CLONAL EXPANSION AND PHENOTYPIC EXPRESSION OF PSEUDOGYMNOASCUS DESTRUCTANS

CLONAL EXPANSION OF THE *PSEUDOGYMNOASCUS DESTRUCTANS* GENOTYPE IN NORTH AMERICA IS ACCOMPANIED BY SIGNIFICANT VARIATION IN PHENOTYPIC EXPRESSION

By: Jordan Khankhet, B. Sc.

A Thesis Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

McMaster University

MASTER OF SCIENCE (2014) McMaster University
(Biology) Hamilton, Ontario

TITLE: Clonal expansion of the *Pseudogymnoascus destructans* genotype in North America is accompanied by significant variation in phenotypic expression

AUTHOR: Jordan Khankhet, B.Sc. (McMaster University)

SUPERVISOR: Professor Dr. Jianping Xu

NUMBER OF PAGES: ix, 88

ABSTRACT

White-nose syndrome is an emerging infectious disease that first appeared in 2006. Since its emergence, it has caused over 5.5 million deaths of bats in North America. *Pseudogymnoascus destructans*, a psychrophilic fungus, is the causative agent of white-nose syndrome. White-nose syndrome is characterized by the growth of *P. destructans* on the nose, wings, and/or ears of the bat causing epidermal erosions and ulcers. Previous studies determined that the population of *P. destructans* in North America is clonal. However, no Canadian isolates of *P. destructans* were used. In this study, multilocus sequence typing and DNA fingerprinting was used to genotype 112 North American isolates. My results showed that the population in Canada had the same genotype as those from the US and there was also evidence of minor genetic variation in three Canadian isolates. Using 16 *P. destructans* isolates representative of various geographical locations in North America, significant phenotypic variations based on mycelial growth, production of exudates, and production of pigments and diffusion into agar media were observed. Overall, my results indicate a clonal genotype expansion of *P. destructans* throughout North America that is accompanied by significant phenotypic variation.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Dr. Jianping Xu for everything he has done for me. I am truly appreciative that I was hired as a research assistant after my second year of undergrad and that he allowed me to be a part of his lab ever since. He was always encouraging and supportive of anything I wanted to do, whether it was in terms of research or my career plans. His door was always open and I felt that our many discussions over the years not only allowed me to become a better scientist but also a better person. He has taught me a great deal and gave me the opportunity to achieve my dreams. For that, I will always be grateful.

Next I would like to thank Aaron Vogan for all of his assistance since I started in the lab. He has taught me nearly all of the experimental techniques I have used and has provided valuable guidance along the way. The discussions we often had allowed me to develop my critical thinking and begin developing my own ideas which were instrumental in completing this thesis. He was always available to discuss my classes, research or just about anything else, so for everything he has done for me I am thankful. Finally, I would like to thank my lab colleagues Zixuan Wang, Howard Chang, Nicholas Pun, and Heather Yoell for their support and assistance. They have made my time at McMaster a wonderful experience and something that I will always cherish.

THESIS FORMAT

This thesis is composed of three chapters. The first chapter provides background information and a brief overview of this study. The second chapter presents my manuscript that has been submitted to a peer-reviewed scientific journal. The third chapter provides an overall conclusion and suggests further research to expand our understanding of this topic.

Chapter 1: **GENERAL INTRODUCTION**

Chapter 2: **Clonal expansion of the *Pseudogymnoascus destructans* genotype in North America is accompanied by significant variation in phenotypic expression**

Authors: Jordan Khankhet, Karen J. Vanderwolf, Donald F. McAlpine, Scott McBurney, David P. Overy, Durda Slavic, Jianping Xu

Comments: This study was conducted by JK under the supervision of JX. Isolates used in this study were provided by KJV, DFM, SM, DPO and DS. The manuscript was written by JK and JX. KJV, DFM, SM and DPO provided feedback and editing.

Chapter 3: **GENERAL CONCLUSIONS AND PERSPECTIVES**

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1:

GENERAL INTRODUCTION

1.1 Emerging Infectious Disease

Emerging infectious diseases are those that have recently been discovered, increased in incidence, spread to new geographical areas, increased in host range, or newly evolved [1]. There are two hypotheses for the origin of these emerging infectious diseases, the novel pathogen hypothesis and the endemic pathogen hypothesis. A novel pathogen is a pathogen that has spread into a new geographical area and encounters a naïve host species vulnerable to the disease. An endemic pathogen is a pathogen that is present in a location but changes in the environment, pathogen or host leads to increased pathogenicity [1].

1.1.1 The Novel Pathogen Hypothesis

Pathogens have been introduced into new geographical areas by various mechanisms with humans having a significant role. Humans have introduced pathogens to new areas through direct contact and have also introduced exotic species causing the emergence of a disease [1]. *Batrachochytrium dendrobatidis* is a fungal pathogen that causes chytridiomycosis in amphibians worldwide. It is considered a novel pathogen in part because of its wide-scale dispersal into many locations including remote locations. Amphibians have been transported throughout the world for research, food, medicine as well many other purposes. Those that show no clinical symptoms such as the American

bullfrog (*Rana catesbeiana*) may have been transporting the pathogen unknowingly to various locations. Similar activities have occurred with other diseases through human transport [1]. Other well-known novel pathogens introduced to new geographical areas include HIV/AIDS, influenza, and SARS.

Novel pathogens show less genetic variation in more recently dispersed geographical areas and more genetic variation in areas in which it is endemic. An example of this is *B. dendrobatidis* which shows low sequence diversity and no evidence of co-evolution with amphibians as well as little association between geography and genotype [2]. These molecular data strongly support the novel pathogen hypothesis of *B. dendrobatidis*.

1.1.2 The Endemic Pathogen Hypothesis

Endemic pathogens emerge due to changes in ecology, immunology, and/or behavior of the host or pathogen [1]. Environmental changes could lead to immunosuppression which allows for disease to occur. For example, a pathogenic fungus (*Saprolegnia ferax*) and UV-B radiation synergistically increase the mortality of amphibian embryos [3]. Changes in the environment could also increase the reproduction or transmission of a pathogen. Altered behavior could also lead to the propagation and spread of a pathogen by causing unfavorable interactions for the host with the pathogen [1].

Endemic pathogens are expected to show a link between geographical distance and genetic distance [1]. Strains should show genetic variation related to the time since they have recently exchanged genetic information. Populations from the same area that have

exchanged genetic information would show more similar genotypes compared to distant populations [1]. Overall, endemic pathogens are expected to show more genetic diversity than novel pathogens.

1.2 White-Nose Syndrome

White-nose syndrome (WNS) is a recently emerging infectious disease that has devastated the North American bat populations. Since it was first observed in Howes Cave near Albany, New York in 2006, it has been estimated to have caused the death of over 5.5 million bats in North America [4,5]. WNS affect many hibernating insectivorous bat species. The following seven bat species have been confirmed to have WNS; big brown bat (*Eptesicus fuscus*), eastern small-footed bat (*Myotis leibii*), gray bat (*Myotis grisescens*), Indiana bat (*Myotis sodalis*), little brown bat (*Myotis lucifgus*), northern long-eared bat (*Myotis septentrionalis*) and tricolored bat (*Perimyotis subflavus*) [6]. Three other species have tested positive for the causative agent of WNS, *Pseudogymnoascus destructans*, however there were no signs of WNS. These three bat species include southeastern bats (*Myotis austroriparius*), silver-haired bats (*Lasionycteris noctivagans*) and Virginia big-eared bats (*Corynorhinus townsendii virginianus*).

1.2.1 Characteristics of WNS

Bats afflicted with WNS can be observed flying outside of their hibernaculum during the daytime. WNS is characterized by white, fluffy fungal growth on the muzzle, wings and ears of bats. Histopathologic confirmation of WNS includes cup-like epidermal erosions, ulcerations and invasion of the connective tissue in the wing membrane.

Elasticity, tone and suppleness of the wing membrane are also reduced [7]. In the muzzle, fungal hyphae enter the hair follicles and sebaceous glands. Despite deep fungal invasion, inflammation is not typically observed in hibernating bats afflicted with WNS [8]. Bats and other hibernating mammals show reduced immune function during hibernation [9], which may allow for fungal invasion by *P. destructans*.

1.2.2 Cause of Mortality

Mortality by WNS may be explained by the dehydration hypothesis. This hypothesis suggests that bats affected by WNS become dehydrated due to infection which leads to starvation. Wings of bats are important for maintaining water balance and gas exchange [7]. Damage to the wings disrupts this balance leading to an increase in evaporative water loss [10]. During hibernation, bats try to conserve their energy to survive the winter. The increase in evaporative water loss may be linked to the increased arousals from topor observed in bats with WNS [11]. Increased arousals lead to a depletion of fat stores and can cause starvation of bats over the winter because of the lack of food available.

There are differences in mortality rates between bat species affected by WNS. This may be related to the different environments in which they hibernate as well as their different susceptibilities to evaporative water loss [7]. Three bat species with a low mortality due to WNS are the eastern small-footed bat, the big brown bat and the Indiana bat. These bats often hibernate in drier environments and are less susceptible to evaporative water loss, whereas, the little brown bat, the Northern long-eared bat and the

tricolored bat are more often diagnosed with WNS and they hibernate in more humid environment suggesting higher susceptibility to evaporative water loss [7].

1.2.3 Mortality Rates

Turner et al. (2011) reviewed the mortality and spread of WNS over five years. They found that the time between detection of *P. destructans* and mass mortality varied by location. For example, in Layton Fire Clay Mine, Pennsylvania, a few bats appeared to have visible white fungal growth but it was not until the following year that deaths from WNS occurred. Whereas, in Shindle Iron Mine, Pennsylvania, the occurrence of large-scale mortality was observed weeks after initial detection. The bat population in 42 sites from five states decreased overall by 88%. The decrease in population of the six bat species examined were Northern long-eared bats – 98%, little brown bats – 91%, tricolored bats – 75%, Indiana bats – 72%, big brown bats – 41% and small footed bats – 12% [12]. The regional extinction of little brown bats is predicted to occur within the next two decades [6]. The Northern long-eared bat and the tricolored bat may also face similar extirpation, however, no estimates have been made. Turner et al. (2011) determined that little brown bats composed 84.5% of all the hibernating bats at 42 sites prior to WNS but after two years, they decreased to only 61% of all hibernating bats [12].

In another survey of various sites in the US, the mortality rate was 30 – 99% with an overall average mortality rate of 73% [6]. In Europe, *P. destructans* is found on bats however, no mass mortality is associated which suggests co-evolution between the bats and *P. destructans* [13,14].

1.2.4 Behavioral Changes

Bats with WNS display various behavioral changes. The proportion of bats roosting individually has increased since the emergence of WNS [15]. During the winter, they have an increase in arousals from topor and are observed emerging from their hibernacula mid-winter and during the daytime [11,15]. Infected bats are also observed roosting closer to the entrance of the hibernacula [16].

Bats affected with WNS showed increased grooming behavior in one study [17], but not in another [18]. Bats displayed decreased activity during arousal when affected by WNS which may be a result of the increased energy expenditure from increased arousals. Despite the dehydration hypothesis providing a probable cause of death in bats with WNS, there was no significant increase in visits to the water dish observed in WNS affected [18]. However, this may be due to differences between artificial and natural hibernacula or the possibility of bats drinking from the accumulation of condensation [18]. Many of these changes in behavior may be adaptive or maladaptive for bats. For example, solidarity roosting may reduce the transmission of WNS to kin or may increase energetic cost by decreasing the surrounding temperature [18]. Differences in behavioral responses between species may also be a factor for the differences in mortality rates.

1.3 *Pseudogymnoascus destructans*

P. destructans, formerly known as *Geomyces destructans,* is the causative agent of WNS [19,20]. It is a psychophilic fungus incapable of growth above 23°C [21]. *P. destructans* is characterized by its asymmetrically curved conidia. The fungus is capable

of invading living bat tissue leading to WNS as previously described [19]. Inoculation of a North American little brown bat with *P. destructans* from Europe caused WNS and mortality [11]. This provides support for the novel pathogen hypothesis of *P. destructans* because there were no mass mortalities associated between *P. destructans* and European bats which is therefore suggestive of co-evolution in Europe and susceptibility in North America [11].

1.3.1 Phylogenetic Analyses

P. destructans was initially placed in the family of *Myxotrichaceae* and in the *Geomyces* genus based off of ITS and SSU sequence [19]. The ITS1, 5.8S and ITS2 and the SSU regions of the rRNA were sequenced and analyzed, placing the fungus within the asexual *Geomyces* genus. *Pseudogymnoascus* was considered a sexual stage of *Geomyces*. One of the most closely related species to *P. destructans* within the *Geomyces* genus at the time, was *Geomyces pannorum* [19]. Numerous *Geomyces* spp. and *P. destructans* were found in North American bat hibernacula [22]. However, in 2013, *P. destructans* was placed in the *Pseudogymnoascus* genus based off of ITS, LSU, rDNA, MCM7, RPB2 and TEF1regions of the genome [23]. In contrast to previous studies, no close relatives of *P. destructans* were found in bat hibernacula when analyzed in further detail. *Geomyces* and allies were reclassified in the *Pseudodeurotiaceae* family. *Geomyces*, *Gymnostellatospora* and *Pseudogymnoascus* were also classified as distinct genra. The most closely related species are those within the *Pseudogymnoascus roseus* species complex. This species complex includes *Geomyces vinaceus, P. roseus,*

Pseudogymnoascus vinaceus and likely *Pseudogymnoascus pannorum* (formely *G. pannorum*). Further taxonomic research must be conducted to separate the identity and relationship of species within this complex [23]. The clades closest to *P. destructans* contain species with known sexual stages suggesting that *P. destructans* may have a sexual stage but has not been documented [23].

1.3.2 Morphology

P. destructans growth on corn meal agar artificial media was originally described as white around the outer edge with "Gray to Gray-Green near Grayish Olive to Andover Green" conidia at the center [19]. *P. destructans* was inoculated on Sabouraud dextrose (SD) agar and potato dextrose agar (PDA) at -10°C, 4°C, 15°C and 25°C in a study by Chaturvedi et al. (2010). No growth was seen at -10°C and 25°C. Colony diameter was larger at 15°C than 4°C. Initial colony appearance of a *P. destructans* isolate at 4°C and 14°C on PDA was white with no pigment on the reverse. 28 days after incubation both colonies turned grayish green but the isolate grown at 15°C also produced diffusible dark brown pigment and exudates on the surface [24]. *P. destructans* growth from conidia exposed to benzothiazole showed increase pigmentation and diffusion of pigments into the media [25].

Colony morphologies of six Czech Republic isolates were studied by Kubátová et al. (2011). Colonies on Czapek agar, soil extract agar, potato carrot agar and synthetic nutrient agar were flat, often whitish with an uncolored reverse after 21 days of incubation at 15°C. Colonies on SD agar, SD agar with cycloheximide, soil extract agar

with glucose and rose bengal and glucose chloramphenicol agar were often white in parts of the colony not sporulating and greyish beige in the sporulating parts. The colonies were also raised with a colored reverse. Growth rates were higher at 12°C compared to 15°C in 7 of the 8 media [26]. Chaturvedi et al. (2010) reported colony diameters between 20-27 mm on SD at 15°C whereas Kubátová et al. (2011) observed an average colony diameter of 14 mm. Individual isolates showed small differences in growth rates in both studies [24,26].

Verant et al. (2012) investigated the optimal growth temperature of six *P. destructans* isolates, three from the US and three from Europe. The optimal growth temperature was determined using performance curves based on a range of nine incubation temperatures. Optimal growth temperatures after 5 weeks of incubation for US isolates ranged from 12.5°C to 13.2°C. Optimal growth temperatures for European isolates ranged from 13.8°C to 15.8°C. Growth did not occur at 21.4°C and the upper critical temperature for growth was determined to be between 19.0 and 19.8°C. Growth rates varied across isolates but were not correlated with geographical origin. Verant et al. (2012) showed that temperature can significantly affect the growth rate *P. destructans* which may affect disease progression in hibernating bats. The majority of little brown bats in a mine in Ohio hibernated at 7.2 ± 2.6°C and the majority of Indiana bats hibernated at 8.4 ± 1.7°C [27]. The hibernating temperatures are below the optimal growth temperature of *P. destructans*. Based off of the measurements in the Verant et al. (2012) study, a temperature decrease from 7.2°C to 4.6°C (one standard deviation),

would reduce the growth of *P. destructans* growth by 40% [27]. The hibernating temperatures of different species may significantly affect the growth of *P. destructans* and may affect the differences in mortality rates as described earlier.

Typical morphology based on previous research was reported by Verant et al. (2012) for *P. destructans* colonies grown between 0 and 7°C. Hyphae were thin and contained asymmetrically curved conidia on branched conidiophore tips. However, at 12°C and above, hyphae were thicker and conidia were pear-shaped to globoid-shaped. The conidia were also produced in short chains. Above 15°C, hyphae were deformed and hyphal tips showed a branched antler-like morphology. Colonies produced arthrospores and chlamydospore-like structures. Colonies were tan to dark brown and the surface of the colony was creased. At 18°C, hyphae were thick and irregular and conidia were not observed [21].

1.3.3 Enzymatic Activity

P. destructans isolates are capable of producing various proteolytic and hydrolytic enzymes [24]. *P. destructans* produced esterase, esterase lipase, lipase, leucine arylmidase, napthol-A-B1-phosphohydrolase, acid phosphatase, alkaline phosphatase, N-acetyl-β-glucosaminidase, β-glucosidase, urease, and vailine arylamidase. It did not produce α-chymotrypsin, trypsin, α-mannosidase, α-galactosidase, α-glucosidase, α-fucosidase, β-galactosidase, β-glucoronidase, and β-glucosidase. When *P. destructans* were grown on albumin, gelatin or casein, it secreted proteinases. *P. destructans* was incapable of growth on egg yolk agar and did not show phospholipase activity. The

secreted proteases in *P. destructans* affect virulence in many other microbial pathogens [24,28]. Protease (aspartyl), urease and superoxide dismutase genes were identified in *P. destructans* and were statistically significant in similarity to the same genes in *C. neoformans* [29]. These genes that are identified in *P. destructans* are dual-use virulence factors [29].

P. destructans persist in hibernacula absent of bats [30]. *P. destructans* is capable of growth on mushroom tissue (*Lentinula edodes*), dead fish (*Poecilia* spp.), shrimp exoskeleton (*Pleoticus muelleri*) and an insect species (*Locusta migratoria*). No keratinolytic activity was observed for *P. destructans* [31]. The fungus was capable of growth on many sources of nitrogen such as nitrate, nitrite, L-asparagine, and ammonium. Isolates of *P. destructans* were identically tolerant to calcium, pH, sodium thiosulfate, sodium sulfite and L-cysteine. Many of these substrates can be found in bat hibernacula allowing for the persistence and spread of *P. destructans* [31].

P. destructans isolates are capable of growth on dissolved organic carbon and show chitinase and cellulase activity [32]. However, these enzymatic activities were reduced compared to other *Pseudogymnoascus* spp. *P. destructans* is capable of hemolysis but only occur when nutrients are limited. This suggests that the hemolysis function does not play a significant role in infection, but is necessary for survival. Overall, *P. destructans* shows reduced saprophytic enzymatic activity compared to *Pseudogymnoascus* spp. found in the same hibernacula and that *P. destructans* is more adapted for growth on the bat host [32].

1.3.4 Genetics of *P. destructans*

The genome of *P. destructans* has been sequenced and is 30.49 Mb with a GC content of 49.8% [33]. *P. destructans* is predicted to have 7,967 proteins compared to 9,689 in *P. pannorum*. 25.8% of the *P. destructans* genome is repeats whereas 5.4% of the *P. pannorum* genome is repeats. There are 458 conserved genes in *P. destructans*. *P. destructans* has fewer proteins with enzymatic function than *P. pannorum* which may be related to the saprophytic nature of *P. pannorum* and the pathogenic nature of *P. destructans* [33].

P. destructans is believed to have been introduced from Europe into North America based on inoculation experiments and genotype data [11,34,35]. Using multilocus sequence typing (MLST) 16 *P. destructans* isolates collected in North America between 2009 and 2010 were clonal [35]. 13 isolates collected between 2010 and 2011 from Midwestern and Southern US were also determined to be clonal using MLST [34]. In both studies, eight gene fragments were sequenced corresponding to the genes *ALR, Bpntase, DHC1, GPHN, PCS, POB3, SRP72 and VPS13*. In another study, six *P. destructans* isolates collected in 2008 in the US were used in DNA fingerprinting and random amplified polymorphic DNA typing. They showed identical banding patterns indicative of a single strain of *P. destructans* [24]. Foster et al. (2012) is currently conducting extensive genetic analysis on *P. destructans* to determine the origin and spread of the fungus. Using PCR assays for 140 microsatellite loci, they found no genetic variation in 21 North American isolates but they found variation in 13 European isolates.

They also used whole genome sequencing for a subset of their isolates and found limited genetic variation in the North American isolates and significant variation among the European isolates. They mention that they found enough variation to determine the dispersal of *P. destructans* but this research is currently unpublished [36].

1.3.5 Potential Treatments

P. destructans is susceptible to many antifungal drugs [37]. The fungus is susceptible to amphotericin B and four azoles (ketoconazole, itraconazole, posaconazole, and voriconazole) at a low minimum inhibitory concentration. Fluconazole produces a susceptible-dose dependent pattern which suggests that maintaining blood levels with a high dose of fluconazole may treat the disease. Five isolates of *P. destructans* showed similar susceptibilities. Using high-throughput screening, 27 compounds out of 1,920 were shown to cause greater than 50% inhibition of *P. destructans* when grown at 15°C. Antifungal drugs (econazole nitrate, chloroacetoxyquinoline, sulconazole nitrate, pyrithione zinc, chloroxine and ciclopriox olamine), biocide (benzalkonium chloride), fungicide (phenylmercuric acetate), fluorouracil and digitonin showed significant inhibition of growth at 6°C and 15°C. Econazole, sulconazole nitrate and phyenylmercuric acetate showed the highest growth inhibition at a low concentration of 0.1 µM [37].

Volatile organic compounds (VOCs) from bacteria were tested for *P. destructans* growth inhibition [25]. The VOCs tested were produced from *Pseduomonas* and *Bacillus* spp. The VOCs tested were: decanal, nonanal, benzothaizole, 2-ethyl-1-1-hexanol, N,N-

dimethyloctylamine and benzaldehyde. The VOCs inhibited the growth of *P. destructans* at a concentration of less than 1 ppm when placed next to media inoculated with *P. destructans* conidia. The VOCs varied in mycelial inhibition of *P. destructans* but all were capable of inhibition at 30 µl of 1.13 ppm to 113 ppm. Some VOCs also synergistically inhibited mycelial growth of *P. destructans* in two or three VOCs combinations. VOCs provide a potential treatment that does not involve contact with *P. destructans* itself [25]. Other biocontrol agents are currently being investigated as a potentially feasible, cost-effective treatment for WNS. *Trichoderma polysporum*, a well-known biocontrol fungus, has been isolated in hibernacula affected by WNS [38]. It was antagonistic to *P. destructans* but not the closely related *P. pannorum*. *T. polysporum* secretes inhibitory metabolites between 6-15°C that can prevent germination and growth of *P. destructans* [38].

Chitosan is a biopolymer derived from chitin [39]. It has many potential biomedical applications such as for wound healing and as artificial skin [39]. It also has strong antimicrobial activity [40]. It is biodegradable, nontoxic, has a low cost and can be aerosolized. Preliminary research shows that it can cause an 84% growth inhibition of *P. destructans;* however, the full study has not currently been published. Further research must be done to determine optimal concentrations to inhibit the growth of *P. destructans* and its effects on bats.

1.4 Project Objectives

Since WNS is a recently emerging infectious disease that appeared in 2006, the numbers of studies in this field is limited. The current literature provides a great deal of information, however, there is much more to learn in order to determine a strategic plan to prevent the spread of WNS and to find possible treatments that may be used. The focus of my research was on the population genetics as well as on the phenotypic variation of *P. destructans* in North America. There have been only a few studies that attempted to determine the genetic relationship between *P. destructans* isolates, however, their sample sizes were small and did not include any Canadian isolates. The phenotype of *P. destructans* has been compared between different growth conditions, however, no extensive studies have been conducted to determine the phenotypic variation between isolates within the same condition.

Using a total of 112 *P. destructans* isolates from four Canadian provinces and five US states, I investigated whether there was any genetic variation using MLST and DNA fingerprinting. I also investigated whether there was any phenotypic variation among isolates based on four phenotypic traits. The details and results of the project are discussed in Chapter 2.

References

1. Rachowicz LJ, Hero J-M, Alford RA., Taylor JW, Morgan JAT, et al. (2005) The Novel and Endemic Pathogen Hypotheses: Competing Explanations for the Origin of Emerging Infectious Diseases of Wildlife. Conserv Biol 19: 1441–1448.

2. Morgan JAT, Vredenburg VT, Rachowicz LJ, Knapp RA, Stice MJ, et al. (2007) Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis.* Proc Natl Acad Sci U S A 104: 13845–13850.

3. Kiesecker JM, Blaustein AR (1995) Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. Proc Natl Acad Sci U S A 92: 11049–11052.

4. Froschauer A, Coleman J (2012) North American bat death toll exceeds 5.5 million from white-nose syndrome. US Fish Wildl Serv: 1–2. Available: http://whitenosesyndrome.org/sites/default/files/files/wns_mortality_2012_nr_final_0.pdf.

5. Blehert DS, Hicks AC, Behr M, Meteyer CU, Berlowski-zier BM, et al. (2009) Bat White-Nose Syndrome : An Emerging Fungal Pathogen? Science 323: 227.

6. Frick WF, Pollock JF, Hicks AC, Langwig KE, Reynolds DS, et al. (2010) An emerging disease causes regional population collapse of a common North American bat species. Science 329: 679–682.

7. Cryan PM, Meteyer CU, Boyles JG, Blehert DS (2010) Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology. BMC Biol 8: 135.

8. Meteyer CU, Buckles EL, Blehert DS, Hicks AC, Green DE, et al. (2009) Histopathologic Criteria to Confirm White-nose Syndrome in Bats. J Vet Diagnostic Investig 21: 411–414. doi:10.1177/104063870902100401.

9. Carey H V, Andrews MT, Martin SL (2003) Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol Rev 83: 1153–1181.

10. Willis CKR, Menzies AK, Boyles JG, Wojciechowski MS (2011) Evaporative water loss is a plausible explanation for mortality of bats from white-nose syndrome. Integr Comp Biol 51: 364–373.

11. Warnecke L, Turner JM, Bollinger TK, Lorch JM, Misra V, et al. (2012) Inoculation of bats with European *Geomyces destructans* supports the novel

pathogen hypothesis for the origin of white-nose syndrome. Proc Natl Acad Sci: 1–5.

12. G. Turner G, D. Reeder M, J. Coleman TH (2011) A Five-year Assessment of Mortality and Geographic Spread of White-nose Syndrome in North American Bats and a Look to the Future. Bat Res News 52: 14–27.

13. Puechmaille SJ, Wibbelt G, Korn V, Fuller H, Forget F, et al. (2011) Pan-European distribution of white-nose syndrome fungus (*Geomyces destructans*) not associated with mass mortality. PLoS One 6: e19167..

14. Martínková N, Bačkor P, Bartonička T, Blažková P, Cervený J, et al. (2010) Increasing incidence of *Geomyces destructans* fungus in bats from the Czech Republic and Slovakia. PLoS One 5: e13853.

15. Langwig KE, Frick WF, Bried JT, Hicks AC, Kunz TH, et al. (2012) Sociality, density-dependence and microclimates determine the persistence of populations suffering from a novel fungal disease, white-nose syndrome. Ecol Lett 15: 1050–1057.

16. McAlpine DF, Vanderwolf KJ, Forbes GJ, Malloch D (2011) Consumption of Bats (*Myotis* spp .) by Raccoons (*Procyon lotor*) During an Outbreak of White-Nose Syndrome in New Brunswick , Canada : Implications for Estimates of Bat Mortality: 257–260.

17. Brownlee-Bouboulis SA, Reeder DM (2013) White-nose syndrome-affected little brown myotis (*Myotis lucifugus*) increase grooming and other active behaviors during arousals from hibernation. J Wildl Dis 49: 850–859.

18. Wilcox A, Warnecke L, Turner JM, McGuire LP, Jameson JW, et al. (2014) Behaviour of hibernating little brown bats experimentally inoculated with the pathogen that causes white-nose syndrome. Anim Behav 88: 157–164.

19. Gargas A, Trest MT, Christensen M, Volk TJ, Blehert DS (2009) Geomyces destructans sp. nov associated with bat white-nose syndrome. Mycotaxon 108: 147–154.

20. Lorch JM, Meteyer CU, Behr MJ, Boyles JG, Cryan PM, et al. (2011) Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. Nature 480: 376–378.

21. Verant ML, Boyles JG, Waldrep W, Wibbelt G, Blehert DS (2012) Temperature-Dependent Growth of *Geomyces destructans*, the Fungus That Causes Bat White-Nose Syndrome. PLoS One 7: e46280.

22. Lindner DL, Gargas A, Lorch JM, Banik MT, Glaeser J, et al. (2011) DNA-based detection of the fungal pathogen *Geomyces destructans* in soils from bat hibernacula. Mycologia 103: 241–246.

23. Minnis AM, Lindner DL (2013) Phylogenetic evaluation of *Geomyces* and allies reveals no close relatives of *Pseudogymnoascus destructans*, comb. nov., in bat hibernacula of eastern North America. Fungal Biol 117: 638–649.

24. Chaturvedi V, Springer DJ, Behr MJ, Ramani R, Li X, et al. (2010) Morphological and molecular characterizations of psychrophilic fungus *Geomyces destructans* from New York bats with White Nose Syndrome (WNS). PLoS One 5: e10783.

25. Cornelison CT, Gabriel KT, Barlament C, Crow SA (2014) Inhibition of *Pseudogymnoascus destructans* growth from conidia and mycelial extension by bacterially produced volatile organic compounds. Mycopathologia 177: 1–10.

26. Kubátová A, Koukol O, Nováková A (2011) *Geomyces destructans*, phenotypic features of some Czech isolates. 63: 65–75.

27. Brack V (2007) Temperatures and locations used by hibernating bats, including *Myotis sodalis* (Indiana bat), in a limestone mine: implications for conservation and management. Environ Manage 40: 739–746. Available: http://www.ncbi.nlm.nih.gov/pubmed/17874161. Accessed 12 May 2014.

28. López-Otín C, Bond JS (2008) Proteases: multifunctional enzymes in life and disease. J Biol Chem 283: 30433–30437.

29. Smyth C, Schlesinger S, Overton BE, Butchkoski C (2013) The alternative host hypothesis and potential virulence genes in *Geomyces destructans*. 54: 17–24.

30. Lorch JM, Muller LK, Russell RE, O'Connor M, Lindner DL, et al. (2013) Distribution and environmental persistence of the causative agent of white-nose syndrome, *Geomyces destructans*, in bat hibernacula of the eastern United States. Appl Environ Microbiol 79: 1293–1301.

31. Raudabaugh DB, Miller AN (2013) Nutritional Capability of and Substrate Suitability for *Pseudogymnoascus destructans*, the Causal Agent of Bat White-Nose Syndrome. PLoS One 8: e78300.

32. Reynolds HT, Barton HA (2014) Comparison of the white-nose syndrome agent *Pseudogymnoascus destructans* to cave-dwelling relatives suggests reduced saprotrophic enzyme activity. PLoS One 9: e86437.

33. Chibucos MC, Crabtree J, Nagaraj S, Chaturvedi S, Chaturvedi V (2013) Draft Genome Sequences of Human Pathogenic Fungus *Geomyces pannorum* Sensu Lato and Bat White Nose Syndrome Pathogen *Geomyces* (*Pseudogymnoascus*) *destructans*. 1: 1–2. doi:10.1093/nar/gks1050.2.

34. Ren P, Haman KH, Last LA, Rajkumar SS, Keel MK, et al. (2012) Clonal spread of *Geomyces destructans* among bats, midwestern and southern United States. Emerg Infect Dis 18: 883–885.

35. Rajkumar SS, Li X, Rudd RJ, Okoniewski JC, Xu J, et al. (2011) Clonal genotype of *Geomyces destructans* among bats with White Nose Syndrome, New York, USA. Emerg Infect Dis 17: 1273–1276.

36. Foster J, Drees K, Lorch J, Blehert D (2012) The origin and spread of *Geomyces destructan*s in North America. 5th Annu White-nose Syndr symp.

37. Chaturvedi S, Rajkumar SS, Li X, Hurteau GJ, Shtutman M, et al. (2011) Antifungal testing and high-throughput screening of compound library against *Geomyces destructans*, the etiologic agent of geomycosis (WNS) in bats. PLoS One 6: e17032.

38. Zhang T, DeFiglio H, Chaturvedi V, Chaturvedi S (2013) Identification of a promising *Trichoderma polysproum* strain for biocontrol of Geomyces Destructans. 6th Annu White-nose Syndr Symp.

39. Rinaudo M (2006) Chitin and chitosan: Properties and applications. Prog Polym Sci 31: 603–632.

40. Muzzarelli R, Tarsi R, Filippini O, Giovanetti E, Biagini G, et al. (1990) Antimicrobial Properties of N-Carboxybutyl Chitosan. Antimicrob Agents and Chemotherpy. 34. doi:10.1128/AAC.34.10.2019.

CHAPTER 2

Clonal expansion of the *Pseudogymnoascus destructans* genotype in North America is accompanied by significant variation in phenotypic expression

Jordan Khankhet[1], Karen J. Vanderwolf[2, 3], Donald F. McAlpine[2], Scott McBurney[4], David P. Overy[5,6,7], Durda Slavic[8], Jianping Xu[1]*

1 Department of Biology, McMaster University, Hamilton, Ontario, Canada, **2** New Brunswick Museum, 277 Douglas Ave., Saint John, New Brunswick E2K 1E5 Canada, **3** Canadian Wildlife Federation, 350 Promenade Michael Cowpland, Kanata, Ontario K2M 2W1 Canada, **4** Canadian Cooperative Wildlife Health Centre, Atlantic Region, Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island, C1A 4P3, **5** Department of Chemistry, University of Prince Edward Island, 550 University Ave., Charlottetown, Prince Edward Island, Canada, C1A 4P3, **6** Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, Prince Edward Island, Canada, C1A 4P3, **7** Nautilus Biosciences Canada, Duffy Research Center, University of Prince Edward Island, 550 University Ave., Charlottetown, Prince Edward Island, Canada, C1A 4P3 **8** Animal Health Laboratory, University of Guelph, Guelph, Ontario, Canada, N1H 6R8

Running title: Rapid expansion and evolution of *P. destructans*

Keywords: white-nose syndrome, bats, multilocus sequence typing, clonal expansion, phenotypic expression

***Corresponding author:** Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, L9H 4G3, Canada. Phone: 1-905-525-9140 ext. 27934; fax: 1-905-522-6066; email: jpxu@mcmaster.ca

Abstract

Pseudogymnoascus destructans is the causative agent of an emerging infectious disease that threatens populations of several North American bat species. The fungal disease was first observed in 2006 and has since caused the death of nearly six million bats. The disease, commonly known as white-nose syndrome, is characterized by a cutaneous infection with *P. destructans* causing erosions and ulcers in the skin of nose, ears and/or wings of bats. Previous studies based on sequences from eight loci have found that isolates of *P. destructans* from bats in the US all belong to one multilocus genotype. Using the same multilocus sequence typing method, we found that isolates from eastern and central Canada also had the same genotype as those from the US, consistent with the clonal expansion of *P. destructans* into Canada. However, our PCR fingerprinting revealed that among the 112 North American isolates we analyzed, three, all from Canada, showed minor genetic variation. Furthermore, we found significant variation among isolates in mycelial growth the production of mycelial exudate; and pigment production and diffusion into agar media. These phenotypic differences were influenced by culture medium and incubation temperature, indicating significant variation in environmental condition - dependent phenotypic expression during the clonal expansion of the *P. destructans* genotype in North America.

Introduction

White-nose syndrome (WNS) is an emerging infectious disease that first appeared in New York in 2006. Since its emergence it has been estimated to have caused the death of over 5.5 million bats in North America [1,2]. The causative agent of WNS is a psychrophilic fungus, *Pseudogymnoascus destructans* (formerly *Geomyces destuctans*) [3,4]. WNS is characterized by the growth of white, fluffy, mycelia of *P. destructans* on the muzzle, wings and ears of hibernating bats, with the hyphae causing erosions and ulcers in the skin of bats and damaging the underlying subcutaneous tissue [5]. The recent rapid spread of WNS has led to the dire prediction that North America's most abundant bat species, the little brown bat (*Myotis lucifugus*), may face regional extinction within two decades [6]. WNS has now been diagnosed in 25 US states and five Canadian provinces (http://www.whitenosesyndrome.org/resources/map). *P. destructans* is also widespread across Europe [7] although the infection is not fatal to European bats [8]. In contrast, infection by both European and North American sourced isolates of *P. destructans* are fatal to the North American little brown bat [9].

Previous studies have used multilocus sequence typing (MLST) and PCR fingerprinting to genotype *P. destructans* isolates from the US. No sequence variation was found among the samples examined and all isolates shared identical PCR fingerprints [10–12]. The apparent clonal genotype of the US population of *P. destructans* is consistent with the hypothesis that a single strain of *P. destructans* was recently introduced into the US. Given the timeline of case reports of WNS in Canada [13,14], it is

believed that Canadian occurrences of *P. destructans* represent the same clonal genotype as that in the US. However, whether the same clonal genotype is responsible for the widespread mortality of bats in Canada remains unconfirmed.

Aside from investigations of genotypic variation, several studies have examined how environmental factors influence the phenotypes of *P. destructans*. For example, Martinkova et al. (2010) showed that *P. destructans* could grow on a variety of media and that different media supported different rates of mycelial growth [15]. Verant et al. (2012) examined incubation temperature and found that it has a significant influence on mycelial growth of *P. destructans*, with optimal growth temperature range between 12.5 and 15.8°C [16]. Verant et al. (2012) also analyzed the growth responses to a variety of temperatures among six independent isolates, three from the US and three from Europe. They found that geographical origin did not influence the growth rate of *P. destructans* on artificial media and that all isolates showed significant responses to small changes in temperature [16]. At present, relatively little is known about the potential phenotypic variations within and among isolates of *P. destructans* from either North America or Europe in their response to differences in growth conditions.

Understanding the genotypic and phenotypic variation of pathogens could help us better track the spread of infectious diseases and develop strategies to prevent and control these diseases. The objectives of this study are to determine whether isolates of *P. destructans* from Canada are members of the same genotype as those from the US, and to examine the variations of four phenotypes (mycelial growth rate, pigment production, pigment secretion and diffusion, and exudate production) at various temperatures and on

a range of media, among a set of geographically diverse North American isolates. While the role of the phenotypic traits we examined in *P. destructans* are not presently known, in other fungi these traits have been associated with vegetative fitness, fungal defense against the host immune system, and the production of fungal toxins and virulence factors in exudates. We hypothesized that if the *P. destructans* population in North America occurred via the recent clonal expansion of a single genotype, then there should be little/no genotypic and phenotype variation among the isolates.

Materials and Methods

Fungal Isolates

A total of 112 *P. destructans* isolates collected from 2008 to 2013 are included in this study (Table S1). The isolates were provided by the Animal Health Laboratory at the University of Guelph (Guelph, Ontario), the New Brunswick Museum (Saint John, New Brunswick), the Canadian Cooperative Wildlife Health Centre (Atlantic Region), and the Mycology Laboratory at Wadsworth Center in Albany, New York. One sample was also isolated at McMaster University. These isolates were collected from four Canadian provinces; Ontario (16 isolates), New Brunswick (71 isolates), Nova Scotia (1 isolate), and Prince Edward Island (9 isolates) and six US states; New York (3 isolates), North Carolina (3 isolates), West Virginia (5 isolates), Ohio (1 isolate), Vermont (2 isolates), and Pennsylvania (1 isolate). New Brunswick isolates originated from live bats, cave associated arthropods, and hibernaculum walls at seven hibernaculum sites. Isolates from

Nova Scotia, Prince Edward Island, Ontario and the US originated from deceased bats confirmed to have died of WNS.

DNA Fingerprinting

Previous genotype analyses of isolates of *P. destructans* have used both multilocus sequence typing (MLST) and PCR fingerprinting [10-12]. PCR fingerprinting, based on changes in repetitive sequences, is easier to perform than MLST and the genomic regions sampled using this method are likely evolving faster than simple nucleotide substitutions. In fungi, two separate primers targeting repetitive elements in the genome have been used extensively for distinguishing closely related strains by PCR fingerprinting. We followed this strategy, using primer $(GACA)_4$ (5'GACAGACAGACAGACA-3') and the M13-phage core sequence (5'-GAGGGTGGCGGTTCT-3') to fingerprint isolates of *P. destructans* obtained from eastern and central Canada and compared them with representative isolates from the US.

P. destructans isolates were first grown on synthetic-dextrose agar plates and genomic DNA was extracted following the protocol outlined in Xu *et al.* (2000). For PCR fingerprinting, each PCR reaction contained 12µL of Ready-to-Go PCR mix (Promega, Madison, WI, USA), 2µL of template DNA, and 2µL of 10µM of primer. PCR conditions were as follows: 5-minute initial denaturation at 94°C, 35 amplification cycles with a 1 minute denaturation at 94°C, 1 minute annealing at 50°C, and 2-minute extension at 72°C, and an 8-minute final extension at 72°C. PCR amplicons were separated on a 1.25% agarose gel stained with ethidium bromide. Gel electrophoresis was run for 1h and

50m at 150V in TBE buffer and visualized under UV light using a Chemi-Imager™ (Alpha InnovTech Corporation, San Leandro, California). PCR fingerprints were scored independently by two investigators and isolates showing identical patterns by both investigators were considered to have the same genotype. Isolates shown to have fingerprint patterns different from the majority were analysed a second time to confirm their distinctiveness. The finalized fingerprint data were used to generate the relationship among isolates using the program PAUP 4.0 [17].

Multilocus Sequence Typing

To confirm genotypes and compare the Canadian isolates with those obtained from the US, 44 of the 112 isolates representing a variety of bat hibernacula, a range of geographic locations, and all distinct PCR fingerprint genotypes were selected for multilocus genotyping, following protocols of Chaturvedi et al. (2010) [12]. Specifically, the following eight gene fragments were amplified and sequenced and compared to those from the US: *ALR*, *Bpntase*, *DHC1*, *GPHN*, *PCS*, *POB3*, *SRP72*, and *VPS13* [10]. PCR reactions contained 13.6µL of Ready-to-Go PCR mix (Promega, Madison, WI, USA), 2µL of template DNA, and 0.4µL of 10µM of primer. The PCR conditions were as follows: 3-minute initial denaturation at 94°C, 40 amplification cycles with a 15-second denaturation at 94°C, 30-second annealing at 55°C, and 1-minute extension at 68°C and a 5-minute final extension at 68°C. For confirmation of successful PCR amplification, 5µL of the amplified product for each gene from each sample was separated on a 1% agarose gel with ethidium bromide in TAE buffer for 25 minutes at 150V. Successfully amplified

PCR products were purified prior to sequencing using MicroClean©. Sequences were aligned in MEGA 5 [18] and genetic relationship among the strain was identified using maximum parsimony based on the concatenated sequences of the eight genes.

Morphological Comparisons

To identify potential phenotypic variations within and among isolates and determine whether different isolates respond similarly to the same set of environmental variables, 16 isolates representing all genotypes and a range of geographic jurisdictions in Canada (Ontario, New Brunswick, Prince Edward Island and Nova Scotia) and the US (New York and North Carolina) were examined. Potential differences in growth and colony morphology on synthetic-dextrose (SD) agar, potato dextrose agar (PDA), and minimal media (MM) agar at temperatures of 4°C, 14°C and 18°C were investigated. The SD agar contained 1% enzymatic digest of casein (Difco™), 2% dextrose and 2% agar; PDA contained 0.4% potato starch (Difco™), 2% dextrose and 1.5% agar; and the MM contained 0.17% yeast nitrogen base without amino acids and ammonium sulphate (Difco™), 0.5% $(NH_4)_2SO_4$, 2% dextrose and 2% agar. Each isolate-medium-temperature combination had three replicates. Colony diameter was measured 28 days after incubation. Qualitative morphological features of fungal colonies were scored visually by the naked eye for three phenotypic traits: colony reverse color, pigment secretion and diffusion into surrounding media, and exudate production, all at 28 days after incubation. Pigment secretion and diffusion into surrounding media was scored as either present or absent. Colony reverse color was recorded as the color of the agar medium directly

underneath each colony. Exudate production was scored on a scale of 0-3, with 0 = no exudate, 1 = little exudate, 2 = intermediate exudate, and 3 = profuse exudate (Fig. 2A-D).

Statistical Analysis

Statistical analyses were performed for the quantitative data of mycelial growth rate using SPSS 20.0 (IBM, Chicago, IL). The colony diameters were analyzed using a three-way factorial analysis of variance (ANOVA). The factors were media, temperature and isolates. P values of <0.05 were considered statistically significant. One-way ANOVAs with Games-Howell or Tukey's HSD post hoc test were conducted to compare diameters of *P. destructans* isolates in different temperature-media combinations. Tukey's HSD was used in conditions with equal variances and Game-Howell was used in conditions with unequal variances.

Results

Genotypes

DNA fingerprinting data based on the two PCR primers revealed four distinct PCR fingerprints among the 112 *P. destructans* isolates. Three of these fingerprints were represented by a single isolate each, while the remaining 109 isolates belonged to the fourth genotype. The three unique isolates included one from Ontario (ON3) and two from New Brunswick (NB26- Dorchester Mine and NB28- Harbell's Cave) (Fig. 1). ON3 was cultured from a deceased bat, NB26 from the hibernaculum wall and NB28 from a

live bat. However, when these three isolates and other 41 representative isolates were subjected to MLST, all 44 isolates from eastern and central Canada (Ontario, New Brunswick, Prince Edward Island, Nova Scotia) were found to have the same MLST genotype identical to those from the US at the eight sequenced loci [10,11]. Our results are thus consistent with the hypothesis of clonal expansion of *P. destructans* from the US to Canada, accompanied by genotypic microevolution.

Mycelial growth

Variation in mycelial growth was found among the 16 isolates on all nine media-temperature combinations. The ANOVA revealed that the isolate, medium and temperature all contributed significantly to the variation in colony diameters ($p<0.05$; Table 1). The three factors also interacted significantly in all three two-way combinations as well as the one three-way combination to influence variation in colony diameter ($p<0.05$; Table 1). The mean colony diameter and the standard deviation for each of the 16 *P. destructans* isolates at 28 days after inoculation are shown in Table S2. Of the three temperatures tested, 14°C was found to be the most conducive for mycelial growth for all 16 isolates tested on all three media. On two of the three media (MM and SD), 4^{o}C was the next most favorable incubation temperature for mycelial growth followed by 18^{o}C. However, on PDA, the reverse was observed.

Interestingly, among the three tested temperatures, the most conducive for growth (14°C) also showed the largest range of colony diameters among the isolates. The two isolates with the smallest colony diameters, US3 and NS1, were consistently the smallest

relative to the other isolates across all environmental conditions, with the exception of PDA/4°C. However, no single isolate had the largest colony diameter across all nine conditions. Pairwise comparisons revealed significant differences in colony diameters between isolates, for all nine conditions (Table S2). The SD/4°C environment showed the largest number of significantly different colony diameters, with 34 pairs. MM/14°C and SD/14°C followed closely, with 30 and 28 pairs respectively. Taken together, our results indicate significant divergence in mycelial growth rate among asexual isolates recently derived from a single ancestor genotype. As mentioned, the observed growth rate variation was influenced by both media and temperature.

Exudate production

Variation in exudate production was found among the 16 *P. destructans* isolates (Fig. 3). All three factors (temperature, medium and isolate) contributed to the observed variation in exudate production. Representatives of scores 0-3 for exudate production are shown in Figures 2A-D. After 28 days of incubation, no exudate was observed on colonies cultured on MM at any of the three incubation temperatures for any of the 16 isolates. In contrast, exudates were often observed among isolates cultured on the two other media and each of the 16 isolates produced exudate in at least two of the six remaining conditions (Fig. 3). Significant differences in exudate production were observed among the isolates within each medium/temperature condition. On SD medium, 13 isolates produced exudates in at least one of the three replicates at 4°C and a slightly different combination of 13 isolates produced exudates at 14^{o}C, while only 5 isolates

produced exudates at 18°C (Fig. 3). Four isolates produced exudates at all three temperatures on SD medium. On PDA, 7, 12, and 15 isolates produced exudates at 4^{o}C, 14^{o}C, and 18^{o}C respectively and only 5 isolates produced exudates at all three temperatures (Fig. 3). NB3 and NB28 were the only isolates that produced exudates on both SD and PDA media at all three temperatures.

Aside from the variation among isolates, variation among repeats for the same isolate under the same conditions was also observed. Overall, of the 144 isolate-by-environment combinations, 79 showed no exudate production in any of the three repeats while 16, 20, and 29 showed exudate production in one, two and all three repeats respectively. In addition, the amount of exudate production also varied among many of the repeats (Fig. 3). For example, on SD/14^{o}C, two replicates produced observable exudates while one replicate did not produce any visible exudates at the time of our observation for each of the following three isolates NB7, ON16 and US3. In contrast, three isolates (NB1, ON3, and NS1) did not produce any exudates in any of the three replicates while the remaining 10 isolates each produced exudates in all three replicates.

Pigment secretion

The secretion and diffusion of soluble pigments from the colony into the surrounding media was observed less frequently than exudate production (Fig. 4 and Fig. 5). Of the 144 isolate-environment combinations, 107 showed no pigment secretion and diffusion while 37 did in at least one of the three repeats. As with mycelial growth rate and exudate production, all three factors (isolate, medium, and incubation temperature)

contributed to differences in pigment secretion and diffusion. Among the 16 isolates, one (US7) did not produce any diffusible pigment in any of the nine environments. Of the remaining 15 isolates, two (NB7 and US3) produced diffusible pigments in five conditions, five (NB26, ON1, ON3, ON16, and NS1) produced diffusible pigments in three conditions and four (NB1, NB4, NB8, and NB25) produced diffusible pigments in two conditions. The remaining four isolates (NB2, NB3, NB28, and PE1) produced diffusible pigments in only one condition (Fig. 4). Among the nine conditions, the number of isolates which produced diffusible pigments varied from 0 (MM/4°C) to 9 (MM/18°C), with both temperature and medium contributing to the observed differences (Fig. 4).

Similar to the observed variation among repeats for exudate production, variation in the production of diffusible pigments were also commonly seen among repeats for the same isolate in the same environmental condition. Among the 37 isolate-environment combinations that showed diffusible pigment production, 8 had all three repeats consistently producing diffusible pigments, while 18 had two repeats and 11 had one that produced diffusible pigments (Fig. 4). Figure 2A and 2C show a non-pigment secreting and a pigment secreting colony respectively.

Colony reverse color

The color of the colony reverse seen from the bottom of the plates for all 16 isolates under the nine environmental conditions is presented in Figure 5. The observed color ranged from white to gray, gray-green, brown, and black. Similar to the other two

qualitative traits examined for these isolates, all three factors (isolate, medium and incubation temperature) were found to influence the colony reverse color. For example, the colony reverse color was brown for all 16 isolates on SD/14°C and SD/18°C while only slight variations were found for three isolates on SD/4^{o}C. In contrast, the remaining two agar media showed a range of colors across these temperatures (Fig. 5). For example, on PDA/4°C, the colony reverse color was gray for all isolates except NS1, which was brown. However, on PDA/14°C, the colony reverse color was mostly brown to black. Compared to the relatively limited or no variation among isolates on SD, the colony reverse colors among the isolates on MM were more varied. For example, two isolates NB4 and NB8 were consistently white on MM at the 4°C condition while other isolates produced a range of colors. Similar to the observed variation among replicates for exudate production and pigment diffusion, variation in colony reverse color were also found among replicates. For example, in the MM/4^{o}C condition, the colony reverse color of NB7 ranged between gray, gray-green and brown among replicates and in the PDA/18^{o}C environment, colony reverse color of isolates ranged from brown to gray or gray to black among replicates. Figures 2E and 2F present a white reverse colony and a black reverse colony respectively.

Discussion

Our analyses indicated that isolates of *P. destructans* from four provinces in eastern and central Canada all had the same multilocus sequence type, identical to that from the US. However, minor PCR fingerprinting variants were observed, consistent with micro-

genotypic changes during the clonal spread of *P. destructans* in North America. In contrast to the limited genotype variation, abundant phenotypic variation was observed within and among 16 representative isolates. Furthermore, the pattern of phenotypic expression differed depending on environmental conditions. Below we discuss the potential mechanisms and implications of these observations.

Clonal expansion of a single *P. destructans* genotype in North America

Two hypotheses have been proposed to explain the emergence of an infectious disease [19]. The novel pathogen hypothesis suggests that a new disease emerges when a pathogen is introduced into a previously unoccupied geographical area and encounters a naïve host population. The endemic pathogen hypothesis suggests that changes in environmental factors or in the behavior of the pathogen and/or host lead to disease emergence. Evidence that a single strain of *P. destructans* was introduced from Europe into North America suggests that the behavior of WNS in North America is best explained by the novel pathogen hypothesis [9]. The MLST data from our study showed 100% DNA sequence identity among *P. destructans* isolates in Canada and the US at the eight sequenced loci. This is consistent with the rapid clonal spread of a single genotype from a site or sites in the US to locations across much of eastern and central Canada.

Our PCR fingerprinting analyses revealed one dominant genotype for the majority of our 112 North American isolates. Three additional genotypes were also observed and each was represented by one isolate. All three unique genotypes originated from Canadian localities. As mentioned above, these three different genotypes were found to

have a MLST genotype at the eight sequenced loci identical to those from sampled isolates originating from both Canada and the US. The presence of one dominant PCR fingerprint genotype from both the US and Canadian samples is consistent with the hypothesis of the clonal spread of a single *P. destructans* genotype throughout North America. However, the identification of three new PCR fingerprinting genotypes suggests that microevolution may be occurring or that there may be more natural genotypic variation in populations of *P. destructans* in North America than previously believed [11, 12]. It is important to note as well that the PCR fingerprinting methodology used in this study may have missed other types of genetic variation. A recent preliminary report based on data from whole-genome sequencing of multiple strains also suggested there was observable genetic variation among isolates of *P. destructans* in North America [20].

Phenotypic variation

In contrast to the observed MLST uniformity and minor PCR fingerprint genotype variation, we found significant phenotypic variation in all four examined traits within and among the 16 isolates that we screened. Given that very limited or no genotypic changes have been detected among North American *P. destructans* isolates using our methods, the large phenotypic differences within and among the isolates are surprising.

In typical assays of genetic contributions to phenotypic differences among fungal isolates, the starting cultures are usually first grown in the same environment to eliminate the potential effects inherited from differences in prior environmental conditions. In this study, the 16 representative isolates were from different geographic locations and/or

ecological niches. Furthermore, different labs might have used slightly different media to isolate and grow them for different lengths of times. As a result, the gene expression patterns and physiologies of these isolates might have been modified by such external environmental factors. To eliminate those effects and to estimate the potential genetic contributions to phenotypic differences among the 16 isolates, all isolates were first cultured on the same medium and incubated at the same temperature (PDA/14^{o}C) for two weeks before being further sub-cultured on the three different media and incubated at three different temperatures.

Rapid phenotypic diversification has been observed in laboratory conditions among replicate lines derived from a single ancestor, in a variety of microorganisms, due to the accumulation of spontaneous genetic mutations and genetic drift [21]. However, we are unaware of any report describing rapid phenotypic divergence among descendants of an asexual lineage in microorganisms in nature. Even though minor genetic differences among isolates were found here and have been reported earlier [20], the relationship between genetic and phenotypic variation among descendants of the North American *P. destructans* asexual lineage remained to be investigated. There are two possibilities to explain the phenotypic variation documented in this study. The first is that there is little genetic basis for these three qualitative phenotypic traits and that these phenotypes are highly sensitive to minute environmental changes. The second is that the genome structures of many of the North American *P. destructans* isolates are not very stable and can change quickly, impacting phenotype expressions. Regardless of the potential mechanisms, the observed phenotypic diversity may have contributed to the rapid spread

and high virulence of *P. destructans* in bats in North America. Below we discuss the potential significance for the four phenotypic traits investigated.

Colony diameter has been commonly used as an indicator of vegetative fitness in filamentous fungi [22]. The *P. destructans* mycelial growth rates between certain isolates differed by as much as two fold in several environmental conditions that we tested (Table S2). However, *in vitro* growth on artificial media may not reflect their mycelial growth in cave soil or *in vivo* in bats. For example, if high growth rate on artificial media is selected for, we should expect the two most recent isolates NS1 (from 2013 in Nova Scotia) and US3 (from 2013 in New York) to be fast growers. Interestingly, these two isolates had among the slowest growth rates but were also among those showing the most consistent pigment production and diffusion, and produced among the least exudates. These results suggest potential trade-offs among some of these traits in *P. destructans*. Specifically, slower growth rates and high pigment production might reflect a different strategy in nutrient allocation and utilization; where carbon and nitrogen resources could have been diverted from primary metabolism required for growth to secondary metabolism involved in pigment generation.

At present, the potential role or roles of exudate production in *P. destructans* are unknown. Exudates are commonly observed in fungal cultures *in vitro*. For example, many *Aspergillius* and *Penicillium* species have been characterized as producing exudate droplets, and this trait has been used for taxonomic differentiation between species within both genera [23]. Such droplets have been found to contain proteins and toxins. High levels of ochratoxins were reported in exudates in several *Penicillium* species [24]. A

strain of *Aspergillus fumigatus* was reported to contain gliotoxin in its exudate [25]. *Metarhizium anisopliae*, an insect pathogen, contained destruxins and was shown to have protease-related enzyme activity in its exudate [26]. At present, the detailed ecological functions of exudates in nature are hotly debated. Jennings [27] believed that the exudates could act as a water reservoir for aerial hyphal growth in unfavourable conditions. McPhee and Colotelo [28] suggested that exudate droplets might function as a reservoir of metabolic by-products, secondary metabolites, or metabolite reserves. The guttation droplets from *Fusarium culmorum* and *Sclerotinia sclerotiorum*, two plant fungal pathogens, have been shown to rapidly degrade plant tissue suggesting that exudate droplets may play a role in pathogenicity [29]. Whether exudates produced by *P. destructans* contain toxins or enzymes capable of damaging bats or degrading organic compounds in their surrounding environment remains to be investigated. However, studies have revealed that *P. destructans* can produce a variety of hydrolytic and proteolytic enzymes and utilize a diversity of carbon and nitrogen sources [12,30]. Some of these proteolytic enzymes are implicated in virulence traits in a variety of pathogenic microbes [31]. The enzymes required to alter the surrounding environment of *P. destructans* may be exuded, permitting growth on various carbon sources and allowing different nitrogen sources to be used. The exudates may also act as a reservoir for secondary metabolites that aid hyphal growth. Interestingly, exudate production in our *P. destructans* isolates was only observed on PDA and SD media; there was no exudate production from any of the isolates on MM agar. Since the major difference between MM agar and the other two media is the lack of free amino acids in MM agar, the results

suggest that the absence of a source of free amino acids has a direct effect upon exudate production in *P. destructans*.

Both pigment production and diffusion varied significantly among isolates and among environmental conditions. While the nature of these pigments and their potential ecological roles are unknown, some of the pigments beneath colonies may be related to melanin, which is typically characterized by its insolubility and grey to brown or black colors tightly associated with mycelial cell walls. Most fungal melanins are produced from 1,8-dihydroxynapthalene though the polyketide pathway or from 3-4 dihyroxyphenylalanine. In fungi, melanin provides enhanced survival and has been linked to virulence in several pathogenic species. For example, strains of *Cryptococcus neoformans* incapable of producing melanin were avirulent to mice [27, 28,29]. A mutant strain of *Aspergillus fumigatus* lacking conidial pigmentation also showed reduced virulence in mice [33]. Melanized strains of *C. neoformans* are less susceptible to nitrogen- and oxygen-derived oxidants, antibody-mediated phagocytosis and macrophages than non-melanized strains [33,34]. Furthermore, melanized *C. neoformans* were also more resistant to heat and cold, enzymatic degradation and UV light [35–37]. If the pigment in *P. destructans* plays a role similar to that demonstrated for melanin in other fungi and *in vitro* observations are consistent with hyphal melanisation *in vivo*, the significant variation among isolates in pigment production in *P. destructans* could contribute to differences in virulence or survival among the isolates.

Conclusion

This study revealed limited genotypic variation among isolates of *P. destructans* from eastern and central Canada. Our results support the currently accepted hypothesis for a single recent introduction for the North American population of *P. destructans*. However, we found large differences in four phenotype traits examined, and that environmental factors significantly influenced the expression of these traits in laboratory culture. The genetic basis for the observed phenotypic differences and their ecological roles in *P. destructans* remain to be investigated.

Acknowledgements

This research was supported by the Ontario Graduate Scholarship and NSERC of Canada. Funds in support of the field collection and initial culturing of *P. destructans* in New Brunswick were provided to DFM and KJV by the New Brunswick Environmental Trust Fund, New Brunswick Wildlife Trust Fund, New Brunswick Department of Natural Resources, National Speleological Society WNS Rapid Response Fund, and Parks Canada. The Canadian Cooperative Wildlife Health Centre, Atlantic Region provided funding for bat necropsies and fungal culture of Nova Scotia and Prince Edward Island samples.

Author Contributions

Conceived and designed the experiments: JK JX. Performed the experiments: JK. Analyzed the data: JK JX. Contributed reagents/materials/analysis tools: JK JX KJV DS SM DO DFM. Wrote the paper: JK JX KJV DO DFM SM.

References

1. Blehert DS, Hicks AC, Behr M, Meteyer CU, Berlowski-zier BM, et al. (2009) Bat White-Nose Syndrome : An Emerging Fungal Pathogen? Science 323: 227.

2. Froschauer A, Coleman J (2012) North American bat death toll exceeds 5.5 million from white-nose syndrome. US Fish Wildl Serv: 1–2.

3. Gargas A, Trest MT, Christensen M, Volk TJ, Blehert DS (2009) *Geomyces destructans* sp. nov associated with bat white-nose syndrome. Mycotaxon 108: 147–154.

4. Minnis AM, Lindner DL (2013) Phylogenetic evaluation of Geomyces and allies reveals no close relatives of *Pseudogymnoascus destructans*, comb. nov., in bat hibernacula of eastern North America. Fungal Biol 117: 638–649.

5. Meteyer CU, Buckles EL, Blehert DS, Hicks a. C, Green DE, et al. (2009) Histopathologic Criteria to Confirm White-nose Syndrome in Bats. J Vet Diagnostic Investig 21: 411–414.

6. Frick WF, Pollock JF, Hicks AC, Langwig KE, Reynolds DS, et al. (2010) An emerging disease causes regional population collapse of a common North American bat species. Science 329: 679–682.

7. Puechmaille SJ, Wibbelt G, Korn V, Fuller H, Forget F, et al. (2011) Pan-European distribution of white-nose syndrome fungus (*Geomyces destuctans*) not associated with mass mortality. PLoS One 6: e19167.

8. Wibbelt G, Puechmaille SJ, Ohlendorf B, Mühldorfer K, Bosch T, et al. (2013) Skin lesions in European hibernating bats associated with *Geomyces destuctans*, the etiologic agent of white-nose syndrome. PLoS One 8: e74105.

9. Warnecke L, Turner JM, Bollinger TK, Lorch JM, Misra V, et al. (2012) Inoculation of bats with European *Geomyces destuctans* supports the novel pathogen hypothesis for the origin of white-nose syndrome. Proc Natl Acad Sci: 1–5.

10. Rajkumar SS, Li X, Rudd RJ, Okoniewski JC, Xu J, et al. (2011) Clonal genotype of *Geomyces destuctans* among bats with White Nose Syndrome, New York, USA. Emerg Infect Dis 17: 1273–1276.

11. Ren P, Haman KH, Last LA, Rajkumar SS, Keel MK, et al. (2012) Clonal spread of *Geomyces destuctans* among bats, midwestern and southern United States. Emerg Infect Dis 18: 883–885.

12. Chaturvedi V, Springer DJ, Behr MJ, Ramani R, Li X, et al. (2010) Morphological and molecular characterizations of psychrophilic fungus *Geomyces destuctans* from New York bats with White Nose Syndrome (WNS). PLoS One 5: e10783.

13. G. Turner G, D. Reeder M, J. Coleman TH (2011) A Five-year Assessment of Mortality and Geographic Spread of White-nose Syndrome in North American Bats and a Look to the Future. Bat Res News 52: 14–27.

14. Canadian Cooperative Wildlife Health Centre (2013) Bat white-nose syndrome confirmed. Available: http://www.whitenosesyndrome.org/sites/default/files/files/bat_white_nose_confirmed_feb_27.final_provincial_press_release.pdf

15. Martínková N, Bačkor P, Bartonička T, Blažková P, Cervený J, et al. (2010) Increasing incidence of *Geomyces destuctans* fungus in bats from the Czech Republic and Slovakia. PLoS One 5: e13853.

16. Verant ML, Boyles JG, Waldrep W, Wibbelt G, Blehert DS (2012) Temperature-Dependent Growth of *Geomyces destuctans*, the Fungus That Causes Bat White-Nose Syndrome. PLoS One 7: e46280.

17. Swofford DL (n.d.) Phylogenetic Analysis Using Parsimony *.

18. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739.

19. Rachowicz LJ, Hero J-M, Alford RA., Taylor JW, Morgan JAT, et al. (2005) The Novel and Endemic Pathogen Hypotheses: Competing Explanations for the Origin of Emerging Infectious Diseases of Wildlife. Conserv Biol 19: 1441–1448.

20. Foster J, Drees K, Lorch J, Blehert D (2012) The origin and spread of *Geomyces destuctans* in North America. 5th Annual White-nose Syndrome symposium.

21. Singh, RS, Xu J, Kulathinal, RJ (2012) Rapidly Evolving Genes and Genetic Systems. Oxford University Press.

22. Pringle A, Taylor JW (2002) The fitness of filamentous fungi. Trends Microbiol 10: 474–481.

23. Frisvad JC, Smedsgaard J, Larsen TO, Samson RA (2004) Mycotoxins , drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*: 201–241.

24. Gareis M, Gareis E-M (2007) Guttation droplets of *Penicillium nordicum* and *Penicillium verrucosum* contain high concentrations of the mycotoxins ochratoxin A and B. Mycopathologia 163: 207–214.

25. Grovel O, Pouchus YF, Verbist J-F (2003) Accumulation of gliotoxin, a cytotoxic mycotoxin from *Aspergillus fumigatus*, in blue mussel (*Mytilus edulis*). Toxicon 42: 297–300.

26. Hutwimmer S, Wang H, Strasser H, Burgstaller W (2009) Formation of exudate droplets by *Metarhizium anisopliae* and the presence of destruxins. Mycologia 102: 1–10.

27. Jennings DH (1991) The role of droplets in helping to maintain a constant growth rate of aerial hyphae. Mycol Res 95: 883–884.

28. McPhee WJ, Colotelo N (1977) Fungal exudates. I. Characteristics of hyphal exudates in *Fusarium culmorum*. Botany 55: 358–365.

29. Colotelo N (1978) Fungal exudates. Can J Microbiol 24: 1173–1181.

30. Raudabaugh DB, Miller AN (2013) Nutritional Capability of and Substrate Suitability for *Pseudogymnoascus destructans*, the Causal Agent of Bat White-Nose Syndrome. PLoS One 8: e78300.

31. López-Otín C, Bond JS (2008) Proteases: multifunctional enzymes in life and disease. J Biol Chem 283: 30433–30437.

32. Kwon-Chung KJ, Rhodes JC (1986) Encapsulation and melanin formation as indicators of virulence in Cryptococcus as Indicators of Virulence in *Cryptococcus neoformans.* Infect and Immun 51: 218-223.

33. Jahn B, Koch A, Schmidt A, Wanner G, Gehringer H, et al. (1997) Isolation and characterization of a pigmentless-conidium mutant of *Aspergillus fumigatus* with altered conidial surface and reduced virulence . Infect and Immun 65: 5110-5117

34. Wang Y, Aisen P, Casadevall A (1995) *Cryptococcus neoformans* melanin and virulence: mechanism of action. Infect and Immun 63: 3131-3136.

35. Rosas L, Casadevall A (2001) Melanization decreases the susceptibility of *Cryptococcus neoformans* to enzymatic degradation. Mycopathol 151: 53–56.

36. Wang Y, Casadevall A, Wang Y (1994) Decreased susceptibility of melanized *Cryptococcus neoformans* to UV light. Appl Env Microbiol 60: 3864-3866.

37. Rosas, Ángel Luis and Casadevall A (1997) Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold. FEMS Microbiol Lett 153: 265–272.

Appendix

Table 1. Three-way ANOVA for isolate-temperature-media interactions on colony diameter for 16 North American *Pseudogymnoascus destructans* isolates 28 days after inoculation.

Source	df	SS	MS	F
Isolate (I)	15	1104.078	73.605	58.078***
Medium (M)	2	1584.962	792.481	625.300***
Temperature (T)	2	5143.753	2571.877	2029.316***
M x T	4	391.764	97.941	77.279***
M x I	30	99.983	3.333	2.630***
T x I	30	262.247	8.742	6.897***
M x T x I	58	174.292	2.905	2.292***
Error	278	365.000	1.267	

df, degrees of freedom; SS, sum of squares; MS, mean squares. *** $p < 0.001$

Table S1. Identification code with collection location and date for 112 North American *Pseudogymnoascus destructans* isolates sampled genetically and for phenotypic variation.

Isolate ID	Province/ State	Location	Collection Date	Isolate ID	Province/ State	Location	Collection Date
NB1	New Brunswick	Markhamville Mine	2/23/2012	NB57	New Brunswick	Glebe Mine	3/1/2012
NB2	New Brunswick	Harbell's Cave	2/28/2012	NB58	New Brunswick	White Cave	2/21/2012
NB3	New Brunswick	Dorchester Mine	4/12/2012	NB59	New Brunswick	White Cave	2/21/2012
NB4	New Brunswick	Glebe Mine	3/1/2012	NB60	New Brunswick	White Cave	2/21/2012
NB5	New Brunswick	Harbell's Cave	2/28/2012	NB61	New Brunswick	Glebe Mine	4/10/2012
NB6	New Brunswick	Markhamville Mine	3/1/2012	NB62	New Brunswick	Markhamville Mine	2/23/2012
NB7	New Brunswick	Berryton Cave	3/6/2012	NB63	New Brunswick	Harbell's Cave	2/28/2012
NB8	New Brunswick	White Cave	2/21/2012	NB64	New Brunswick	Markhamville Mine	3/1/2012
NB9	New Brunswick	Glebe Mine	4/10/2012	NB65	New Brunswick	White Cave	2/21/2012
NB10	New Brunswick	Berryton Cave	3/6/2012	NB66	New Brunswick	Markhamville Mine	3/1/2012
NB11	New Brunswick	Berryton Cave	3/6/2012	NB67	New Brunswick	Markhamville Mine	2/23/2012
NB12	New Brunswick	Berryton Cave	3/6/2012	NB68	New Brunswick	White Cave	2/21/2012
NB13	New Brunswick	Berryton Cave	3/6/2012	NB69	New Brunswick	Markhamville Mine	2/23/2012
NB14	New Brunswick	Berryton Cave	3/6/2012	NB70	New Brunswick	White Cave	2/21/2012
NB15	New Brunswick	White Cave	2/21/2012	NB71	New Brunswick	White Cave	2/21/2012
NB16	New Brunswick	Berryton Cave	4/6/2012	PE1	Prince Edward Island	Rocky Point, Queens County	3/3/2013
NB17	New Brunswick	Berryton Cave	4/6/2012	PE2	Prince Edward Island	Rocky Point, Queens County	3/3/2013
NB18	New Brunswick	Berryton Cave	3/6/2012	PE3	Prince Edward Island	Uigg, Queens County	3/8/2013

NB19	New Brunswick	Berryton Cave	3/6/2012
NB20	New Brunswick	Berryton Cave	3/6/2012
NB21	New Brunswick	Berryton Cave	3/6/2012
NB22	New Brunswick	Berryton Cave	2011
NB23	New Brunswick	Glebe Mine	4/10/2012
NB24	New Brunswick	Markhamville Mine	2/23/2012
NB25	New Brunswick	Howes Cave	2/24/2012
NB26	New Brunswick	Dorchester Mine	4/12/2012
NB27	New Brunswick	Dorchester Mine	4/12/2012
NB28	New Brunswick	Harbell's Cave	2/28/2012
NB29	New Brunswick	White Cave	2/21/2012
NB30	New Brunswick	Markhamville Mine	3/1/2012
NB31	New Brunswick	Harbell's Cave	2/28/2012
NB32	New Brunswick	Markhamville Mine	2/23/2012
NB33	New Brunswick	Glebe Mine	3/1/2012
NB34	New Brunswick	White Cave	2/21/2012
NB35	New Brunswick	Dorchester Mine	4/12/2012
NB36	New Brunswick	Harbell's Cave	2/28/2012
NB37	New Brunswick	Harbell's Cave	2/28/2012
NB38	New	Markhamville	3/1/2012
PE4	Prince Edward Island	Vernon Bridge, Queens County	3/14/2013
PE5	Prince Edward Island	Prim Point, Queens County	3/18/2013
PE6	Prince Edward Island	Caledonia, Queens County	4/10/2013
PE7A	Prince Edward Island	Murray River, Kings County	4/15/2013
PE7B	Prince Edward Island	Murray River, Kings County	4/15/2013
PE9	Prince Edward Island	Panmure Island, Kings County	4/23/2013
NS1	Nova Scotia	Falmouth, Hants County	4/4/2013
ON1	Ontario		
ON2	Ontario		
ON3	Ontario		
ON4	Ontario		
ON5	Ontario		
ON6	Ontario		
ON7	Ontario		
ON8	Ontario		
ON9	Ontario		
ON10	Ontario		
ON11	Ontario		
ON12	Ontario		
ON13	Ontario		

	Brunswick	Mine					
NB39	New Brunswick	Markhamville Mine	2/23/2012	ON14	Ontario	Rattlesnake Point, Milton	3/16/2012
NB40	New Brunswick	Markhamville Mine	3/1/2012	ON15	Ontario		
NB41	New Brunswick	Glebe Mine	4/10/2012	ON16	Ontario		
NB42	New Brunswick	White Cave	2/21/2012	US1	Vermont	Greely Mine	2013
NB43	New Brunswick	Markhamville Mine	3/1/2012	US2	Vermont	Greely Mine	2013
NB44	New Brunswick	Glebe Mine	4/10/2012	US3	New York	Williams Lake Mine	2013
NB45	New Brunswick	White Cave	2/21/2012	US4	New York	Williams Lake Mine	2013
NB46	New Brunswick	White Cave	2/21/2012	US5	West Virginia	Greenbrier	12/3/2010
NB47	New Brunswick	Glebe Mine	4/10/2012	US6	Pennsylvania	Woodward	3/25/2010
NB48	New Brunswick	Dorchester Mine	4/12/2012	US7	North Carolina	Avery	3/2/2011
NB49	New Brunswick	Dorchester Mine	4/12/2012	US8	North Carolina	Avery	3/2/2011
NB50	New Brunswick	Glebe Mine	4/10/2012	US9	West Virginia	Tucker	3/23/2011
NB51	New Brunswick	Harbell's Cave	2/28/2012	US10	North Carolina	Yancey	8/2/2011
NB52	New Brunswick	White Cave	2/21/2012	US11	Ohio	Lawrence	3/22/2011
NB53	New Brunswick	Berryton Cave	4/6/2012	US12	West Virginia	Fayette	3/23/2011
NB54	New Brunswick	White Cave	2/21/2012	US13	West Virginia	Randolph	11/3/2011
NB55	New Brunswick	Markhamville Mine	3/1/2012	US14	West Virginia	Pendleton	3/23/2011
NB56	New Brunswick	White Cave	2/21/2012	US15	New York	Williams Hotel Mine	3/28/2008

Table S2. Mean colony diameter (n=3; mm) 28 days after inoculation of 16 North American *Pseudogymnoascus destructans* isolates grown on minimal media (MM), potato dextrose agar (PDA) and synthetic-dextrose (SD) in 4°C, 14°C and 18°C.

	MM 4°C[2]	MM 14°C[1]	MM 18°C[2]	PDA 4°C[2]	PDA 14°C[2]	PDA 18°C[1]	SD 4°C[1]	SD 14°C[2]	SD 18°C[1]
NB1	9.3 ± 1.5 (abc)	14.3 ± 2.1 (abef)	8.0 ± 1.0 (ab)	14.3 ± 1.2 (abc)	21.7 ± 0.6 (d)	15.0 ± 1.7 (a)	14.8 ± 1.3 (e)	22.7 ± 0.6 (c)	9.7 ± 0.6 (ab)
NB2	8.0 ± 0.0 (abc)	15.7 ± 2.5 (ade)	7.2 ± 1.3 (ab)	13.3 ± 1.5 (abc)	19.3 ± 0.6 (ade)	14.0 ± 1.7 (ab)	13.2 ± 0.8 (adef)	19.3 ± 0.6 (ade)	9.5 ± 0.9 (ab)
NB3	8.0 ± 0.0 (abc)	15.7 ± 1.2 (ade)	7.5 ± 0.5 (a)	12.7 ± 1.2 (abc)	20.3 ± 0.6 (ad)	14.0 ± 1.0 (ab)	13.7 ± 1.5 (aef)	21.3 ± 1.2 (acd)	9.3 ± 1.2 (ab)
NB4	12.0 ± 1.0 (ac)	14.7 ± 0.6 (abe)	8.0 ± 0.0 (ac)	12.0 ± 0.0 (ac)	21.0 ± 1.0 (ade)	12.3 ± 1.5 (ab)	14.0 ± 1.0 (ae)	20.7 ± 0.6 (acd)	9.7 ± 0.6 (ab)
NB7	8.0 ± 0.0 (abc)	15.7 ± 1.2 (ade)	7.2 ± 0.8 (ab)	10.7 ± 0.6 (ab)	18.3 ± 0.6 (ae)	13.3 ± 2.1 (ab)	10.7 ± 1.2 (fg)	20.0 ± 1.0 (acde)	7.7 ± 1.5 (a)
NB8	9.8 ± 0.3 (c)	20.7 ± 2.1 (d)	7.7 ± 0.6 (a)	12.7 ± 0.6 (ac)	20.3 ± 0.6 (ad)	14.0 ± 1.7 (ab)	12.5 ± 0.9 (acdef)	21.7 ± 0.6 (cd)	9.5 ± 1.5 (ab)
NB25	8.3 ± 0.6 (abc)	15.3 ± 0.6 (ae)	6.7 ± 0.6 (ab)	12.7 ± 1.2 (abc)	19.7 ± 0.6 (ad)	13.3 ± 1.2 (ab)	13.3 ± 1.5 (aef)	21.3 ± 0.6 (acd)	9.7 ± 1.5 (ab)
NB26	10.0 ± 1.0 (acd)	15.7 ± 1.5 (ade)	7.7 ± 0.6 (a)	13.7 ± 0.6 (c)	20.3 ± 1.2 (acde)	14.3 ± 0.6 (a)	13.7 ± 0.6 (aef)	21.7 ± 0.6 (cd)	9.5 ± 1.8 (ab)
NB28	9.7 ± 1.5 (abc)	17.3 ± 2.1 (ad)	7.8 ± 0.3 (a)	14.0 ± 0.0 (ac)	18.7 ± 0.6 (ae)	13.8 ± 1.9 (ab)	14.3 ± 1.2 (ae)	22.0 ± 1.0 (acd)	11.3 ± 1.4 (b)
ON1	7.3 ± 0.6 (abc)	12.0 ± 1.0 (ef)	6.7 ± 1.5 (ab)	10.0 ± 0.0 (abc)	17.3 ± 2.1 (abde)	13.0 ± 1.0 (ab)	10.0 ± 0.0 (bdg)	18.7 ± 0.6 (ae)	9.0 ± 1.0 (ab)
ON3	9.0 ± 1.0 (abc)	16.7 ± 2.1 (ade)	6.0 ± 0.0 (ab)	12.0 ± 1.0 (abc)	19.7 ± 1.5 (abde)	13.7 ± 0.6 (ab)	11.0 ± 1.0 (abg)	18.0 ± 1.0 (acde)	8.3 ± 1.2 (ab)
ON16	8.2 ± 0.3 (ab)	15.2 ± 2.8 (abe)	5.7 ± 0.6 (ab)	11.8 ± 1.0 (abc)	16.3 ± 1.5 (abde)	13.3 ± 1.2 (ab)	12.0 ± 1.0 (abef)	18.7 ± 0.6 (ae)	9.3 ± 1.2 (ab)
NS1	5.2 ± 0.8 (b)	10.0 ± 0.0 (bcf)	4.2 ± 0.3 (b)	8.5 ± 0.5 (b)	14.0 ± 0.0 (bcf)	10.0 ± 1.0 (b)	9.3 ± 1.2 (bcg)	15.0 ± 1.0 (be)	6.7 ± 0.6 (a)
PE1	8.7 ± 1.5 (abc)	18.7 ± 1.5 (ad)	7.3 ± 1.2 (ab)	12.3 ± 0.6 (ac)	18.0 ± 1.0 (acde)	14.3 ± 1.2 (a)	13.3 ± 1.5 (aef)	19.7 ± 0.6 (ade)	10.0 ± 1.7 (ab)
US3	6.3 ± 0.6 (bd)	9.2 ± 0.3 (cf)	4.7 ± 0.6 (bc)	10.2 ± 0.7 (abc)	11.0 ± 0.0 (b)	11.0 ± 1.0	9.0 ±	12.3 ± 1.2 (b)	7.7 ± 0.6 (a)

						(ab)	1.0 (bg)		
US7	8.0 ± 1.0 (abc)	15.3 ± 2.5 (ae)	5.7 ± 0.6 (ab)	12.3 ± 0.6 (ac)	16.7 ± 0.6 (ef)	12.7 ± 0.6 (ab)	14.8 ± 0.3 (ae)	17.3 ± 2.9 (abc)	10.0 ± 1.0 (ab)
Average	8.5 ± 1.7	15.1 ± 3.1	6.7 ± 1.3	12.1 ± 1.7	18.3 ± 2.8	13.3 ± 1.7	12.5 ± 2.1	19.4 ± 2.8	9.2 ± 1.5
Significant Pairs	6	30	9	7	25	3	34	28	3

Different letters denote significant differences within each condition ($p < 0.05$). Data are presented as mean ± SD.
[1] Tukey's Honest Significant Difference
[2] Games-Howell

Table S3. Raw DNA fingerprint scores for 112 *Pseudogymnoascus destructans* isolates using $(GACA)_4$ primer. WAC5705 was *P. pannorum.*

ON1	1	1	0	0
ON2	1	1	0	0
ON3	1	1	1	1
ON4	1	1	0	0
ON5	1	1	0	0
ON6	1	1	0	0
ON7	1	1	0	0
ON8	1	1	0	0
ON9	1	1	0	0
ON10	1	1	0	0
ON11	1	1	0	0
ON12	1	1	0	0
ON13	1	1	0	0
ON14	1	1	0	0
ON15	1	1	0	0
ON16	1	1	0	0
NB1	1	1	0	0
NB2	1	1	0	0
NB3	1	1	0	0
NB4	1	1	0	0
NB5	1	1	0	0
NB6	1	1	0	0
NB7	1	1	0	0
NB8	1	1	0	0
NB9	1	1	0	0
NB10	1	1	0	0
NB11	1	1	0	0
NB12	1	1	0	0
NB13	1	1	0	0
NB14	1	1	0	0
NB15	1	1	0	0
NB16	1	1	0	0
NB17	1	1	0	0
NB18	1	1	0	0
NB19	1	1	0	0
NB20	1	1	0	0
NB21	1	1	0	0

NB22	1	1	0	0
NB23	1	1	0	0
NB24	1	1	0	0
NB25	1	1	0	0
NB26	1	1	0	0
NB27	1	1	0	0
NB28	1	1	0	0
NB29	1	1	0	0
NB30	1	1	0	0
NB31	1	1	0	0
NB32	1	1	0	0
NB33	1	1	0	0
NB34	1	1	0	0
NB35	1	1	0	0
NB36	1	1	0	0
NB37	1	1	0	0
NB38	1	1	0	0
NB39	1	1	0	0
NB40	1	1	0	0
NB41	1	1	0	0
NB42	1	1	0	0
NB43	1	1	0	0
NB44	1	1	0	0
NB45	1	1	0	0
NB46	1	1	0	0
NB47	1	1	0	0
NB48	1	1	0	0
NB49	1	1	0	0
NB50	1	1	0	0
NB51	1	1	0	0
NB52	1	1	0	0
NB53	1	1	0	0
NB54	1	1	0	0
NB55	1	1	0	0
NB56	1	1	0	0
NB57	1	1	0	0
NB58	1	1	0	0
NB59	1	1	0	0
NB60	1	1	0	0

NB61	1	1	0	0
NB62	1	1	0	0
NB63	1	1	0	0
NB64	1	1	0	0
NB65	1	1	0	0
NB66	1	1	0	0
NB67	1	1	0	0
NB68	1	1	0	0
NB69	1	1	0	0
NB70	1	1	0	0
NB71	1	1	0	0
NS1	1	1	0	0
PE1	1	1	0	0
PE2	1	1	0	0
PE3	1	1	0	0
PE4	1	1	0	0
PE5	1	1	0	0
PE6	1	1	0	0
PE7A	1	1	0	0
PE7B	1	1	0	0
PE9	1	1	0	0
US1	1	1	0	0
US2	1	1	0	0
US3	1	1	0	0
US4	1	1	0	0
US5	1	1	0	0
US6	1	1	0	0
US7	1	1	0	0
US8	1	1	0	0
US9	1	1	0	0
US10	1	1	0	0
US11	1	1	0	0
US12	1	1	0	0
US13	1	1	0	0
US14	1	1	0	0
US15	1	1	0	0
WAC	0	0	1	1

Table S4. Raw DNA fingerprint scores for 112 *Pseudogymnoascus destructans* isolates using M13 primer. WAC5705 was *P. pannorum.*

ON1	1	1	0	0	0	0	1	0	1
ON2	1	1	0	0	0	0	1	0	1
ON3	1	1	0	0	1	0	1	0	1
ON4	1	1	0	0	0	0	1	0	1
ON5	1	1	0	0	0	0	1	0	1
ON6	1	1	0	0	0	0	1	0	1
ON7	1	1	0	0	0	0	1	0	1
ON8	1	1	0	0	0	0	1	0	1
ON9	1	1	0	0	0	0	1	0	1
ON10	1	1	0	0	0	0	1	0	1
ON11	1	1	0	0	0	0	1	0	1
ON12	1	1	0	0	0	0	1	0	1
ON13	1	1	0	0	0	0	1	0	1
ON14	1	1	0	0	0	0	1	0	1
ON15	1	1	0	0	0	0	1	0	1
ON16	1	1	0	0	0	0	1	0	1
NB1	1	1	0	0	0	0	1	0	1
NB2	1	1	0	0	0	0	1	0	1
NB3	1	1	0	0	0	0	1	0	1
NB4	1	1	0	0	0	0	1	0	1
NB5	1	1	0	0	0	0	1	0	1
NB6	1	1	0	0	0	0	1	0	1
NB7	1	1	0	0	0	0	1	0	1
NB8	1	1	0	0	0	0	1	0	1
NB9	1	1	0	0	0	0	1	0	1
NB10	1	1	0	0	0	0	1	0	1
NB11	1	1	0	0	0	0	1	0	1
NB12	1	1	0	0	0	0	1	0	1
NB13	1	1	0	0	0	0	1	0	1
NB14	1	1	0	0	0	0	1	0	1
NB15	1	1	0	0	0	0	1	0	1
NB16	1	1	0	0	0	0	1	0	1
NB17	1	1	0	0	0	0	1	0	1
NB18	1	1	0	0	0	0	1	0	1
NB19	1	1	0	0	0	0	1	0	1
NB20	1	1	0	0	0	0	1	0	1
NB21	1	1	0	0	0	0	1	0	1

NB22	1	1	0	0	0	0	1	0	1
NB23	1	1	0	0	0	0	1	0	1
NB24	1	1	0	0	0	0	1	0	1
NB25	1	1	0	0	0	0	1	0	1
NB26	1	1	0	1	0	0	1	0	1
NB27	1	1	0	0	0	0	1	0	1
NB28	1	1	1	0	0	1	1	1	1
NB29	1	1	0	0	0	0	1	0	1
NB30	1	1	0	0	0	0	1	0	1
NB31	1	1	0	0	0	0	1	0	1
NB32	1	1	0	0	0	0	1	0	1
NB33	1	1	0	0	0	0	1	0	1
NB34	1	1	0	0	0	0	1	0	1
NB35	1	1	0	0	0	0	1	0	1
NB36	1	1	0	0	0	0	1	0	1
NB37	1	1	0	0	0	0	1	0	1
NB38	1	1	0	0	0	0	1	0	1
NB39	1	1	0	0	0	0	1	0	1
NB40	1	1	0	0	0	0	1	0	1
NB41	1	1	0	0	0	0	1	0	1
NB42	1	1	0	0	0	0	1	0	1
NB43	1	1	0	0	0	0	1	0	1
NB44	1	1	0	0	0	0	1	0	1
NB45	1	1	0	0	0	0	1	0	1
NB46	1	1	0	0	0	0	1	0	1
NB47	1	1	0	0	0	0	1	0	1
NB48	1	1	0	0	0	0	1	0	1
NB49	1	1	0	0	0	0	1	0	1
NB50	1	1	0	0	0	0	1	0	1
NB51	1	1	0	0	0	0	1	0	1
NB52	1	1	0	0	0	0	1	0	1
NB53	1	1	0	0	0	0	1	0	1
NB54	1	1	0	0	0	0	1	0	1
NB55	1	1	0	0	0	0	1	0	1
NB56	1	1	0	0	0	0	1	0	1
NB57	1	1	0	0	0	0	1	0	1
NB58	1	1	0	0	0	0	1	0	1
NB59	1	1	0	0	0	0	1	0	1
NB60	1	1	0	0	0	0	1	0	1

NB61	1	1	0	0	0	0	1	0	1
NB62	1	1	0	0	0	0	1	0	1
NB63	1	1	0	0	0	0	1	0	1
NB64	1	1	0	0	0	0	1	0	1
NB65	1	1	0	0	0	0	1	0	1
NB66	1	1	0	0	0	0	1	0	1
NB67	1	1	0	0	0	0	1	0	1
NB68	1	1	0	0	0	0	1	0	1
NB69	1	1	0	0	0	0	1	0	1
NB70	1	1	0	0	0	0	1	0	1
NB71	1	1	0	0	0	0	1	0	1
NS1	1	1	0	0	0	0	1	0	1
PE1	1	1	0	0	0	0	1	0	1
PE2	1	1	0	0	0	0	1	0	1
PE3	1	1	0	0	0	0	1	0	1
PE4	1	1	0	0	0	0	1	0	1
PE5	1	1	0	0	0	0	1	0	1
PE6	1	1	0	0	0	0	1	0	1
PE7A	1	1	0	0	0	0	1	0	1
PE7B	1	1	0	0	0	0	1	0	1
PE9	1	1	0	0	0	0	1	0	1
US1	1	1	0	0	0	0	1	0	1
US2	1	1	0	0	0	0	1	0	1
US3	1	1	0	0	0	0	1	0	1
US4	1	1	0	0	0	0	1	0	1
US5	1	1	0	0	0	0	1	0	1
US6	1	1	0	0	0	0	1	0	1
US7	1	1	0	0	0	0	1	0	1
US8	1	1	0	0	0	0	1	0	1
US9	1	1	0	0	0	0	1	0	1
US10	1	1	0	0	0	0	1	0	1
US11	1	1	0	0	0	0	1	0	1
US12	1	1	0	0	0	0	1	0	1
US13	1	1	0	0	0	0	1	0	1
US14	1	1	0	0	0	0	1	0	1
US15	1	1	0	0	0	0	1	0	1
WAC	0	0	0	0	1	0	0	0	0

Table S5. P-values from Tukey's HSD and Games-Howell post hoc tests comparing colony diameters of 16 *Pseudogymnoascus destructans* isolates in nine conditions. P-values less than 0.05 are highlighted in grey.

Isolate		MM 4°C	MM 14°C	MM 18°C	PDA 4°C	PDA 14°C	PDA 18°C	SD 4°C	SD 14°C	SD 18°C
NB1	NB2	.902	1.000	.998	.998	.106	1.000	.854	.032	1.000
	NB25	.987	1.000	.763	.852	.169	.969	.927	.458	1.000
	NB26	1.000	1.000	1.000	.997	.835	1.000	.991	.719	1.000
	NB28	1.000	.721	1.000	1.000	.046	.999	1.000	.994	.935
	NB3	.902	1.000	.999	.852	.458	1.000	.991	.835	1.000
	NB4	.570	1.000	1.000	.398	.994	.522	1.000	.169	1.000
	NB7	.902	1.000	.986	.157	.032	.969	.003	.238	.793
	NB8	1.000	.006	1.000	.679	.458	1.000	.390	.719	1.000
	NS1	.224	.183	.118	.052	.012	.005	.000	.012	.207
	ON1	.721	.939	.971	.141	.379	.882	.000	.016	1.000
	ON16	.951	1.000	.320	.473	.134	.969	.140	.016	1.000
	ON3	1.000	.939	.404	.520	.721	.996	.009	.054	.990
	PE1	1.000	.183	1.000	.532	.106	1.000	.927	.046	1.000
	US3	.420	.050	.137	.110	.006	.053	.000	.010	.793
	US7	.971	1.000	.320	.532	.007	.722	1.000	.454	1.000
NB2	NB1	.902	1.000	.998	.998	.106	1.000	.854	.032	1.000
	NB25	.988	1.000	1.000	1.000	1.000	1.000	1.000	.169	1.000
	NB26	.404	1.000	1.000	1.000	.953	1.000	1.000	.106	1.000
	NB28	.795	.997	.995	.998	.949	1.000	.991	.238	.875
	NB3	-	1.000	1.000	1.000	.719	1.000	1.000	.532	1.000
	NB4	.126	1.000	.973	.902	.584	.969	1.000	.458	1.000
	NB7	-	1.000	1.000	.505	.719	1.000	.287	.994	.875
	NB8	.052	.066	1.000	1.000	.719	1.000	1.000	.106	1.000
	NS1	.144	.021	.302	.171	.025	.053	.009	.067	.281
	ON1	.761	.414	1.000	.355	.882	1.000	.061	.949	1.000
	ON16	.988	1.000	.804	.947	.420	1.000	.991	.949	1.000
	ON3	.842	1.000	.877	.971	1.000	1.000	.509	.763	.997
	PE1	.998	.721	1.000	.987	.763	1.000	1.000	1.000	1.000
	US3	.225	.004	.419	.393	.010	.337	.003	.029	.875
	US7	1.000	1.000	.804	.987	.069	.996	.854	.971	1.000
NB25	NB1	.987	1.000	.763	.852	.169	.969	.927	.458	1.000
	NB2	.988	1.000	1.000	1.000	1.000	1.000	1.000	.169	1.000
	NB26	.584	1.000	.719	.953	.997	1.000	1.000	1.000	1.000
	NB28	.934	.983	.412	.761	.719	1.000	.998	.994	.935
	NB3	.988	1.000	.807	1.000	.949	1.000	1.000	1.000	1.000
	NB4	.106	1.000	.325	.988	.763	1.000	1.000	.949	1.000
	NB7	.988	1.000	.998	.532	.458	1.000	.204	.763	.793
	NB8	.249	.038	.719	1.000	.949	1.000	1.000	1.000	1.000

	NS1	.075	.038	.072	.120	.022	.196	.006	.022	.207
	ON1	.719	.567	1.000	.325	.802	1.000	.039	.069	1.000
	ON16	1.000	1.000	.719	.997	.350	1.000	.971	.069	1.000
	ON3	.994	1.000	.761	1.000	1.000	1.000	.390	.137	.990
	PE1	1.000	.567	.997	1.000	.584	1.000	1.000	.277	1.000
	US3	.169	.008	.169	.390	.010	.722	.002	.014	.793
	US7	1.000	1.000	.719	1.000	.046	1.000	.927	.653	1.000
NB26	NB1	1.000	1.000	1.000	.997	.835	1.000	.991	.719	1.000
	NB2	.404	1.000	1.000	1.000	.953	1.000	1.000	.106	1.000
	NB25	.584	1.000	.719	.953	.997	1.000	1.000	1.000	1.000
	NB28	1.000	.997	1.000	.988	.679	1.000	1.000	1.000	.875
	NB3	.404	1.000	1.000	.953	1.000	1.000	1.000	1.000	1.000
	NB4	.590	1.000	.988	.225	1.000	.882	1.000	.719	1.000
	NB7	.404	1.000	.998	.046	.532	1.000	.094	.584	.875
	NB8	1.000	.066	1.000	.719	1.000	1.000	.991	1.000	1.000
	NS1	.046	.021	.029	.005	.069	.025	.002	.019	.281
	ON1	.238	.414	.987	.052	.697	.996	.015	.046	1.000
	ON16	.462	1.000	.169	.532	.273	1.000	.854	.046	1.000
	ON3	.979	1.000	.225	.584	1.000	1.000	.204	.106	.997
	PE1	.971	.721	1.000	.458	.520	1.000	1.000	.169	1.000
	US3	.106	.004	.046	.054	.033	.196	.001	.013	.875
	US7	.590	1.000	.169	.458	.157	.969	.991	.596	1.000
NB28	NB1	1.000	.721	1.000	1.000	.046	.999	1.000	.994	.935
	NB2	.795	.997	.995	.998	.949	1.000	.991	.238	.875
	NB25	.934	.983	.412	.761	.719	1.000	.998	.994	.935
	NB26	1.000	.997	1.000	.988	.679	1.000	1.000	1.000	.875
	NB3	.795	.997	.994	.761	.277	1.000	1.000	1.000	.793
	NB4	.684	.852	.988	-	.320	.988	1.000	.763	.935
	NB7	.795	.997	.934	.063	1.000	1.000	.015	.590	.049
	NB8	1.000	.567	1.000	.325	.277	1.000	.754	1.000	.875
	NS1	.188	.001	.002	.018	.033	.075	.000	.016	.004
	ON1	.606	.038	.951	-	.984	1.000	.002	.137	.583
	ON16	.861	.966	.104	.381	.606	1.000	.390	.137	.793
	ON3	1.000	1.000	.052	.404	.987	1.000	.039	.110	.207
	PE1	.999	1.000	.999	.225	.994	1.000	.998	.320	.990
	US3	.350	.000	.038	.082	.012	.425	.000	.007	.049
	US7	.904	.983	.104	.225	.169	.999	1.000	.558	.990
NB3	NB1	.902	1.000	.999	.852	.458	1.000	.991	.835	1.000
	NB2	-	1.000	1.000	1.000	.719	1.000	1.000	.532	1.000

	NB25	.988	1.000	.807	1.000	.949	1.000	1.000	1.000	1.000
	NB26	.404	1.000	1.000	.953	1.000	1.000	1.000	1.000	1.000
	NB28	.795	.997	.994	.761	.277	1.000	1.000	1.000	.793
	NB4	.126	1.000	.842	.988	.994	.969	1.000	.997	1.000
	NB7	-	1.000	1.000	.532	.169	1.000	.094	.927	.935
	NB8	.052	.066	1.000	1.000	1.000	1.000	.991	1.000	1.000
	NS1	.144	.021	.019	.120	.018	.053	.002	.032	.370
	ON1	.761	.414	.996	.325	.634	1.000	.015	.319	1.000
	ON16	.988	1.000	.182	.997	.248	1.000	.854	.319	1.000
	ON3	.842	1.000	.210	1.000	1.000	1.000	.204	.236	.999
	PE1	.998	.721	1.000	1.000	.320	1.000	1.000	.679	1.000
	US3	.225	.004	.047	.390	.008	.337	.001	.011	.935
	US7	1.000	1.000	.182	1.000	.023	.996	.991	.685	1.000
NB4	NB1	.570	1.000	1.000	.398	.994	.522	1.000	.169	1.000
	NB2	.126	1.000	.973	.902	.584	.969	1.000	.458	1.000
	NB25	.106	1.000	.325	.988	.763	1.000	1.000	.949	1.000
	NB26	.590	1.000	.988	.225	1.000	.882	1.000	.719	1.000
	NB28	.684	.852	.988	-	.320	.988	1.000	.763	.935
	NB3	.126	1.000	.842	.988	.994	.969	1.000	.997	1.000
	NB7	.126	1.000	.795	.325	.238	1.000	.039	.994	.793
	NB8	.354	.011	.988	.761	.994	.969	.927	.719	1.000
	NS1	.014	.113	.012	.043	.043	.722	.001	.031	.207
	ON1	.054	.852	.902	-	.515	1.000	.006	.169	1.000
	ON16	.118	1.000	.123	1.000	.173	1.000	.634	.169	1.000
	ON3	.251	.983	-	1.000	.971	.996	.094	.238	.990
	PE1	.382	.283	.988	.988	.251	.882	1.000	.719	1.000
	US3	.031	.028	.063	.306	.021	.996	.000	.018	.793
	US7	.110	1.000	.123	.988	.067	1.000	1.000	.773	1.000
NB7	NB1	.902	1.000	.986	.157	.032	.969	.003	.238	.793
	NB2	-	1.000	1.000	.505	.719	1.000	.287	.994	.875
	NB25	.988	1.000	.998	.532	.458	1.000	.204	.763	.793
	NB26	.404	1.000	.998	.046	.532	1.000	.094	.584	.875
	NB28	.795	.997	.934	.063	1.000	1.000	.015	.590	.049
	NB3	-	1.000	1.000	.532	.169	1.000	.094	.927	.935
	NB4	.126	1.000	.795	.325	.238	1.000	.039	.994	.793
	NB8	.052	.066	.998	.169	.169	1.000	.754	.584	.875
	NS1	.144	.021	.103	.111	.038	.196	.971	.053	.999
	ON1	.761	.414	1.000	.761	.998	1.000	1.000	.763	.990
	ON16	.988	1.000	.502	.866	.721	1.000	.971	.763	.935

	ON3	.842	1.000	.577	.763	.934	1.000	1.000	.590	1.000
	PE1	.998	.721	1.000	.277	1.000	1.000	.204	1.000	.583
	US3	.225	.004	.151	.998	.013	.722	.854	.016	1.000
	US7	1.000	1.000	.502	.277	.277	1.000	.003	.909	.583
NB8	NB1	1.000	.006	1.000	.679	.458	1.000	.390	.719	1.000
	NB2	.052	.066	1.000	1.000	.719	1.000	1.000	.106	1.000
	NB25	.249	.038	.719	1.000	.949	1.000	1.000	1.000	1.000
	NB26	1.000	.066	1.000	.719	1.000	1.000	.991	1.000	1.000
	NB28	1.000	.567	1.000	.325	.277	1.000	.754	1.000	.875
	NB3	.052	.066	1.000	1.000	1.000	1.000	.991	1.000	1.000
	NB4	.354	.011	.988	.761	.994	.969	.927	.719	1.000
	NB7	.052	.066	.998	.169	.169	1.000	.754	.584	.875
	NS1	.036	.000	.029	.012	.018	.053	.061	.019	.281
	ON1	.072	.000	.987	.096	.634	1.000	.287	.046	1.000
	ON16	.032	.028	.169	.975	.248	1.000	1.000	.046	1.000
	ON3	.936	.283	.225	.994	1.000	1.000	.927	.106	.997
	PE1	.951	.983	1.000	1.000	.320	1.000	1.000	.169	1.000
	US3	.029	.000	.046	.151	.008	.337	.024	.013	.875
	US7	.462	.038	.169	1.000	.023	.996	.390	.596	1.000
NS1	NB1	.224	.183	.118	.052	.012	.005	.000	.012	.207
	NB2	.144	.021	.302	.171	.025	.053	.009	.067	.281
	NB25	.075	.038	.072	.120	.022	.196	.006	.022	.207
	NB26	.046	.021	.029	.005	.069	.025	.002	.019	.281
	NB28	.188	.001	.002	.018	.033	.075	.000	.016	.004
	NB3	.144	.021	.019	.120	.018	.053	.002	.032	.370
	NB4	.014	.113	.012	.043	.043	.722	.001	.031	.207
	NB7	.144	.021	.103	.111	.038	.196	.971	.053	.999
	NB8	.036	.000	.029	.012	.018	.053	.061	.019	.281
	ON1	.223	.983	.535	.210	.546	.337	1.000	.106	.583
	ON16	.103	.050	.249	.155	.577	.196	.204	.106	.370
	ON3	.096	.003	.052	.124	.144	.105	.854	.251	.935
	PE1	.324	.000	.234	.016	.126	.025	.006	.054	.105
	US3	.723	1.000	.953	.382	-	1.000	1.000	.402	.999
	US7	.225	.038	.249	.016	.096	.522	.000	.951	.105
ON1	NB1	.721	.939	.971	.141	.379	.882	.000	.016	1.000
	NB2	.761	.414	1.000	.355	.882	1.000	.061	.949	1.000
	NB25	.719	.567	1.000	.325	.802	1.000	.039	.069	1.000
	NB26	.238	.414	.987	.052	.697	.996	.015	.046	1.000
	NB28	.606	.038	.951	-	.984	1.000	.002	.137	.583

	NB3	.761	.414	.996	.325	.634	1.000	.015	.319	1.000
	NB4	.054	.852	.902	-	.515	1.000	.006	.169	1.000
	NB7	.761	.414	1.000	.761	.998	1.000	1.000	.763	.990
	NB8	.072	.000	.987	.096	.634	1.000	.287	.046	1.000
	NS1	.223	.983	.535	.210	.546	.337	1.000	.106	.583
	ON16	.679	.645	.987	.483	1.000	1.000	.634	1.000	1.000
	ON3	.584	.113	.998	.404	.911	1.000	.998	.994	1.000
	PE1	.934	.003	1.000	.123	1.000	.996	.039	.719	.999
	US3	.719	.791	.721	1.000	.205	.882	.998	.038	.990
	US7	.994	.567	.987	.123	1.000	1.000	.000	.998	.999
ON16	NB1	.951	1.000	.320	.473	.134	.969	.140	.016	1.000
	NB2	.988	1.000	.804	.947	.420	1.000	.991	.949	1.000
	NB25	1.000	1.000	.719	.997	.350	1.000	.971	.069	1.000
	NB26	.462	1.000	.169	.532	.273	1.000	.854	.046	1.000
	NB28	.861	.966	.104	.381	.606	1.000	.390	.137	.793
	NB3	.988	1.000	.182	.997	.248	1.000	.854	.319	1.000
	NB4	.118	1.000	.123	1.000	.173	1.000	.634	.169	1.000
	NB7	.988	1.000	.502	.866	.721	1.000	.971	.763	.935
	NB8	.032	.028	.169	.975	.248	1.000	1.000	.046	1.000
	NS1	.103	.050	.249	.155	.577	.196	.204	.106	.370
	ON1	.679	.645	.987	.483	1.000	1.000	.634	1.000	1.000
	ON3	.936	.999	.988	1.000	.510	1.000	.998	.994	.999
	PE1	1.000	.489	.679	1.000	.904	1.000	.971	.719	1.000
	US3	.157	.011	.719	.675	.161	.722	.094	.038	.935
	US7	1.000	1.000	1.000	1.000	1.000	1.000	.140	.998	1.000
ON3	NB1	1.000	.939	.404	.520	.721	.996	.009	.054	.990
	NB2	.842	1.000	.877	.971	1.000	1.000	.509	.763	.997
	NB25	.994	1.000	.761	1.000	1.000	1.000	.390	.137	.990
	NB26	.979	1.000	.225	.584	1.000	1.000	.204	.106	.997
	NB28	1.000	1.000	.052	.404	.987	1.000	.039	.110	.207
	NB3	.842	1.000	.210	1.000	1.000	1.000	.204	.236	.999
	NB4	.251	.983	-	1.000	.971	.996	.094	.238	.990
	NB7	.842	1.000	.577	.763	.934	1.000	1.000	.590	1.000
	NB8	.936	.283	.225	.994	1.000	1.000	.927	.106	.997
	NS1	.096	.003	.052	.124	.144	.105	.854	.251	.935
	ON1	.584	.113	.998	.404	.911	1.000	.998	.994	1.000
	ON16	.936	.999	.988	1.000	.510	1.000	.998	.994	.999
	PE1	1.000	.983	.761	1.000	.904	1.000	.390	.584	.935
	US3	.238	.001	.325	.567	.065	.522	.634	.047	1.000

	US7	.979	1.000	.988	1.000	.420	1.000	.009	1.000	.935
PE1	NB1	1.000	.183	1.000	.532	.106	1.000	.927	.046	1.000
	NB2	.998	.721	1.000	.987	.763	1.000	1.000	1.000	1.000
	NB25	1.000	.567	.997	1.000	.584	1.000	1.000	.277	1.000
	NB26	.971	.721	1.000	.458	.520	1.000	1.000	.169	1.000
	NB28	.999	1.000	.999	.225	.994	1.000	.998	.320	.990
	NB3	.998	.721	1.000	1.000	.320	1.000	1.000	.679	1.000
	NB4	.382	.283	.988	.988	.251	.882	1.000	.719	1.000
	NB7	.998	.721	1.000	.277	1.000	1.000	.204	1.000	.583
	NB8	.951	.983	1.000	1.000	.320	1.000	1.000	.169	1.000
	NS1	.324	.000	.234	.016	.126	.025	.006	.054	.105
	ON1	.934	.003	1.000	.123	1.000	.996	.039	.719	.999
	ON16	1.000	.489	.679	1.000	.904	1.000	.971	.719	1.000
	ON3	1.000	.983	.761	1.000	.904	1.000	.390	.584	.935
	US3	.606	.000	.319	.223	.043	.196	.002	.025	.583
	US7	1.000	.567	.679	1.000	.763	.969	.927	.937	1.000
US3	NB1	.420	.050	.137	.110	.006	.053	.000	.010	.793
	NB2	.225	.004	.419	.393	.010	.337	.003	.029	.875
	NB25	.169	.008	.169	.390	.010	.722	.002	.014	.793
	NB26	.106	.004	.046	.054	.033	.196	.001	.013	.875
	NB28	.350	.000	.038	.082	.012	.425	.000	.007	.049
	NB3	.225	.004	.047	.390	.008	.337	.001	.011	.935
	NB4	.031	.028	.063	.306	.021	.996	.000	.018	.793
	NB7	.225	.004	.151	.998	.013	.722	.854	.016	1.000
	NB8	.029	.000	.046	.151	.008	.337	.024	.013	.875
	NS1	.723	1.000	.953	.382	-	1.000	1.000	.402	.999
	ON1	.719	.791	.721	1.000	.205	.882	.998	.038	.990
	ON16	.157	.011	.719	.675	.161	.722	.094	.038	.935
	ON3	.238	.001	.325	.567	.065	.522	.634	.047	1.000
	PE1	.606	.000	.319	.223	.043	.196	.002	.025	.583
	US7	.584	.008	.719	.223	.022	.969	.000	.514	.583
US7	NB1	.971	1.000	.320	.532	.007	.722	1.000	.454	1.000
	NB2	1.000	1.000	.804	.987	.069	.996	.854	.971	1.000
	NB25	1.000	1.000	.719	1.000	.046	1.000	.927	.653	1.000
	NB26	.590	1.000	.169	.458	.157	.969	.991	.596	1.000
	NB28	.904	.983	.104	.225	.169	.999	1.000	.558	.990
	NB3	1.000	1.000	.182	1.000	.023	.996	.991	.685	1.000
	NB4	.110	1.000	.123	.988	.067	1.000	1.000	.773	1.000
	NB7	1.000	1.000	.502	.277	.277	1.000	.003	.909	.583

NB8	.462	.038	.169	1.000	.023	.996	.390	.596	1.000
NS1	.225	.038	.249	.016	.096	.522	.000	.951	.105
ON1	.994	.567	.987	.123	1.000	1.000	.000	.998	.999
ON16	1.000	1.000	1.000	1.000	1.000	1.000	.140	.998	1.000
ON3	.979	1.000	.988	1.000	.420	1.000	.009	1.000	.935
PE1	1.000	.567	.679	1.000	.763	.969	.927	.937	1.000
US3	.584	.008	.719	.223	.022	.969	.000	.514	.583

Figure 1. PCR fingerprint banding patterns of four *Pseudogymnoascus destructans* isolates using M13 primer. Three distinct fingerprints are represented by NB26, NB28 and ON3. NB53 represents the dominant fingerprint of 109 isolates. C is the H20 control.

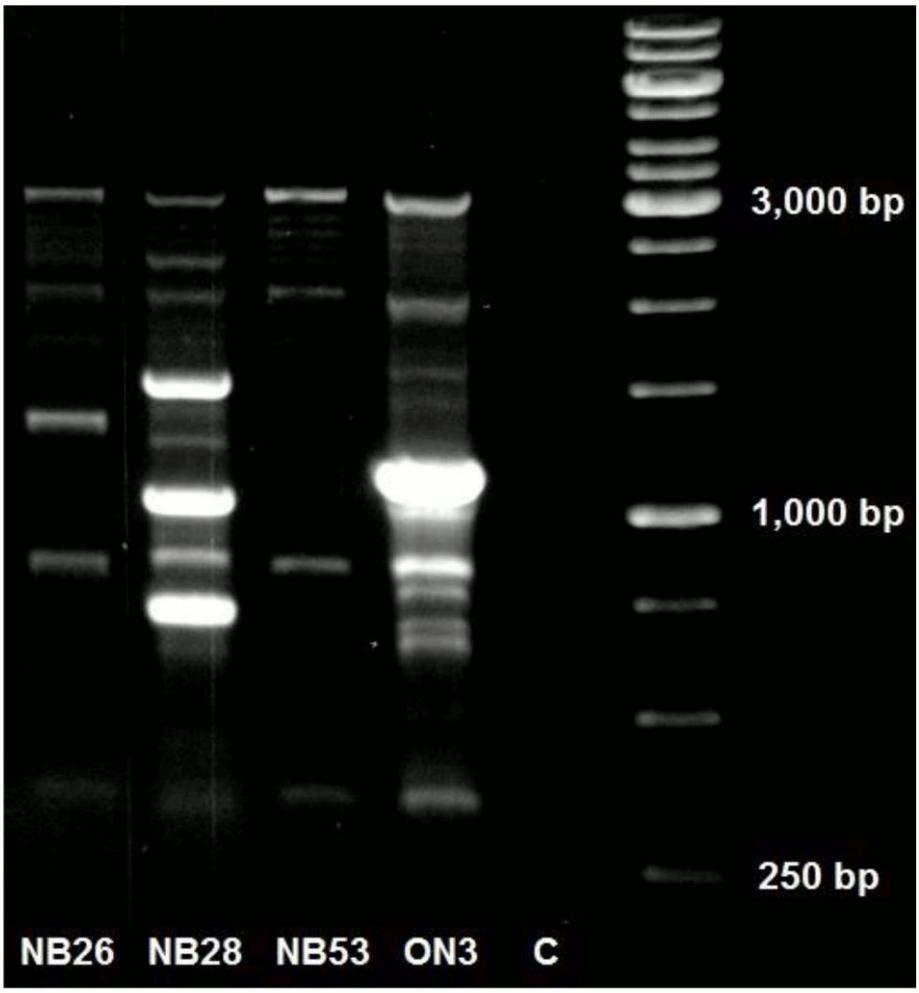

3,000 bp
1,000 bp
250 bp
NB26
NB28
NB53
ON3
C

Figure 2. Morphological characteristics of *Pseudogymnoascus destructans* isolates following 28 days of incubation. (A) Typical isolate colony morphology on PDA/14°C represented by NB7. A score of 0 for exudate production was given. (B) Colony morphology of NB28 on SD/4°C with a score of 1 for exudate production. (C) Colony morphology of ON16 on SD 14°C. A score of 2 was given for exudate production and pigment secretion into medium was recorded. (D) Colony morphology of PE1 on PDA/18°C with a score of 3 for exudate production. (E) White reverse colony color of NB8 on MM/4°C. (F) Black reverse colony color for ON16 on PDA/14°C. Scale bar indicates 5mm. SD = synthetic-dextrose, PDA = potato dextrose agar, MM= minimal media.

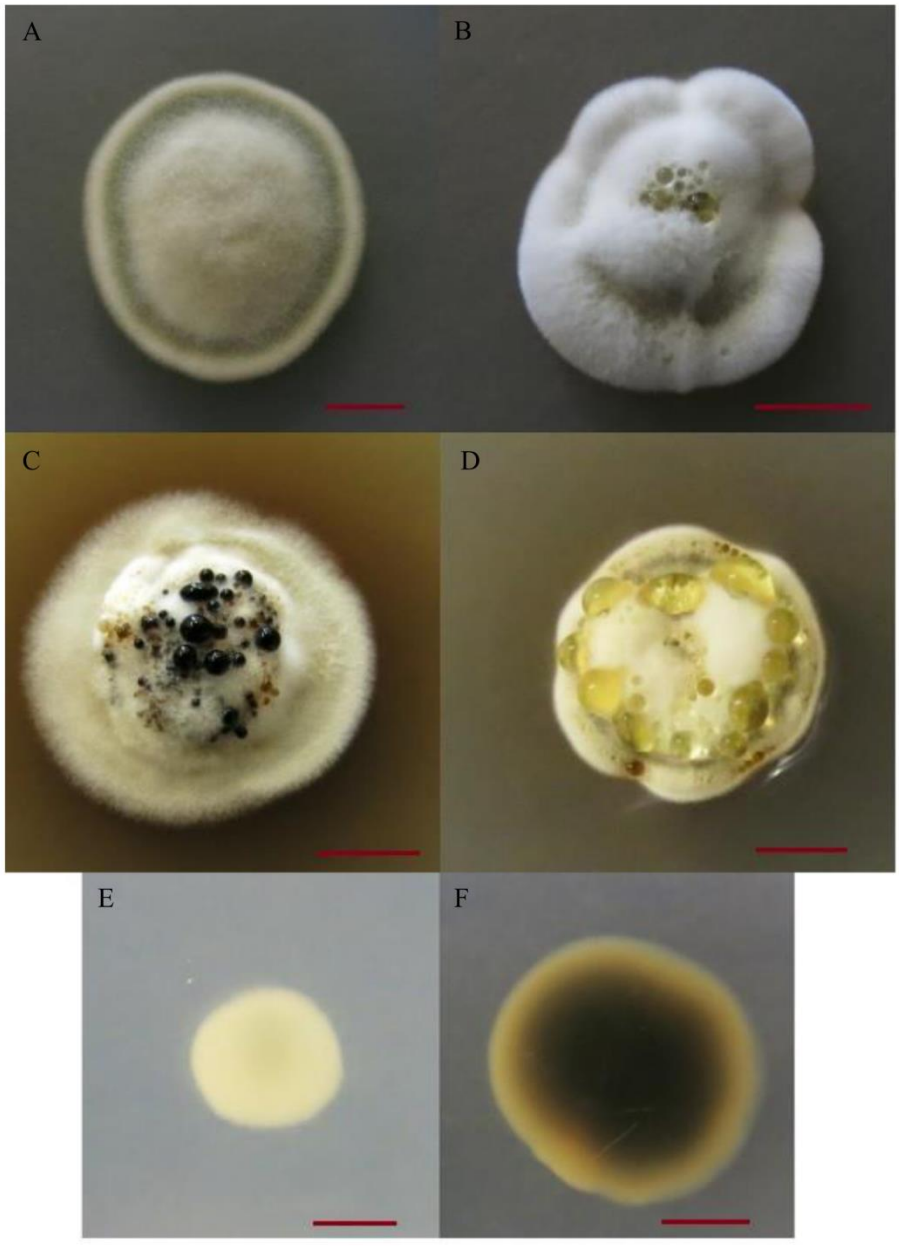

A
B
C
D
E
F

Figure 3. Variations in exudate production within and among 16 North American *Pseudogymnoascus destructans* isolates in nine conditions. 0 = no exudate production; 1 = small but visible exudate production; 2 = intermediate exudate production; 3 = profuse exudate production (for representative figures, please see Figure 2B-D). SD = synthetic-dextrose, PDA = potato dextrose agar, MM= minimal media. Three replicates are shown for each medium-temperature condition for each isolate.

Isolate	MM 4°C	MM 14°C	MM 18°C	PDA 4°C	PDA 14°C	PDA 18°C	SD 4°C	SD 14°C	SD 18°C
NB1	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	3/0/0	0/1/0	0/0/0	0/0/0
NB2	0/0/0	0/0/0	0/0/0	0/0/0	1/1/2	2/1/1	2/1/0	3/2/3	0/0/0
NB3	0/0/0	0/0/0	0/0/0	1/1/0	1/1/2	3/0/0	2/1/0	2/3/3	0/1/0
NB4	0/0/0	0/0/0	0/0/0	1/2/1	0/0/1	3/2/2	3/3/1	1/2/3	0/0/0
NB7	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/1	0/0/0	1/1/0	0/0/0
NB8	0/0/0	0/0/0	0/0/0	0/2/2	0/0/0	0/3/3	3/3/1	3/2/2	0/0/0
NB25	0/0/0	0/0/0	0/0/0	0/0/0	1/1/2	3/3/1	1/2/0	3/3/3	1/1/0
NB26	0/0/0	0/0/0	0/0/0	0/0/0	1/1/1	1/2/2	2/1/1	3/3/3	1/1/0
NB28	0/0/0	0/0/0	0/0/0	1/1/1	1/1/1	1/2/2	1/1/0	3/3/3	2/1/2
ON1	0/0/0	0/0/0	0/0/0	0/0/0	1/1/1	0/1/0	1/1/0	3/1/3	0/0/0
ON3	0/0/0	0/0/0	0/0/0	0/0/3	0/0/0	2/0/1	1/1/0	0/0/0	0/0/0
ON16	0/0/0	0/0/0	0/0/0	0/1/0	0/1/0	0/1/2	0/0/0	2/1/0	0/0/0
NS1	0/0/0	0/0/0	0/0/0	0/0/0	1/0/0	0/0/0	0/0/3	0/0/0	0/0/0
PE1	0/0/0	0/0/0	0/0/0	0/0/0	0/1/0	0/3/3	2/1/0	3/3/3	0/0/0
US3	0/0/0	0/0/0	0/0/0	0/0/0	0/1/1	0/0/1	1/0/0	0/1/1	0/0/0
US7	0/0/0	0/0/0	0/0/0	0/3/2	2/3/2	3/3/3	0/0/0	3/3/3	0/3/0

= 3 replicates producing exudate; = 2 replicates producing exudate; = 1 replicate producing exudate

Figure 4. Variations in pigment diffusion within and among 16 North American *Pseudogymnoascus destructans* isolates in nine conditions. 0 = no visible pigment diffusion; 1=visible pigment diffusion. SD = synthetic-dextrose, PDA = potato dextrose agar, MM= minimal media. Three replicates are shown for each medium-temperature condition for each isolate.

Isolate	MM 4°C	MM 14°C	MM 18°C	PDA 4°C	PDA 14°C	PDA 18°C	SD 4°C	SD 14°C	SD 18°C
NB1	0/0/0	0/0/0	0/1/1	0/0/0	0/0/0	0/0/0	0/0/0	1/1/1	0/0/0
NB2	0/0/0	0/0/0	0/1/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
NB3	0/0/0	0/0/0	0/1/1	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
NB4	0/0/0	0/0/0	0/1/1	0/0/0	0/0/1	0/0/0	0/0/0	0/0/0	0/0/0
NB7	0/0/0	0/0/0	0/1/1	1/1/1	0/0/0	1/1/1	1/1/0	1/1/0	0/0/0
NB8	0/0/0	1/1/1	0/0/1	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
NB25	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	1/1/0	0/0/0	0/0/0	0/1/1
NB26	0/0/0	0/0/0	0/1/1	0/0/0	0/0/0	1/1/0	0/0/0	0/0/0	0/1/0
NB28	0/0/0	0/0/0	0/1/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
ON1	0/0/0	0/0/0	0/1/1	1/1/0	0/0/0	1/1/1	0/0/0	0/0/0	0/0/0
ON3	0/0/0	1/1/0	0/0/0	0/1/0	0/0/0	1/0/0	0/0/0	0/0/0	0/0/0
ON16	0/0/0	1/1/0	0/0/0	0/1/0	0/0/0	0/0/0	0/0/0	1/1/1	0/0/0
NS1	0/0/0	0/0/0	0/0/0	1/1/1	0/1/1	0/0/0	1/0/1	0/0/0	0/0/0
PE1	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/1/1	0/0/0	0/0/0	0/0/0
US3	0/0/0	0/0/0	0/0/0	1/1/1	1/0/0	0/1/0	0/1/1	0/0/1	0/0/0
US7	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0

= 3 replicates secreting pigments; = 2 replicates secreting pigments; = 1 replicate secreting pigments

Figure 5. Variation in colony reverse color among 16 North American *Pseudogymnoascus destructans* isolates in nine conditions. SD = synthetic-dextrose, PDA = potato dextrose agar, MM= minimal media. Three replicates are shown for each medium-temperature condition for each isolate.

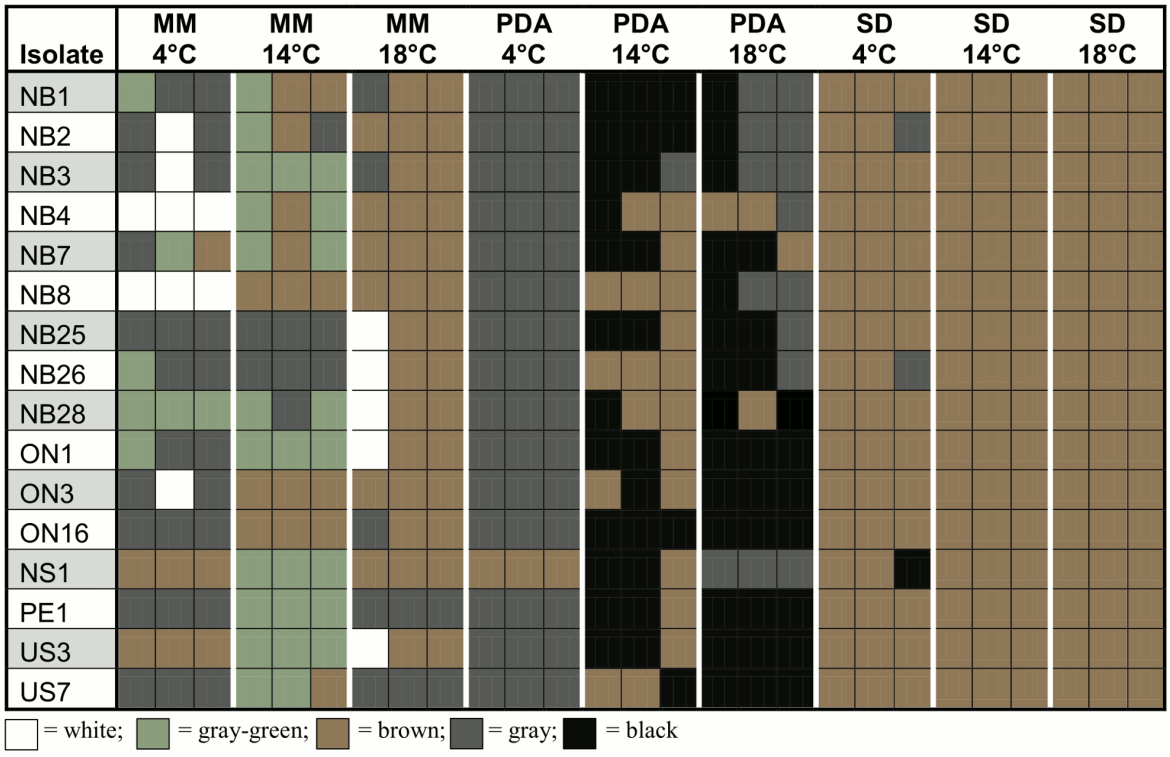

Isolate
MM 4°C
MM 14°C
MM 18°C
PDA 4°C
PDA 14°C
PDA 18°C
SD 4°C
SD 14°C
SD 18°C
NB1
NB2
NB3
NB4
NB7
NB8
NB25
NB26
NB28
ON1
ON3
ON16
NS1
PE1
US3
US7
= white; = gray-green; = brown; = gray; = black

Figure S1. Dendrogram created in PAUP 4.0 using UPGMA, based on conservative scoring of fingerprinting patterns from GACA4 and M13 primers for 112 North American isolates of *Pseudogymnoascus destructans*. *P. pannorum*, WAC5705, was used as an outgroup.

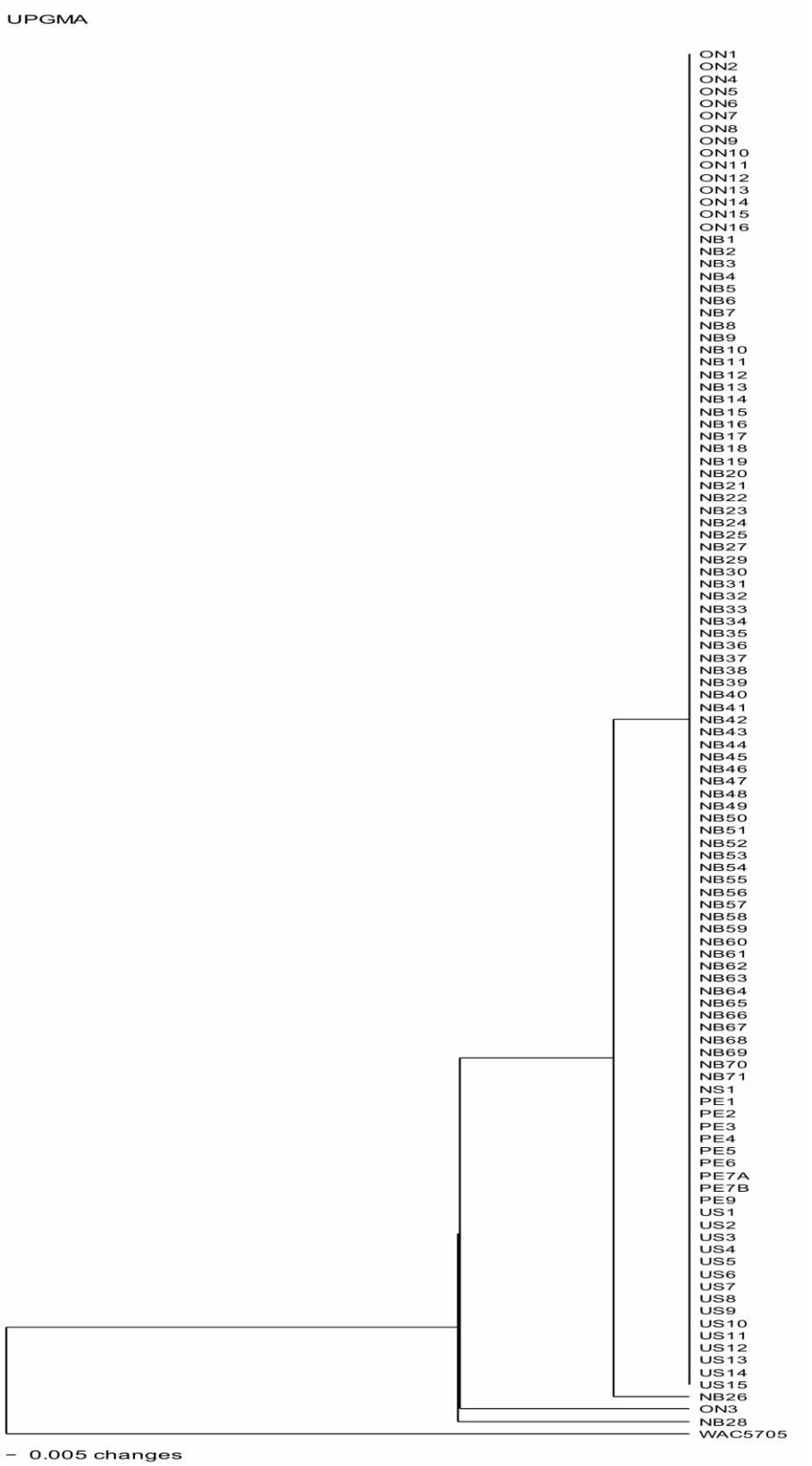
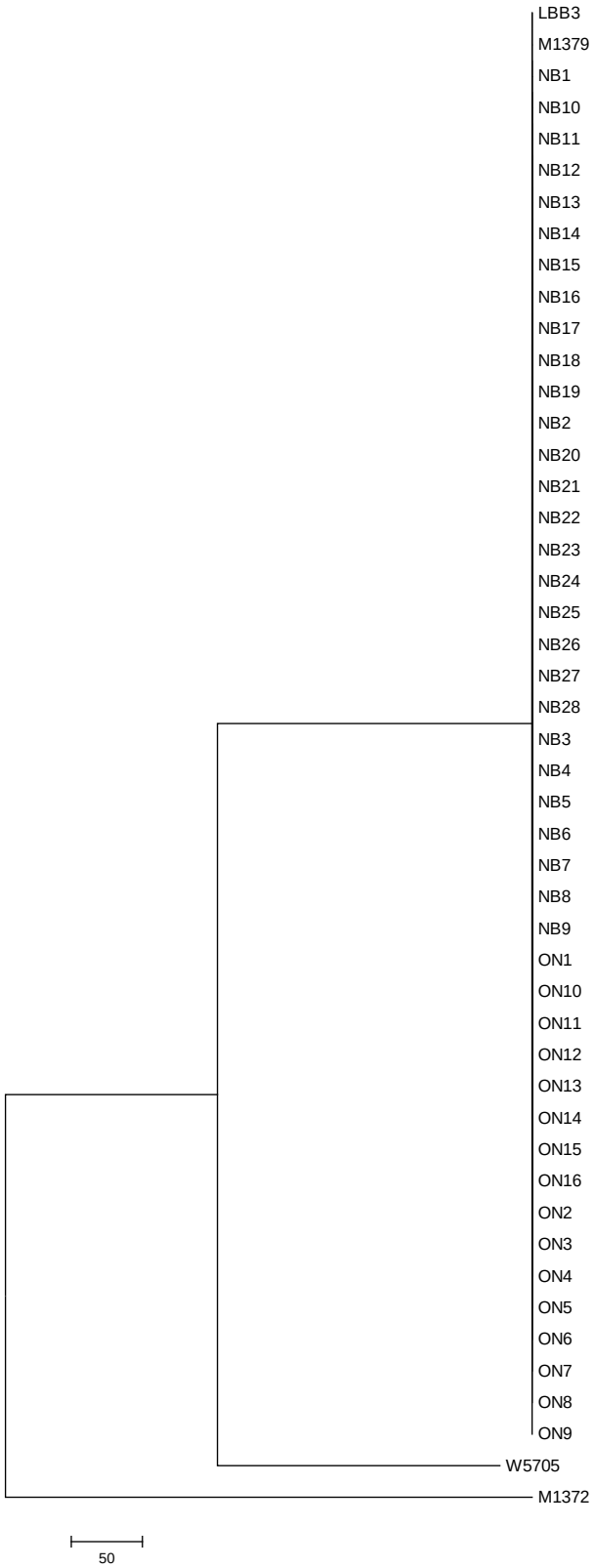

UPGMA
ON1
ON2
ON4
ON5
ON6
ON7
ON8
ON9
ON10
ON11
ON12
ON13
ON14
ON15
ON16
NB1
NB2
NB3
NB4
NB5
NB6
NB7
NB8
NB9
NB10
NB11
NB12
NB13
NB14
NB15
NB16
NB17
NB18
NB19
NB20
NB21
NB22
NB23
NB24
NB25
NB27
NB29
NB30
NB31
NB32
NB33
NB34
NB35
NB36
NB37
NB38
NB39
NB40
NB41
NB42
NB43
NB44
NB45
NB46
NB47
NB48
NB49
NB50
NB51
NB52
NB53
NB54
NB55
NB56
NB57
NB58
NB59
NB60
NB61
NB62
NB63
NB64
NB65
NB66
NB67
NB68
NB69
NB70
NB71
NS1
PE1
PE2
PE3
PE4
PE5
PE6
PE7A
PE7B
PE9
US1
US2
US3
US4
US5
US6
US7
US8
US9
US10
US11
US12
US13
US14
US15
NB26
ON3
NB28
WAC5705
0.005 changes

Figure S2. Maximum parsimony tree using data from eight concatenated gene fragments from 28 New Brunswick and 16 Ontario *Pseudogymnoascus destructans* isolates. *P. destructans* LBB3 and M1379, and *P. pannorum* M1372 from previous studies was included in data set. *P. pannorum* W5705 was used as a reference control. *VPS13* gene fragment for W5705 was not obtained. Number of nucleotide substitutions per site is indicated with scale bar.


LBB3
M1379
NB1
NB10
NB11
NB12
NB13
NB14
NB15
NB16
NB17
NB18
NB19
NB2
NB20
NB21
NB22
NB23
NB24
NB25
NB26
NB27
NB28
NB3
NB4
NB5
NB6
NB7
NB8
NB9
ON1
ON10
ON11
ON12
ON13
ON14
ON15
ON16
ON2
ON3
ON4
ON5
ON6
ON7
ON8
ON9
W5705
M1372
50

CHAPTER 3

GENERAL CONCLUSIONS AND PERSPECTIVES

In this thesis I began by providing background information on WNS such as where it occurs, how it causes mortality in bats as well as behavioral changes that accompany the disease. I then focused on the causative agent of WNS, *P. destructans*, and described key findings relating to its morphology, genetics and other various features of the fungus. Chapter 1 consisted of the main results from the majority of publications on WNS and *P. destructans.* Despite all of the knowledge gained in the past 8 years, it is clear that there is still much to study. We currently do not know the full life cycle of *P. destructans* that may exist in nature and the genotype of the fungus in Canada was unknown prior to this study. There is also currently no feasible treatment for WNS and the long term effects of the disease are unknown.

My study focused on the genotypic and phenotypic variation in the North American population of *P. destructans*. MLST revealed no genetic variation between 16 Ontario and 28 New Brunswick isolates which is indicative of a clonal population. DNA fingerprinting did show minor genetic variation in two New Brunswick and one Ontario isolate. There is currently no published evidence of any genetic variation between *P. destructans* isolates in North America. Foster et al. (2012) did find genetic variation using whole-genome sequencing but did not find any variation in 140 microsatellite loci. Their study is unpublished but will provide further insight in the population genetics of *P. destructans*. Interestingly, they found no variation in 140 microsatellite loci and in my

study I found some variation using DNA fingerprinting. Microsatellites have higher mutation rates than gene sequences and also likely higher than the M13 marker used in my research. Differences in our studies may be due to random genetic drift in the isolates used and may also be a result of the differences in sample size with Foster et al.'s (2012) study using 21 North American isolates whereas 112 were used in this study. Since their study is unpublished, the specific differences between our studies are unknown.

The second major finding of my study is that there is significant phenotypic variation between and within the North American isolates of *P. destructans*. A clonal population of a fungus is expected to show little phenotypic variation between isolates when grown within identical conditions. However, my results show that the clonal population of *P. destructans* has significant phenotypic variation. As previously discussed in Chapter 2, this may be due to the phenotypes studied being sensitive to micro-environmental changes or the genome is not stable and can rapidly change.

The implications of my study are currently unknown. Significant phenotypic variation may affect the pathogenicity, but further studies must first be conducted to determine the role of pigments and exudates in *P. destructans*. Exudates could contain enzymes that aid in the digestion of the surrounding environments or have antimicrobial activity. Differences in exudate production could affect its pathogenicity in bats or its survival in hibernacula. Studies investigating the chemical structure of the pigments would provide insight for its potential role in *P. destructans*. The pigments could be melanin which is a virulence factor in other pathogenic fungi. Comparing isolates significantly different in phenotypes for their pathogenicity could also yield interesting

results. If phenotypic differences of *P. destructans* resulted in different mortality rates of bats, this could potentially determine new drug targets to be used to treat WNS. Overall, much more research needs to be done to fully understand *P. destructans* and to find feasible treatments to help the bats.