The Journal of Experimental Microbiology & Immunology+

Vol 2:26-32

Identification of fungi from soil in the Nakimu caves of Glacier National Park

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The Nakimu caves of Glacier National Park, British Columbia, CA are a remote, oligotrophic karst environment. These caves are swarming sites of endangered little brown myotis (*Myotis lucifugus)* and northern myotis (*Myotis septentrionalis*) bat populations. This project is the first mycological investigation of cultivable fungi in Nakimu Caves, and is intended to focus on the presence of *Pseudogymnoascus* species. *Pseudogymnoascus destructans* is the deadly fungus that causes white nose syndrome (WNS) infection in bats in eastern North America and was first discovered in 2006. With the westward advance of this disease, it is important to determine the fungal flora of the cave, to detect any fungal pathogens, and to have baseline information of the common fungal cave inhabitants. This will assist monitoring the impacts of cave use, and visitation policy to the cave would be impacted by the detection of *Pseudogymnoascus destructans*. A total of 29 soil samples and 26 environmental swabs from the caves were plated onto Sabouraud and Rose Bengal agar. Samples were cultured at 4°C for eight to ten weeks to capture the diversity of cultivable species from the cave's dark zone. Out of 272 fungal isolates, 50 representative samples were identified using ITS sequencing of fungal DNA to the genus and species level. From these, 10 genera of fungi were identified, consistent with common cave fungal flora from around the world. The *Pseudogymnoascus* genus was common in the major cave system, but *Pseudogymnoascus destructans* was not amongst the sequenced samples.

Karst ecosystems are unique, each isolated, with little to no light and limited nutrients. Life existing in caves have extraordinary adaptations to not only survive, but thrive in oligotrophic conditions. Microorganisms that grow in these environments can play critical roles in the nutrient cycle of caves (1), such as fungi acting as decomposers of tree litter that washes into caves, or even in the formation of cave structures themselves, as fungal hyphae can act as attachment and nucleation sites for calcium carbonate precipitation in soda straw formations (1). The investigation of cave fungi has been limited and infrequent in the past, but with the emergence and spread of *Pseudogymnoascus destructans (P. destructans),* (2) formerly *Geomyces destructans* (3), there is growing attention to fungal cave communities (4).

Received: 12/02 2015 **Accepted:** 04/18 2016 **Published:** 06/08 2016

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P. destructans came to the attention of scientists in 2007 when the affliction known as White Nose Syndrome (WNS) appeared in the caves of northeastern USA (2). It was determined that the fungus was not an opportunistic infection as originally believed, after Lorch and colleagues found that exposing healthy bats to the fungus in hibernating conditions resulted in the symptoms (5). WNS is an inflammatory cutaneous infection, affecting the nose, ear and wing membranes of hibernating bats. Infected bats deplete their stores of body fat at a faster rate during hibernation than normally occurs over winter, and emerge from hibernation too early. Without insects to feed on, the bats starve to death before spring (5). This has caused the death of up to 6 million bats in North America, causing population declines of 70-80% in the hibernating bat populations of infected areas (6). Bats are a primary insectivore and reduce populations of insect pests among crops and populated centres, and are a keystone species for cave ecosystems (7).

There is evidence to indicate *P. destructans* originated in Europe (6) and was introduced to North America; though the method of migration is unknown, human-assisted transmission is a suspect (8,9). As *P. destructans* is a

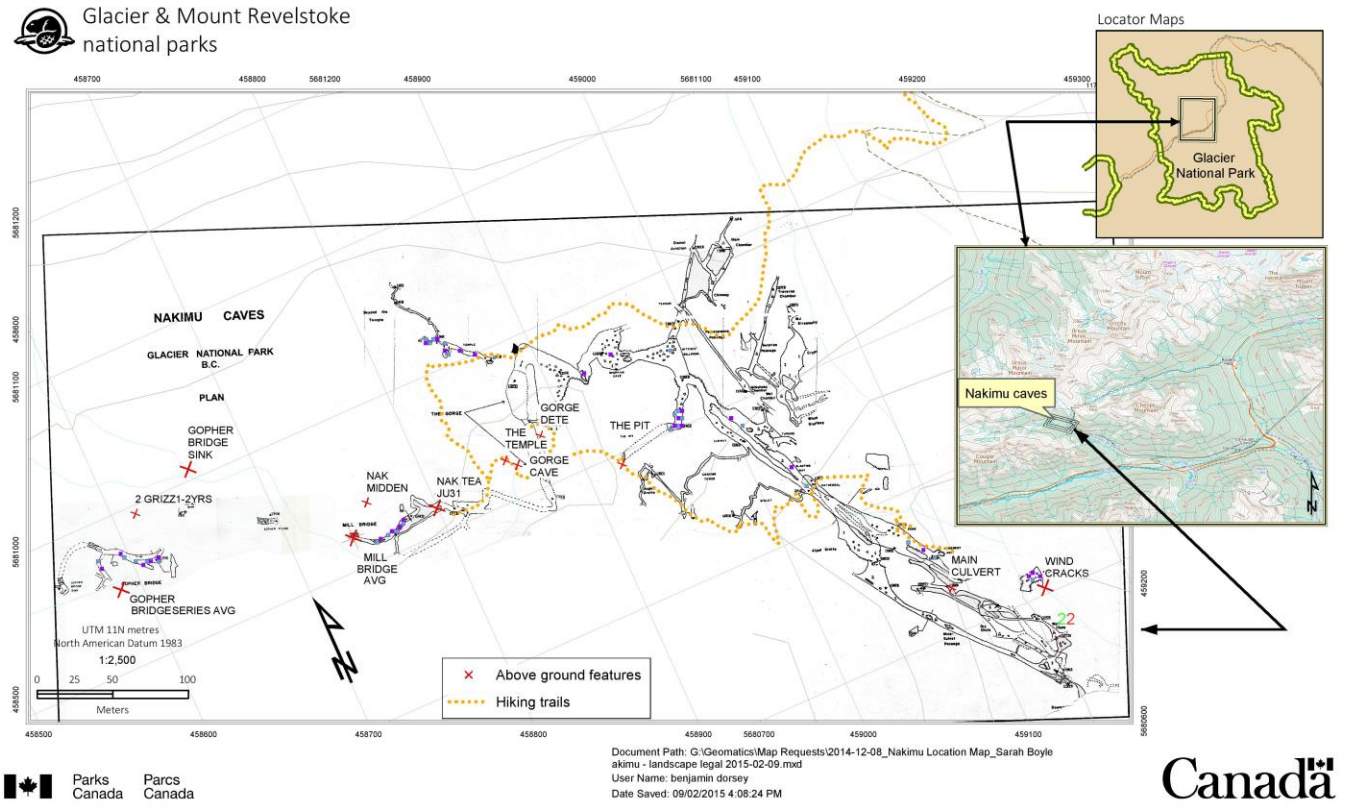

FIG 1. Map of Nakimu Cave system annotated with blue squares (soil sampling sites) and purple squares (environmental swab sample sites). Map courtesy of Parks Canada

psychrophilic fungus, growing best between 4-15°C, it is able to persist between hibernation cycles within cave soil before infecting bat roosts, causing WNS (10, 11). *P. destructans* can not only persist in caves, but can also propagate in cave sediments, especially in areas where there is shed hair to provide keratin as a nutrient source (11).

Early studies that isolated *Pseudogymnoascus* from environmental samples had success with selective agar cultivation methods, especially with Rose Bengal agar and Sabouraud agar infused with antimicrobials to reduce bacterial contamination (9, 10, 12, 13). These studies have also focused on distinguishing *P. destructans* from other closely related species by looking at the distinct morphology of the *P. destructans* spores, which appear curved (12, 13). PCR identification of soil fungi from environmental sources is difficult, but *P. destructans* is especially difficult, due to its close relation to other *Pseudogymnoascus* and *Geomyces* species. However, ITS sequence in fungi can be used to distinguish as the universal barcode sequence for identification of fungal species. The 18S, 5.8S and 28S ribosomal subunit genes are separated by two internal transcribed spacers (ITS) that are universally found in fungi. These ITS regions are of interest due to being non-transcribed intergenic spaces that are subject to low evolutionary pressure. Being non-functional, they tend to have high variability between fungal species, which is useful for species identification. ITS regions 1 and 4 (14) are commonly used for the identification of fungal samples, including *P. destructans*.

The human impact on the Nakimu cave system is relevant as human-assisted transmission of spores is a potential mechanism of transplanting *P. destructans* to new territory. The Nakimu caves of Glacier National Park have a history of extensive visitation between 1904 and 1935 with tourist use via the Glacier House rail stop in Rogers Pass, British Columbia. Currently, exploration of the caves is limited to less than 50 cavers a year, which has allowed the caves to regress to more natural conditions. The Nakimu Caves are one of the largest cave systems in British Columbia, and are a key feature of Glacier National Park (15). The subterranean passages stretch for six kilometers, formed by Cougar Creek cutting through limestone strata in the Balu Pass (Fig. 1), with all cave entrances emerging within the Englemann Spruce-Subalpine Fir Biogeoclimatic zone. The mouths of the caves can be submerged in snow for up to 8 months of the year, creating an isolated, nutrient limited zone (16). The cave is a designated Zone 1 cave, restricted to permit access only for exploration, research and surveillance for the

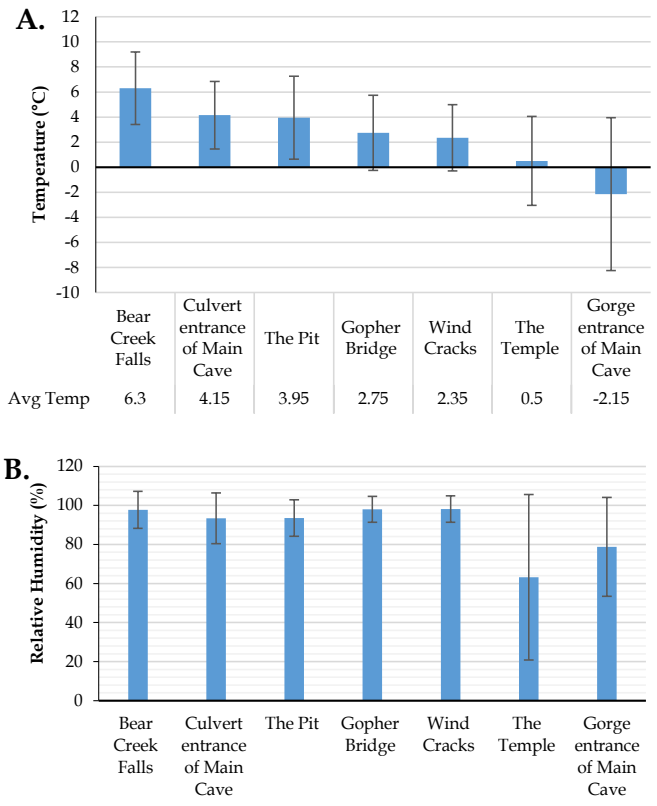

FIG 2. (A) Average Temperature (°C) recorded in caves over the course of one year with error bars of standard deviation and **(B)** Average relative humidity (RH) (%) values recorded inside caves over the course of one year with error bars of standard deviation. (12).

benefit of the cave system (17). The caves have a myriad of features unique in the area, extensive waterfalls, plunge pools, stalactite grottoes, moonmilk and both seasonal and permanent ice deposits deep underground. Life in these caves are relegated to the fringes, with trogloxenes such as Bushy-tailed woodrats, marmots and American Pikas denning near the cave entrances, but rarely venturing into the depths (16). From 1904, the caves became a showcase piece for the National Park, with broad wooden boardwalks and concrete stairs installed to ease tourist access. The main entrance to the largest passage was blasted open to install a culvert, which allowed greater human access to the cave, but the additions only served to damage the natural cave features. In 1935, tourist visits declined, and the cave was closed to the public. In recent years, the decaying boardwalks and concrete stairs have been removed, and the area preserved by Parks Canada with the intention of allowing the slow recovery of natural features from the damage that occurred while it was a tourist attraction (15). The caves are extensive and well mapped (Fig. 1), however, there has been no previous mycological investigation of the cave's inhabitants.

The Nakimu caves of Glacier National Park are documented swarming sites with appropriate conditions for roosting (above freezing temperatures over winter and running water) for two bat species that are susceptible to WNS: little brown myotis (*Myotis lucifugus)* and northern myotis (*Myotis septentrionalis*) (17). The Nakimu caves are also a swarming site for four non-threatened species: big brown bat *(Eptesicus fuscus),* Yuma myotis (*Myotis yumanensis*), long-eared myotis (*Myotis evotis*) and long legged myotis (*Myotis volans*) (17). Identification of fungal species residing in the caves, particularly at the cave entrances, assists long-term conservation efforts for these bats, and contributes to the future management of the caves access for exploration by cavers. The purpose of this investigation was to provide a baseline estimate of the current fungal flora of the cave system and to determine if *P. destructans* was present, by providing microbial analysis of 29 soil samples and 27 environmental swabs from the cave systems. Positive identification would influence the park policy for cave exploration and conservation of hibernaculum; taxonomic classification of cave flora and no detection of *P. destructans* would act as baseline data for future disease surveillance efforts.

MATERIALS AND METHODS

Sample Collection. Over the course of a two-day expedition into the caves, 29 soil samples and 26 environmental swabs were aseptically collected from the various substrates of the Nakimu caves, including gravel mixed with decayed wood, limestone grit, moonmilk, and dust. Sample sites are marked on the map with blue squares noting locations where soil samples were collected and purple squares noting environmental swabbing sites (Fig. 1).

Traces of habitation by small mammals were found at cave entrances, such as guano and nesting materials from Bushytailed woodrats. Some evidence of adult woodrats was found up to 50 m into the Temple Cave, and 5 m into Bear Creek Falls Cave. The Gopher Bridge cave had guano from American Pikas 36 m into the cave (measurements in collaboration with Parks Canada, 17). Cave crickets and a bat of unidentified species were also observed during the expedition (observations in collaboration with Parks Canada, 17). Collection of soil samples was attempted at each cave, however, the substrates of some caves were too rocky for appropriate soil sampling. Sampling locations in the cave such as crevices, ledge or floor, were chosen from areas along the historical cavern foot-passages but away from the surfaces tread by the guide, camera crew and invited Parks Canada staff and were left as undisturbed by the investigative crew as possible to ensure a distributed range of cave surfaces and soils were sampled from the accessible lengths of the caves, as well as to prevent collecting samples that had been deposited by our caving group. Where possible, up to 200 g (wet weight) of soil was collected into an autoclaved screw-cap glass vial using a sterile metal scoopula, which was flame sterilized by wiping with 70% ethanol and burning the ethanol off with a butane torch lighter after use to maintain sterility between samples. Swab sampling approximately followed the procedure of Ogórek (18), with the following changes: swabbing was performed aseptically using dry sterile cotton tip wooden swabs on a surface of approximately

Table 1. Representative isolated fungal colonies. Identities were obtained using the BLAST database and NCBI.

Sample ID	Accession No.	Identity	%
Gopher Hole			
C2 S3 F S	KP411563	*Pseudogymnoascus* sp.	100
C2-2 10^{-2} WS	KP411564	*Mortierella* sp.	99
C2-4 10^{-3} 1	KP411565	*Thelebolus* sp.	98
C2-4 10^{-3} 2	KP411566	*Thelebolus* sp.	98
C2-5 10^{1} F	KP411567	*Mortierella fimbricystis*	99
C2-5 10^{1} S1	KP411568	*Mortierella fimbricystis*	97
C2-5 10^{1} S2	KP411569	*Penicillium crustosum*	100
Mill Bridge			
C1 S1 10^{1}	KP411552	*Cladosporium* sp.	100
C1 S2 10^{1}	KP411553	*Thelebolus ellipsoideus*	99
C1-2 10^{1}	KP411554	*Mortierella sclerotiella*	99
C1-2 10^{-2} 1	KP411555	*Cylindrocarpon obtusisporum*	100
C1-3 10^{1}	KP411556	*Mucor strictus*	99
C1-3 10^{-3}	KP411557	*Pseudodeurotium* sp.	99
C1-4 10^{1}	KP411558	*Cylindrocarpon obtusisporum*	100
C1-4 10^{-1}	KP411559	*Mortierella* sp.	100
C1-4 10^{-2}	KP411560	*Cadophora fastigiata*	99
C1-4 10^{-2} 2	KP411561	*Mucor strictus*	99
C1-S2 10^{1}	KP411562	*Pseudogymnoascus pannorum var. pannorum*	99
Bear Creek			
B3_10_1	KP411551	*Mucor racemosus*	99
B1_10_1	KP411550	*Pseudogymnoascus pannorum*	99
Wind Crack			
W1_10_1	KP411591	*Mortierella* sp.	99
W1_10_2	KP411592	*Pseudeurotium* sp.	99
The Pit			
P1 10^{1}	KP411575	*Penicillium crustosum*	100
P4 10^{1} F	KP411576	*Mortierella* sp.	100
P5 10^{-1}	KP411577	*Mucor racemosus*	100
P5 10^{-1} S2	KP411578	*Mortierella polygonia*	99
P5 10^{1} F	KP411579	*Mortierella fimbricystis*	99
P5 10^{-2}	KP411580	*Mortierella polygonia*	99
PS3 F	KP411581	*Tetracladium* sp.	99
Main Cave			
M S2	KP411570	*Mortierella hyaline*	100
M S3 1	KP411571	*Pseudogymnoascus pannorum*	100
M1 10^{1}	KP411572	*Pseudogymnoascus pannorum*	100
M2 10^{1}	KP411573	*Mortierellaceae* sp.	98
M4 10^{-2}	KP411574	*Mortierellaceae* sp.	100
Temple			
T S1 F	KP411582	*Penicillium commune*	100
T S4	KP411583	*Mortierella* sp.	99
T S5	KP411584	*Penicillium brevicompactum*	99
T2 10^{-3}	KP411585	*Pseudogymnoascus pannorum*	100
T3 10^{1}	KP411586	*Pseudogymnoascus pannorum*	100
T3 10^{-2}	KP411587	*Pseudogymnoascus pannorum*	100
T3 10^{-3}	KP411588	*Penicillium echinulatum*	99
T5 10^{-1}	KP411590	*Pseudeurotium* sp.	99
T5 10^{1} F	KP411589	*Pseudeurotium* sp.	99

5 cm^2 on cave walls or ceiling (between 1-2 m off ground surface, away from running water or signs of tourist damage). Swabs were sealed in autoclaved glass vials with screw-cap lids for transportation. All samples were maintained between 4-10°C and then shipped to the TRU Microbiology lab.

In partnership with Parks Canada, environmental data was collected by placing eight HOBO U23 Pro v2 Temperature/Relative Humidity data loggers (Onset Computer Corporation, Massachusetts, USA) into the caves at distances varying between 10-40 m depending on suitable shelter (17). These logs were able to confirm our temperature condition for fungal culture growth (up to 4°C) was with-in range of the cave system temperature average (Fig. 2a).

Cultivation of samples. To cultivate the swab samples, each swab was lightly dampened with a 100 µl drop of sterile deionised water and gently streaked as a lawn onto Sabouraud 4% D-Glucose agar plates (pH 5.6). The agar was infused with tetracycline (5 µg/ml) and streptomycin (50 µg/L) to deter bacterial contamination. Each soil sample was subject to serial dilutions to determine the fungal community of cave surfaces. Following the methods of Lorch (5), a 200 mg portion of soil from each sample was resuspended in 0.5 ml sterile deionized water in a 1.5 ml Snap-Cap tube. The original suspension was serial diluted to 10^{-2} and a 150 µl aliquot from each 10^{0}, 10^{-1} and 10^{-2} was transferred to antibiotic infused Sabouraud 4% D- Glucose agar plates as well. An additional plate per sample was enriched with approximately 1 g/L powdered sterile peacock feather in addition to the antibiotics (see concentration above) on the advice of Dr. Diana Northup (personal communication) to encourage growth of fungi able to digest keratin. These plates were sealed with parafilm to prevent contamination and water loss, and incubated in dark conditions at 4°C for 8-10 weeks to mimic in situ temperatures on average ~2.5°C (17). During this incubation period fungal growth was monitored on a weekly basis using a light microscope.

Colonies were aseptically isolated using a sterile transfer needle and transferred to a mini plate (4.5 cm diameter) of Sabouraud 4% D-glucose agar and a second plate of Rose Bengal Agar also containing tetracycline and streptomycin to continue growth. These plates were incubated at 4°C for 6 weeks to allow pure cultures to mature and form spores.

As a positive control, two strains of *P. destructans* were also aseptically plated onto each agar type and incubated under the same conditions, to ensure our method did not inhibit its growth, and to provide a positive control for sequencing.

Sabouraud 4% D-glucose agar is a common medium for mycological investigations due to its simple, easily digested components that fungi thrive on (40 g/L dextrose, 10 g/L peptone, 20 g/L agar, pH 5.6). Rose Bengal agar is composed of 5 g/L Enzymatic Digest of Soybean Meal, 10 g/L Dextrose, 1 g/L Monopotassium Phosphate, 0.5 g/L Magnesium Sulfate. 0.5 g/L Rose Bengal, and 15 g/L Agar (pH 7.2). Rose Bengal is useful for long-term investigations as it is a minor growth inhibitor that slows the rate of fungal colonization on the surface area of the agar, allowing for longer storage and ease of collecting isolates of slow-growing *P. destructans* by inhibiting fungal overgrowth or lawn formation.

Sequencing. Following morphological comparisons of the fungi on Rose Bengal agar and Sabouraud 4% D-glucose agar, 272 samples of fungi were aseptically sampled for staining with lactophenol cotton blue and characterizing spore and hyphae

morphology. Following spore staining, 16 tentative classes of fungi were isolated according to morphology and spore characteristics. Fifty samples of fungi representative of these 16 classes were chosen, double wrapped with parafilm, and shipped in a cooled container to Macrogen Korea for sequencing of the ITS region. ITS1 and ITS4 primers were used to sequence both ITS regions in full. From the returned sequence data, contigs were assembled using DNA Baser, aligned and sequence ends trimmed. BLAST searches were performed on quality-trimmed sequences against the Nucleotide collection (nr/nt) database to determine identity.

RESULTS

From the 50 samples sent for sequencing, only 42 returned usable molecular data due to contamination or lack of affinity to the ITS primers. These samples demonstrated the variety of fungal flora in each cave passage (Table 1). Eight of the isolates fell within the *Pseudogymnoascus* genus (Table 1). From these sequence identifications, the remaining members of the 16 isolate groups can be tentatively identified, as seen in Table 2.

Species diversity in each cave is difficult to estimate due to sample bias from limited soil samples collected and culture bias; however, using the Shannon-Weiner index, it can be seen in Table 3 that the composition of fungal species in each cave is variable, but of low diversity.

A phylogenetic tree was constructed to demonstrate the relationship between the isolates identified within the genus *Pseudogymnoascus*. The tree was constructed using alignment of sequences using the NCBI nucleotide BLAST following the greedy algorithm of aligning DNA sequences (19). Sequences from two known *P. destructans* ITS1 to ITS4 sequences were included (20, 21). The tree shows that isolates from this study are more closely related to *P. pannorum* species and *P. pannorum var. pannorum* than *P. destructans.*

DISCUSSION

Our goal was to act as a preliminary investigation to provide background information on the fungal community in the Nakimu caves, we were able to successfully cultivate species from 10 genera of fungi. These data provided a basic snapshot of fungal presence in the cave system; however, our focus on the bat pathogen *P. destructans* returned no matches

Samples of fungi from the *Pseudogymnoascus* genus were successfully cultivated in each of the larger caves the Main Cave, Temple, Mill Bridge and in Bear Creek Falls. However, the only members of the *Pseudogymnoascus* genus successfully identified to the species level were samples of *Pseudogymnoascus pannorum*, and samples that were unable to be identified to the species level are more closely related to *P. pannorum* than *P. destructans* (Fig. 3). These two members of Pseudogymnoascus are very similar, in studies comparing the two fungal genomes, fifty-two percent of *P. destructans* reference DNA shares similarity with *P. pannorum* query DNA, with 45% of it being identical (22). Like *P. destructans*, *P. pannorum* is adapted to grow successfully between 4-15°C and prefers keratinaceous substrates for growth such as shed hair (23). Both *P. destructans* and *P. pannorum* expresses keratinolytic enzymes (21)

Table 2. Indication of relative diversity of isolates recovered from each represented by portion of total identified per cave

Species	Mill Bridge	Gopher Hole	Wind Crack	Bear Creek Falls	The Pit	The Temple	Main Cave
Thelebolus sp.	1	1	0	0	0	0	0
Thelebolus ellipsoideus	1	0	0	0	1	0	0
Tetracladium sp.	0	0	0	0	2	0	0
Mucor racemosus	5	1	1	5	2	0	0
Mucor strictus	5	1	1	1	0	0	0
Cladosporium sp.	3	0	0	2	2	1	3
Mortierella sp.	3	7	3	4	18	14	9
Mortierella hyalina	0	0	0	0	0	0	1
Mortierella sclerotiella	0	0	0	3	0	0	0
Mortierella fimbricystis	2	4	0	0	2	4	3
Mortierella polygonia	1	1	0	0	5	2	0
Pseudogymnoascus sp.	8	2	0	3	2	4	2
Pseudogymnoascus pannorum	1	0	0	9	0	8	7
Cylindrocarpon obtusisporum	2	2	0	0	0	0	0
Cadophora fastigiata	1	1	0	0	1	1	0
Pseudeurotium sp.	1	5	2	2	1	9	4
Penicillium crustosum	0	3	0	0	0	0	0
Common *Penicillium* spp.	6	3	0	0	11	7	6
Total	40	31	7	29	47	50	35

Table 3. Species richness and diversity of cultivated fungi from each cave site

Cave	Total isolates	Richness	Shannon-Wiener Index
Mill Bridge	40	14	2.37
Gopher Hole	31	12	2.25
Wind Crack	7	4	1.28
Bear Falls	29	8	1.89
The Pit	47	11	1.86
The Temple	50	9	1.92
Main Cave	35	8	1.91

that contribute to pathogenicity resulting in skin and nail geomycosis; though it is extremely rare, *P. pannorum* causes opportunistic infections in human patients with weakened immune systems (24). *P. pannorum* is common to arctic and low-temperature soils worldwide, and it is not unusual to find in these low-temperatures conditions (23).

Caves that neighbour each other along Cougar Creek, namely Gopher Hole and Mill Bridge, had similar fungal isolates. This was not surprising as the caves are fed by the same stream, and spores from the first cave in a series would certainly be introduced to the latter via water and air currents. Caves inhabited by trogloxenes, such as Bushytailed woodrats, identified by scat traces and den materials by Sarah Boyle in the Mill Bridge and Gopher Hole caves, had fungi from the Order Thelebolales, which are known to inhabit the dung of warm-blooded animals and can survive cold, dry environments (25, 26).

The fungal inhabitants of Nakimu caves were all relatively well characterized fungi with many similar genera reportedly found in other cave systems. Most caves samples had isolates of *Mucor* sp., a group of saprophytic soil fungi that is common in caves and soil (27; 28). *Cladosporium* sp., common in the Nakimu cave system, are amongst the most common genera to be identified in caves, and are capable of degrading lignocellulose (28). *Cladosporium* members were found in all caves except the Gopher Hole and Wind Cracks. Each cave except Mill Bridge had *Mortierella* spp., which are common saprotrophic fungi that grow on decaying leaves and other organic material (28, 29, 30), such as the vegetation piles left by American Pikas and Bushytailed woodrats. Every cave except the Bear Creek Falls cavern and the Wind Cracks had some species of *Penicillium* fungi, which are among the most common fungi cultivated from caves and soil globally (28, 31). Both the Mill Bridge and the Gopher hole were found to contain *Cylindrocarpon obtusisporum,* a fungus that opportunistically can cause root rot in a wide variety of plants including pine trees (32). The spores are 'slimy'

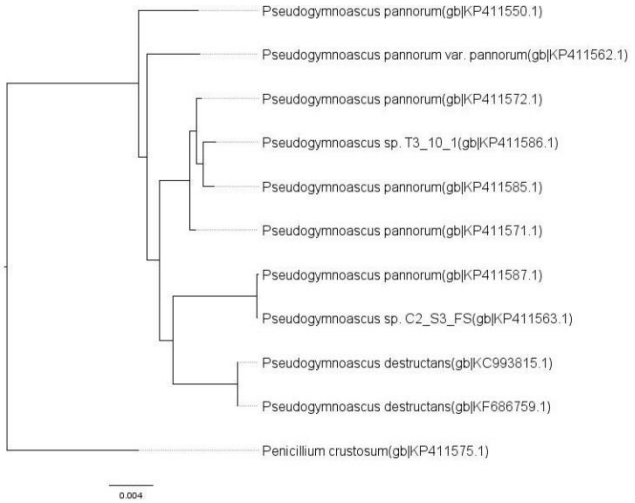

FIG 3. Phylogenetic tree of identified Pseudogymnoascus isolates from this study (Labelled with species and accession number from Genbank) in relation to two Pseudogymnoascus destructans strains from literature: KC993815 (19) and KF686759 (20). This figure shows the closer relationship of the isolates cultivated in this study to *P. pannorum* than to *P. destructans*. The ITS sequence of *Penicillium crustosum* (P1 10^1, KP411575) in this study was used as an outgroup to root the tree.

and are spread mostly through water run-off (32) which is expected as these caves are the first two in series (Fig. 1) to receive runoff water from the surrounding boreal forest.

Researchers have successfully cultivated *P. destructans* from cave soils using methods we have employed. However, these studies usually originate from caves that have already housed bats with symptoms of WNS. Our results provide a preliminary assessment of the fungal taxa present in the cave. Future studies should account for cultivation bias using metagenomic approaches to identify the uncultivable fungal community. Other sampling and culturing methods, including air-filter spore sweeps, water sampling or using bait such as sterile horse hair could also be expanded upon to census rarer and slow growing fungi adapted to oligotrophic conditions.

ACKNOWLEDGEMENTS

A great many thanks are owed to Dr. JP Xu of McMaster University for kindly shipping two essential positive control strains of *P. destructans* to our lab, as well as for comments for our manuscript and for sharing his knowledge and experience in WNS research with us. Additionally, thanks go to Danielle Backman of Mount Revelstoke and Glacier National Parks for her time and assistance. Additionally, we would like to express our gratitude to Drs. Cori Lausen and Karen Vanderwolf for their insightful comments in manuscript preparation. Thanks go to the CUEF UREAP Research Fund and Parks Canada for their generous funding and permission for protected area research for this project. The funders had no role in study design, data

collection and interpretation, or the decision to submit the work for publication.

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