1-1 isolate and a MAT1-2 isolate were enumerated with a hemocytometer and used to
inoculate 50 ml liquid cultures of Champe’s medium at a concentration of $1 \times 10^5$ conidia per ml. Cultures were incubated in a shaker at 15º and 200 rpm for 14 days. Mycelium was collected from each strain by sterile filtration over Miracloth (CalBiochem) and subsequently transferred to the surface of solid GMM medium agar plates: one plate for each mating type as well as one that was a 1:1 mixture of mycelium from MAT1-1 and MAT1-2 strains. The plates were wrapped in Parafilm-M (Bemis) and aluminum foil and incubated at 15º for an additional 14 days. Mycelium was then scrapped off the surface of the plates using a sterile glass slide, immediately frozen in liquid nitrogen, and lyophilized overnight. Total RNA was extracted from the lyophilized tissue using Isol-RNA Lysis Reagent (5 Prime) following manufacturer’s recommendations, treated with DNase I (NEB) according to the manufacturer’s protocol, and subsequently used to make cDNA using the iScript cDNA Synthesis Kit (Biorad). Genes involved in sexual reproduction in other filamentous fungi were identified through BLASTp searching of the reference genome and primers were designed for the mating-type genes (MAT1-1-I – GMDG_01209.1, MAT1-1-3 – GMDG_01208.1, and MAT1-2-I - KJ938434), the pheromone pathway (ppg1 – GMDG_06142.1, pre1 – GMDG_00660.1, and pre2 – GMDG_08410.1), the G-protein signaling pathway (fad1 – GMDG_04604.1, sfa4 – GMDG_08182.1, gpg1 – GMDG_01954.1, mpk2 – GMDG_04404.1, and ste1 – GMDG_05416.1), and the velv complex (vel1 – GMDG_00043.1, vel2 – GMDG_08054.1, and lae1 – GMDG_07817.1); actin (act1 – GMDG_01001.1) was used as a loading control. Between 32 and 42 amplification cycles were used to detect transcription of genes putatively involved in sexual reproduction.

RESULTS

Identification of the Mating-Type Locus - The MAT locus of P. destructans was identified by a BLASTp (Altschul et al. 1997) search of the P. destructans draft genome assembly with the MAT α-box (MAT1-1-I) protein sequence from Aspergillus nidulans AN2755 (Paoletti et al. 2007). This resulted in identification of a single hit on Supercontig 14, corresponding to GMDG_01209.1. In other filamentous fungi conserved primary metabolism genes apn2 and sla2 flank the MAT locus (Figure 1); thus we looked at flanking genes on Supercontig 14 and identified GMDG_01207.1 as apn2 and GMDG_01210.1 as sla2. Using the Conserved Domain Database (CDD) search (Marchler-Bauer et al. 2011) with GMDG_01209.1, we identified the MAT α-box domain (pfam04769). Interestingly, GMDG_01208.1 is also located in the MAT locus and has a predicted HMG-box domain (cd01389). A BLASTp search using GMDG_01208.1 of the non-redundant protein database (nr) at NCBI revealed the top hits to be MAT1-2-I proteins (ACA51904.1, AFY11134.2, AGH03115.1, CBY44653.1). Therefore, we initially thought that P. destructans could be homothallic because the MAT locus harbored both MAT1-1-I (α-box) and MAT1-2-I (HMG-box) genes. However, since we have never observed fruiting bodies from P. destructans 20631-21 in culture and the MAT locus of some fungi contains two HMG-box domain genes, we could not rule out that P. destructans 20631-21 was a MAT1-1 genotype.

Cloning and sequencing of Pseudogymnoascus homothallic MAT loci - Several species of Pseudogymnoascus are known to be homothallic and thus produce sexual fruiting bodies in culture (Rice and Currah 2006; Tsuneda 1982). Since homothallic ascomycetes typically have both MAT idiomorphs at the MAT locus, we reasoned that comparison of the MAT locus from a closely related homothallic species would aid in characterization of the P. destructans mating system. We selected two unnamed homothallic isolates from a recent study: Pseudogymnoascus sp. WSF 3629 (clade G – P. roseus complex) and Pseudogymnoascus sp. 23342-1-II (clade D) (Minnis and Lindner 2013). Pseudogymnoascus sp. WSF 3629 does not produce conidia in culture; however it produces visible gymnothecia (Figure 2A), which are composed of loosely woven, pigmented peridial hyphae (Figure 2B), asci (Figure 2C), and ascospores (Figure 2D). We have observed a similar sexual state for Pseudogymnoascus sp. 23342-1-II; formal identification and/or description of these species will be presented elsewhere.

Following confirmation of homothallism in two Pseudogymnoascus species, PCR primers for the P. destructans MAT1-1-1 were used to amplify, clone, and sequence a portion of the MAT1-1-I gene from both Pseudogymnoascus species (WSF 3629 and 23342-1-II). Subsequent rounds of fusion primer and nested integrated PCR (FPNI-PCR) (Wang et al. 2011) were used to obtain sequence of the flanking regions in each direction, yielding the entire sequence between the apn2 and sla2 genes from WSF 3629 and 23342-1-II (13.2 kB and 12.4 kB, respectively) (Figure 2). Using the ab-initio gene prediction programs AUGUSTUS 2.7 (Stanke et al. 2004) and
FGENESH (www.softberry.com), we deduced that the homothallic MAT locus from both WSF 3629 and 23342-1-I1 contain a nearly identical gene structure consisting of five predicted open reading frames (ORFs). A combination of BLAST (Altschul et al. 1997), CDD (Marchler-Bauer et al. 2011), and InterProScan (Quevillon et al. 2005) searches identified a clear MAT α-box protein (MAT1-1-1) and two high mobility group (HMG) domain containing proteins (MAT1-2-1 and MAT1-1-3) (Figure 2). This analysis also identified two additional ORFs (MAT1-1-6 and MAT1-2-5); however BLAST search did not reveal any significant homology with other known proteins, suggesting that these predicted ORFs are either novel MAT genes unique to the Pseudeurotiaceae or perhaps pseudogenes. Pairwise comparison of the MAT locus from the homothallic Pseudogymnoascus species to the P. destructans genome sequenced reference strain (20631-21) indicated that the genome reference strain was a MAT1-1 (α-box) mating type while the MAT locus of MAT1-2 strains is depicted by the Czech strain of P. destructans CCF3942.

Figure 2. Homothallic species of Pseudogymnoascus produced gymnothecia and contain a MAT locus consisting of the conserved regulators MAT1-2-1, MAT1-1-3, and MAT1-1-1. (A) Gymnothecia of Pseudogymnoascus WSF 3629 grown at 25º for 4 weeks on solid oatmeal medium in the dark. Scale bars are drawn on each of the images. (B) Gymnothecia of WSF 3629 are composed of loosely woven, pigmented peridial hyphae and among the peridial hyphae there are asci. (C and D) Higher magnification of asci containing ascospores and ascospores liberated from asci. (E) Schematic of the mating-type locus (MAT) for the homothallic species Pseudogymnoascus sp. WSF 3629 and Pseudogymnoascus sp. 23342-1-I1). The North American genome reference strain of P. destructans (20631-21) is the MAT1-1 mating type while the MAT locus of MAT1-2 strains is depicted by the Czech strain of P. destructans CCF3942.
Identification of the *P. destructans* MAT idiomorph -

The *P. destructans* genome reference strain (20631-21) is a North American isolate that has been hypothesized to be spreading clonally (Ren et al. 2012; Rajkumar et al. 2011), which has recently been substantiated because analysis of whole genome sequencing data of 26 North American isolates of *P. destructans* revealed that they all are the MAT1-1 genotype (Chibucos et al. 2013; K. Drees and J. Foster, unpublished data). While diversity studies of *P. destructans* isolates collected from Europe have not been conducted, it has been hypothesized that the fungus may have originated from Europe (Warnecke et al. 2012), therefore we looked for alternative mating types in *P. destructans* isolates from central Europe (Czech Republic and Slovakia). We screened 23 isolates of *P. destructans* for the presence of the MAT1-1-1 gene via Southern blotting and found that 5 of the isolates (CMF2584, CCF3942, CCF4124, CCF4131, and CCF4351) were missing MAT1-1-1 (Figure 3A). These isolates were confirmed to be *P. destructans* by morphology as well as sequencing of the ITS, LSU, and TEF regions (see Table 1). We next cloned and sequenced the entire MAT locus from CCF3942 as well as the genome reference strain 20631-21 as a control (see Table 1). Consistent with its Southern blot, CCF3942 did not contain the MAT1-1-1 sequence; instead this isolate harbors a HMG box domain isolate is the opposite mating type (Figure 3B). Moreover, a Southern blot using a probe for MAT1-2-1 identified the remaining 4 isolates as being identical to CCF3942 (Figure 3A). There is also an additional faint band in the Southern blot of MAT1-1 isolates when probed with MAT1-2-1, which could be due to homology in the HMG-box domain of MAT1-1-3. Moreover, it has recently been recognized that the MAT transcription factors share an evolutionary history, as even the MAT1-1-1 α-box is derived from the HMG gene family (Martin et al. 2010).

![Image](image_url)

**Figure 3. Central European isolates of *P. destructans* have two mating types (MAT1-1 or MAT1-2).** (A) Southern blot of the MAT locus of the North American isolate (20631-21) and 23 isolates from central Europe. Expected banding patterns for an EcoRI digestion of MAT1-1 strains is a single band of 3.183 kb. Expected banding pattern for EcoRI digestion using MAT1-2 as a probe is three bands of 2.6 kb, 2.063 kb, and 0.699 kb. European isolates of *P. destructans* collected from the same hibernaculum and date, different individual bats, but opposite mating types are demarcated with a red line above the isolate name. (B) Schematic of the two MAT idiomorphs in *P. destructans* illustrating the gene prediction structure and restriction enzyme cut sites. Radio-labeled probes used in the Southern blot are indicated by a black line.
We also cloned and sequenced the MAT locus from CCF4124, which was a MAT1-2 isolate that was collected on a different date and location. These data corroborate that there are two MAT idiomorphs for the isolates examined: MAT1-1 and MAT1-2. Interestingly, both mating types were isolated from samples taken at distinct times from different individual bats from the same hibernaculum, even though only 23 isolates of European P. destructans were screened in this study (Figure 3A).

Analysis of genes involved in sexual reproduction

While this is the first molecular characterization of sexual reproduction in Pseudogymnoascus, much is known about the molecular pathways in other model fungal systems such as Saccharomyces cerevisiae, Neurospora crassa, Aspergillus nidulans, etc (Dyer and O'Gorman 2012). Using data from the aforementioned model systems, we sought to examine expression of several conserved genes involved in sexual reproduction by semi-quantitative reverse transcriptase PCR of P. destructans mRNA from two mating type isolates grown alone or in mixed culture (Figure 4A). These data are consistent with a typical heterothallic mating system in other fungi, where the MAT1-1 locus controls the expression of the precursor of α-pheromone (ppg1), which is involved in production of the α-mating pheromone. The α-pheromone is recognized by the G-protein coupled receptors (PRE1 and/or PRE2), which our data suggests are in turn under the control of the MAT1-2 locus in P. destructans (Figure 4A). In P. destructans, it appears that co-cultivation of MAT1-1 and MAT1-2 strains results in the weak induction of the MAT1-1-3 HMG domain containing gene (Figure 4A). Expression of genes in the signal transduction pathway (fad1, sfa4, gpg1, mpk2, and ste1) as well as in the velvet complex (vel1, vel2, and lae1) is not drastically altered in either of the mating-types or in mixed culture (Figure 4A). These data taken together indicate that P. destructans has the necessary genetics for sexual reproduction and allow us to propose a heterothallic sexual reproduction pathway (Figure 4B).

DISCUSSION

In order to gain insight into the molecular components of sexual reproduction in the Pseudeurotiaceae, we selected two homothallic (self-

![Figure 4. Putative genes involved in sexual reproduction are expressed in laboratory culture.](image-url)

(A) Semi-quantitative reverse transcriptase PCR was used to measure gene expression of genes predicted to be involved in sexual reproduction. All PCR reactions were conducted with 32 amplification cycles except for those marked with an asterisk (*), where 42 amplification cycles were used. Mating type MAT1-1 is required for expression of the precursor to alpha-pheromone (ppg1), while on the other hand MAT1-2 is required for expression of the G-protein coupled receptors pre1 and pre2. Expression of MAT1-1-3 is only found when both mating types were co-cultured. (B) Proposed diagram of genes involved in sexual reproduction in P. destructans based on homology and expression in laboratory culture.
fertile) isolates from a recent study characterizing species related to *P. destructans* (Minnis and Lindner 2013). Cloning and sequencing of the *MAT* locus in each of these species revealed that this locus was nearly identical between these species and encodes a conserved α-box domain protein (*MAT1-1-1*) and two conserved HMG box domain proteins (*MAT1-1-3* and *MAT1-2-1*). This is consistent with the *MAT* locus of other well-studied homothallic Ascomycota species such as *Sordaria macrospora* (Klix et al. 2010), *Fusarium graminearum* (Yun et al. 2000), and *Sclerotinia sclerotiorum* (Amselem et al. 2011), where the mating genes are located in one conserved locus flanked by the primary metabolism genes *sla2* and *apn2*. Comparison of the homothallic *MAT* locus to the genome reference strain of *P. destructans* (20631-21) revealed that it was missing the *MAT1-2-1* HMG box protein, suggesting it was heterothallic. Interestingly, there are two more predicted ORFs in the homothallic *MAT* locus, *MAT1-1-6* and *MAT1-2-5*, which appear to have no known functional domains or homology to other known proteins and thus may represent novel *MAT* genes in the Pseudurotiales.

Pertinent to WNS management, we found isolates of both mating types of *P. destructans* coexisting in European hibernacula, indicating that in central Europe there is the potential for mating populations. While these data suggest that in our limited sampling, the *MAT1-1* mating type is found more frequently on *Myotis myotis* (18 out of 23), more sampling of European fungal isolates is necessary to understand the prevalence of mating types in *P. destructans*. Preliminary experiments inducing sexual reproduction in the lab have not yielded results to date; this is not surprising, as *P. destructans* is slow growing and sexual reproduction may not occur for long time periods as exemplified by other members of the genus (Rice and Currah 2006). Moreover, finding the appropriate cultural conditions for fungi with cryptic sexual cycles is time consuming. For example, although the heterothallic *MAT* locus of *Aspergillus fumigatus* was characterized in 2005 (Paolletti et al. 2005), it took another four years to find cultural conditions conducive to sexual reproduction (O’Gorman et al. 2009). Molecular characterization of the *MAT* locus of isolates will hasten the progress in finding the sexual cycle of *P. destructans*.

In the absence of sexual structures of *P. destructans*, we sought to further investigate genetic pathways involved in sex that have been well studied in other fungi (Dyer and O’Gorman 2012). Consistent with other Ascomycota, our expression data suggest that *MAT1-1* and *MAT1-2* are likely responsible for determination of mating type, as the precursor to α-pheromone (*ppg1*) was only expressed in the *MAT1-1* background. Moreover, both of the G-coupled protein receptors (*PRE1* and *PRE2*) hypothesized to recognize the α-pheromone are only expressed in the *MAT1-2* background. While we did not detect differences in expression of genes involved in sexual development in other fungi, which included the signal transduction cascade (*fad1, sfa4, gpg1, mpk2, ste1*) (Dyer and O’Gorman 2012) and the velvet complex of proteins (*lae1, vel1, vel2*) (Bayram and Braus 2012; Bayram et al. 2008), this was not surprising given the central importance of these genes for normal growth of the fungus. Interestingly, *MAT1-1-3*, the HMG-box domain protein of the *MAT1-1* idiomorph, is only expressed at low levels when both mating types are grown in co-culture, suggesting that it could be involved in downstream transcriptional activation of sexual reproduction.

Given the apparent clonality of *P. destructans* in North America, this important discovery of heterothallic mating types highlights the need for continued vigilance in preventing additional introductions of this pathogen in North America. Further work is needed to find and characterize the cryptic sexual cycle of *P. destructans*, although determination of the mating-types of isolates will be crucial to successfully characterizing sexual reproduction in this fungal pathogen under laboratory conditions. Sexual recombination may allow *P. destructans* to quickly adapt to its environment and hosts, despite its slow growth. Pertinent to pathogenicity of *P. destructans*, mating types in other fungi have been correlated to virulence (Cheema and Christians 2011; Nielsen et al. 2005; Kwon-Chung et al. 1992), and therefore this will be an important consideration in elucidating pathogenicity factors of WNS.

**ACKNOWLEDGMENTS**

This study was funded by the U.S. Forest Service (Northern Research Station), the U.S. Fish & Wildlife Service (FWS-R5-ES-12-001, Agreement # F11AP0078), and the Czech Science Foundation (Project No. P506/12/1064). We are grateful to Kevin Drees and Jeff Foster for sharing genome-sequencing data prior to publication. We would like to thank Nancy Keller (University of Wisconsin) for support as well as Jessie Glaeser and Mark Banik for critical review of this manuscript prior to submission.
LITERATURE CITED


TABLE 1. Isolates of *Pseudogymnoascus* used in this study

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<th>Citation</th>
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<th>LSU</th>
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<td><strong>MAT1-1</strong></td>
<td>USA, New York</td>
<td>2008</td>
<td><em>M. lucifugus</em></td>
<td>(Gargas et al. 2009)</td>
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<td>KF017865</td>
<td>KF017806</td>
<td>KJ938437</td>
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<td>USA, Wisconsin</td>
<td>1960</td>
<td>Amorphous peat</td>
<td>(Christensen and Whittingham 1965)</td>
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1. ITS = internal transcribed spacer region
2. LSU = nuclear large subunit region
3. TEF = translation elongation factor EF-1α
4. MAT = mating-type locus
5. CR = Czech Republic