White Nose Syndrome & Illinois Bat Populations: Biology & Monitoring

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Introduction

We implemented a monitoring program to detect *Pseudogymnoascus destructans*, the fungus responsible for fatal white-nose syndrome in cave-hibernating bats in Illinois. Using molecular and culture-based approaches, we evaluated dead and live-caught bats and cave and mine substrates in order to detect *P. destructans* and to better understand the microbial community context for the integration and spread of *P. destructans* into cave ecosystems. Our objectives for this work were:

1. To establish a monitoring program for the rapid and early detection of the fungus, *P. destructans*, on bat specimens from around Illinois.
2. Characterize the background microbial load present in the fur of potential bat vectors of *P. destructans*.
3. Characterize the establishment potential of *P. destructans* in Illinois cave environments.

To accomplish these objectives, we surveyed 11 Illinois caves during the winters of 2012 and 2013, when hibernating bats were present. Because *P. destructans* is known to persist in caves even after the total loss of bat populations from those caves, it is important to understand the environmental and ecological factors related to *P. destructans* establishment and survival in various cave habitats. Therefore, we applied cultivation-based microbiological approaches and cultivation-independent DNA-based approaches to characterize the microbial load of bat fur, cave ceilings with and without roosting bats, and cave soils in hibernacula and bat-free portions of caves. In this report we describe the microbial communities of sampled animals and caves. This study provides crucial data on the occurrence and distribution of *P. destructans* and the microbial ecosystems in which it must compete to become established.

Methods

Study Area

In consultation with IDNR, USFWS, and USFS we selected a total of eleven sites to visit for field sampling of hibernating bats **(Table 1**). Magazine Mine was only visited during the first winter, as liability concerns precluded subsequent visits. White Rock Mine was

only visited to count bats and check for signs of WNS, with no sampling planned for this site.

Field Sampling

We utilized standardized field forms (**Appendix 1**) to collect data about the cave, about samples, and about the bats. At each site, three live or fresh dead bats (**Figure 1**,a) were sampled using sterile technique. For each bat, species, age, sex, condition, and weight were recorded. Two wing punch samples were collected from each bat using biopsy punches[1], and additional samples from wings and fur were collected using sterile swabs[2]. Additional swab samples were collected from the cave/mine wall/ceiling at the location where the bats were roosting (**Figure 1**,b), and airborne fungal samples were collected by waving a petri dish with agar in the air for 5-10 seconds near the roost location. Below each of the three bats (**Figure 1**,c), an associated soil/substrate sample was collected into a sterile whirl-pak bag using a sterile spoon[3]. At three additional sites away from the bats (to the extent feasible), other soil/substrate samples were collected (**Figure 1**,d). At each site, we also collected soil/substrate samples associated with "other" habitats - these typically included woody debris, old raccoon feces, or soil beneath leaf litter (**Figure 1**,e). We also collected three soil samples just outside of each/cave mine. Finally, prior to leaving each site we swabbed the boots of three researchers using the sterile swabs. Controls for swabs and air samples were brought into each cave, pulled out as if sampling (but not opened), and then returned to the laboratory. Occasionally, when dead/dying bats were found we collected additional dead bats to bring to the laboratory. University IACUC and Biosafety protocols were in place for all field activities, as were state and federal permits for listed bats, and state and federal permits for research activities on various types of lands (state, federal, private, nature preserves, etc.).

In association with many of the above sample types (excluding swabs of boots), temperature, humidity, and light data were collected to better characterize the habitats. Numerous photographs were taken to document all aspects of the work.

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[1] Sterile disposable biopsy punches, 2mm (Miltex Biopsy Dermal Punch Sterilized Disposable Punches for Dermal Anchors, Punch Size: 2.0mm~12g; Catalog Number MED-033 (Painful Pleasures, Inc., 7410 Coca Cola Dr., Unit # 214, Hanover MD 21076)).

[2] Sterile swabs in tubes with labels. Copan Diagnostics Sterile Plain Swabs in labeled tubes (Copan Diagnostics, Inc., No.: 159C), Catalog Number 14-906-19 (Fisher Scientific, Fisher Scientific, 300 Industry Dr., Pittsburgh PA 15275).

[3] Sterile disposable spoons, 1 tablespoon. Fisherbrand Disposable Sterile Spoons Capacity: 1 Tbs.; Length: 7 in.; Catalog Number 14-375-257 (Fisher Scientific, 300 Industry Dr., Pittsburgh PA 15275)

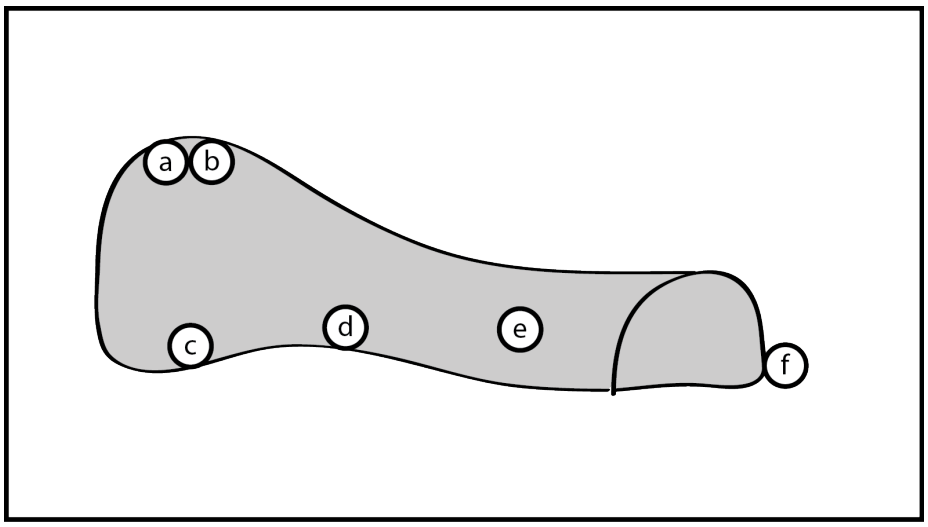

Figure 1. Diagrammatic representation of a cave/mine hibernaculum, showing locations of various samples: a) live bats [swab samples, wing punches]; b) swab samples of roost area substrate; c) soil/guano samples near roost site; d) soil samples away from bat roosts; e) other substrate and swab samples, including swabs from researcher's boots just before exiting cave/mine; f) soil sample from just outside of cave/mine.

We utilized USFWS approved WNS decontamination protocols, altering these as changes to protocols were implemented/recommended at a national level.

Samples, including occasionally euthanized bats, were placed on ice in coolers for transportation back to the laboratories in Champaign/Urbana, Illinois, where they then fell under different biosafety protocols. Histopathological confirmation of WNS was carried out by the University of Illinois Veterinary Diagnostic Laboratory (Adam Stern), fungal cultures were grown in the Miller Mycology Lab (University of Illinois, Prairie Research Institute, Illinois Natural History Survey), and whole-community microbial analyses were conducted in the Yannarell Lab (University of Illinois, Department of Natural Resources and Environmental Sciences).

Laboratory Methods

Isolating and culturing fungi

All glassware/utensils utilized in the isolation process had the openings covered with aluminum foil, followed by one cycle through the autoclave at standard temperature and pressure. All plates were sealed with Parafilm and incubated at 7°C or 14°C. After inoculation, plates were examined for fungal growth every 3 days for up to 4 weeks. After 6 weeks, all inoculation plates were re-examined for slow growing fungi prior to

sterilization. Axenic cultures were obtained from the inoculation plates by transferring individual colonies or conidia to individual 60 mm plates containing PDA. Colonies were selected based on differences in their culture mat morphology, color, and exudate production. For short-term backup, each axenic culture was later transferred to several PDA plates. For long-term backup, each culture was transferred to one cryogenic vial containing autoclaved water and 4-6 (5 mm) agar plugs containing actively growing mycelium using sterile techniques.

Isolation from soil samples

Approximately 1 gram of each substrate sample was diluted in 9 mL sterile distilled water and serially diluted to 10^{-3}. A 400 µL aliquot of each of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions was pipetted onto the center of PDA and SDA plates (90 mm diam.) containing streptomycin sulfate and penicillin G (0.015 g/L and 0.0075 g/L respectively). A sterile bent glass spreader was used to distribute the liquid inoculation evenly over the entire plate surface (lawn plating) and the resulting plate was processed as previously stated. Throughout each set of isolations, controls were obtained at the end following the same procedure with the exception of adding an inoculum.

Isolation from punches, swabs and suspect bat samples

Fungal isolates were also obtained from biopsy punches (2 mm diam.), Conpan swabs and directly from bat samples. Bat wing tissue was dislodged from each disposable biopsy punch and submerged in a SDA plate. Swab samples were streaked on PDA or SDA plates and further processed as above. Several isolates were obtained from WNS suspected bats. The suspected bats were inspected under magnification for fungal growth. Flame sterilized metal picks were used to remove small portions of fungal mycelium to SDA plates and processed as above.

DNA extraction, PCR and sequencing of fungal cultures

DNA was extracted by adding fresh mycelium to 200 µL 0.5 M NaOH, ground, centrifuged at 14,000 RPM for 2 minutes, and 5 µL of the resulting supernatant added to 495 µL 100 mM Tris-HCl buffered with NaOH to pH 8.5-8.9 (Tris-HCl-DNA extraction solution, Osmundson et al. 2013). PCR was completed on a Bio-Rad PTC 200 thermal cycler. The total reaction volume was 25 µL (12.5 µL GoTaq® Green Master Mix, 1 µL of each 10 µL primers ITS4 and ITSIF, 3 µL of the Tris-HCl-DNA extraction solution and 7.5 µL DNA free water). The following thermal cycle parameters were used: initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 1 minute with a final extension step of 72°C for 10 minutes. Gel electrophoresis (1% TBE agarose gel stained with ethidium bromide) was used to verify the presence of PCR product and the resulting PCR product was purified using a Wizard® SV Gel and PCR Clean-Up System (Promega). A BigDye® Terminator 3.1 cycle sequencing kit (Applied Biosystems Inc.) was used to sequence the ITS5 region on an Applied

Biosystems 3730XL high-throughput capillary sequencer at the W.M. Keck Center for Comparative and Functional Genomics at UIUC. Identity was confirmed through nBLAST analysis using the NCBI database at GenBank.

If the above PCR protocol was unsuccessful, DNA was extracted using the DNeasy® Plant Mini kit (Qiagen) following the instructions supplied by the manufacturer and PCR was completed using PuReTaq®Ready-To-Go® PCR beads (GE Healthcare). The total PCR reaction volume was 25 µL (20 µL of DNA free water, 1 µL of each 10 µL primers ITS4 and ITSIF, 3 µL of purified DNA) and the following thermal cycle parameters were used: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 47°C for 15 seconds, 72°C for 1 minute with a final extension step of 72°C for 10 minutes. PCR verification, purification and sequencing were as above.

Table 1. List of winter hibernacula visited during the present study. Site names ending in "Cave" or "Mine" may have these words eliminated elsewhere in this report to facilitate easy communication.

Landowner	Land Status	County	Site
IDNR	Illinois Nature Preserve	La Salle	Blackball Mine
IDNR	Illinois Nature Preserve	Adams	Burton Cave
US Forest Service	National Forest	Pope	Ellis Cave
US Forest Service	National Forest, Research Natural Area	Saline	Equality Cave
IDNR	Illinois Nature Preserve	Monroe	Fogelpole Cave
Private	Private Land	Hardin	Griffith Cave
IDNR	State Natural Area	Monroe	Illinois Caverns
Unimin Mine Corporation	National Forest (corporate mineral rights)	Alexander	Magazine Mine
The Nature Conservancy	Illinois Nature Preserve	Pike	Twin Culvert Cave
Clifftop NFP	Illinois Nature Preserve	Monroe	White Rock Mine

Whole-community microbial DNA extraction

We extracted bulk microbial DNA for cultivation-independent characterization of cave microbial communities. We used the FastDNA SPIN kit for soil (M.P. Biomedicals, Solon OH) to process all samples so that we could compare the results from different sample types without introducing methodological extraction bias. We lyophilized all soil samples prior to DNA extraction of a 500 mg subsample of each soil collected in 2012 and 2013. For swabs (of bat fur, cave ceilings, and researcher boots), we broke the stem of each swab directly into a separate extraction tube and then added extraction buffer directly to the tube. We used bead-beating (6.0 m/s maximum velocity, for 40 seconds)

to liberate DNA from all soil and swabs, and then we proceeded with DNA extraction according the kit manufacturer's protocol.

PCR for microbial community fingerprinting

We characterized bacterial and fungal community composition of soil and swabs using automated ribosomal intergenic spacer analysis (ARISA). The ARISA technique uses PCR to amplify the hypervariable internal transcribed spacer region of bacterial and fungal ribosomal RNA-encoding regions. Length heterogeneity of these regions is used to differentiate microbial taxa and compare the relative abundances of these taxa within and between samples. For bacterial ARISA, we used the forward primer 1406f with a fluorescent 6-FAM molecule attached to the 5' end and the reverse primer 23Sr. For fungal ARISA, we used the forward primer 3126T with a fluoresecent HEX molecule attached to the 5' end and the reverse primer 2234C. Otherwise, for all PCR reactions the total reaction volume was 25 µL (2.5 µL Tris buffer (pH 8.3), 0.625 µL of bovine serum albumin (10 mg/ml), 2.5 µL $MgCl_2$ (25 mM), 1.25 µL dNTP (5 mM of each dATP, dTTP, dCTP, dGTP), 1 µL of the appropriate forward and reverse primer, 0.25 µL of GoTaq® DNA polymerase (5 U/µL), and 13.88 µL DNA free water). The following thermal cycle parameters were used for all ARISA PCR: initial denaturation at 94°C for 2 minutes, followed by 26 cycles of 94°C for 35 seconds, 55°C for 45 seconds, 72°C for 2 minute with a final extension step of 72°C for 2 minutes. We diluted all ARISA PCR products 1:1 with DNA-free water and visualized the ARISA profile fluorescence signal by capillary gel electrophoresis on an Applied Biosystems 3730XL high-throughput capillary sequencer at the W.M. Keck Center for Comparative and Functional Genomics at UIUC. We included a custom-designed internal ROX size standard in each separate ARISA sample.

PCR for high-throughput DNA sequencing of cave soils

To provide a more in-depth view of cave soil microbial communities, we employed high-throughput DNA sequencing using the Illumina MiSeq® platform. We used whole-community DNA extracts to sequence the V3-V4 region of bacterial 16S rRNA genes, a widely-used taxonomic marker. We applied a dual-indexing approach, including separate "barcode" DNA sequences on both the forward and reverse PCR primers, the unique combination of each allowing us to unambiguously assign each DNA sequence to a specific cave soil sample. We used the PCR primers 515F and 926R, modified on the 5' end for dual-index Illumina sequencing (Illumina adapter sequence, unique 12-nt barcode, primer pad, linker, PCR primer). The total reaction volume of PCR reactions was 50 µL (25 µL Kapa HiFi Library Amplification buffer, 1 µL of each primer, 2 µL of template DNA, and 21 µL DNA free water). The following thermal cycle parameters were used: initial denaturation at 98°C for 45 seconds, followed by 25 cycles of 98°C for 15 seconds, 65°C for 30 seconds, 72°C for 30 seconds, with a final extension step of 72°C for 2 minutes. We purified PCR products with AMPure® XP beads (Agencourt, CA) to remove unincorporated primers, dNTPs, polymerase, etc., and then we quantified the concentration of PCR products using the Quant-it® HS DNA Assay kit (Invitrogen, CA)

according to the manufacturers' protocols. We pooled equimolar concentrations of PCR products from 48 different samples, using a unique combination of (one of) 2 barcoded forward primers and 24 barcoded reverse primers for each sample in the pool. Each pool was then paired-end sequenced on one flow cell using the Illumina MiSeq v3 platform by the W.M. Keck Center for Comparative and Functional Genomics at UIUC.

DNA data analysis

We performed size-calling and profile processing of ARISA data using Genemaker® software (SoftGenetics, PA). This software translates the fluorescent signal of ARISA profiles into a series of peaks, based on the size (in bp) of the DNA fragment estimated in comparison to the internal size standard. We performed automatic binning of similar-sized fragments, and then we manually corrected the computer bins to eliminate overlap between bins and to add bins for small peaks missed by the software. Here, we take each of these bins to represent distinct microbial taxa (hereafter referred to as "operation taxonomic units," or OTUs), with the signal intensity of each OTU's peak in a given sample taken to reflect the overall population size for that OTU in that sample.

We used a custom-made bioinformatics pipeline to process the Illumina sequencing data. The W.M. Keck Center used the combination of forward and reverse barcodes to sort DNA sequences based on their sample of origin. We used the open-source software FLASH (Fast Length Adjustment of Short reads) to assemble the paired-end reads of each sequence into a single DNA sequence, and we also used FLASH to quality-filter the assembled reads. We used the open-source software QIIME to remove singleton sequences, cluster DNA sequences into OTUs based on 97% similarity, select a representative sequence for each OTU, and assign taxonomy to each OTU using the Ribosomal Database Project's Classifier.

Results

Publications, Presentations and Student Training

In addition to the specific data collected and analyzed during this study, our work has also contributed to two publications thus far, provided support and experience in student training, and has resulted in a variety of presentations (**Appendix 2**).

Site Visits

A wide variety of individuals, primarily from state and federal agencies, assisted with fieldwork, with a total of 223.28 hours spent in-cave (or in-mine) conducting fieldwork (**Table 2**).

Table 2. Summary of site visits winter 2011-2012 and winter of 2012-2013. Full site names given in **Table 1**.

Site	Date	Crew	Person- Hours in Cave/Mine
Blackball	2 Feb 2012	Joe Merritt (JM), Ed Heske (EH), Steve Taylor (SJT), Joe Kath (JK, IDNR Endangered Species Specialist), Dan Kirk (IDNR District Heritage Biologist), Stefanie Fitzsimons (IDNR Intern working with Dan Kirk)	28.20
Burton	8 Feb 2012	JM, EH, SJT, JK, Dean Corgiat (DC, IDNR District Heritage Biologist)	15.07
Twin Culvert	9 Feb 2012	JM, EH, SJT, JK, DC, Ray Geroff (intern for Angella Moorehouse, INPC Area 4 Natural Areas Preservation Specialist)	12.75
Equality	15 Feb 2012	JM, EH, SJT, JK, Rod McClanahan (RM, USFS Wildlife Biologist), Nick Hausen (WSIL-TV 3)	21.40
Magazine	16 Feb 2012	JM, EH, SJT, JK. Gary Slusher (Unimin Corporation) cleared the road, opened gates for us, but did not go in mine.	11.27
Fogelpole	22 Feb 2012	JM, EH, SJT, JK	12.53
White Rock	22 Feb 2012	JM, EH, SJT	3.15
Illinois Caverns	23 Feb 2012	JM, EH, SJT, JK	10.80
Griffith	29 Feb 2012	JM, EH, SJT, JK, JK	13.17
Ellis	29 Feb 2012	JM, EH, SJT, JK	5.27

(Table concluded on following page)

Table 2. Concluded

Site	Date	Crew	Person-Hours in Cave/Mine
Blackball	30 Jan 2013	JM, EH, SJT, JK	15.67
Burton	5 Feb 2013	JM, EH, SJT, JK	8.00
Twin Culvert	6 Feb 2013	JM, EH, SJT, JK	9.67
Illinois Caverns	12 Feb 2013	JM, SJT, JK	6.10
White Rock	12 Feb 2013	JM, SJT, JK, Pen Daubach (Clifftop NFP), Carl Daubach (Clifftop NFP)	7.50
Fogelpole	13 Feb 2013	JM, SJT, JK	7.25
Griffith	20 Feb 2013	JM, EH, SJT, JK, JK	12.92
Ellis	20 Feb 2013	JM, EH, SJT, JK, JK	12.92
Equality	21 Feb 2013	JM, EH, SJT, JK	9.67

We collected data on 79 individual bats, with *Perimyotis subflavus* and *Myotis lucifugus* being the most frequent bats sampled (**Figure 2**). Of these, 13 (17%) were found dead, 1 was likely dead but was euthanized as a precaution, 7 (9%) others were euthanized, and 58 (73%) were sampled live and released back to their roost locations. Most of the bats (75) were adults, the remaining 4 were too badly decomposed to determine age. Our samples included 29 female bats, 42 male bats, and 8 for which sex could not be determined (decomposing dead bats), and the bats sampled were approximately equally distributed among sample sites (**Figure 3**).

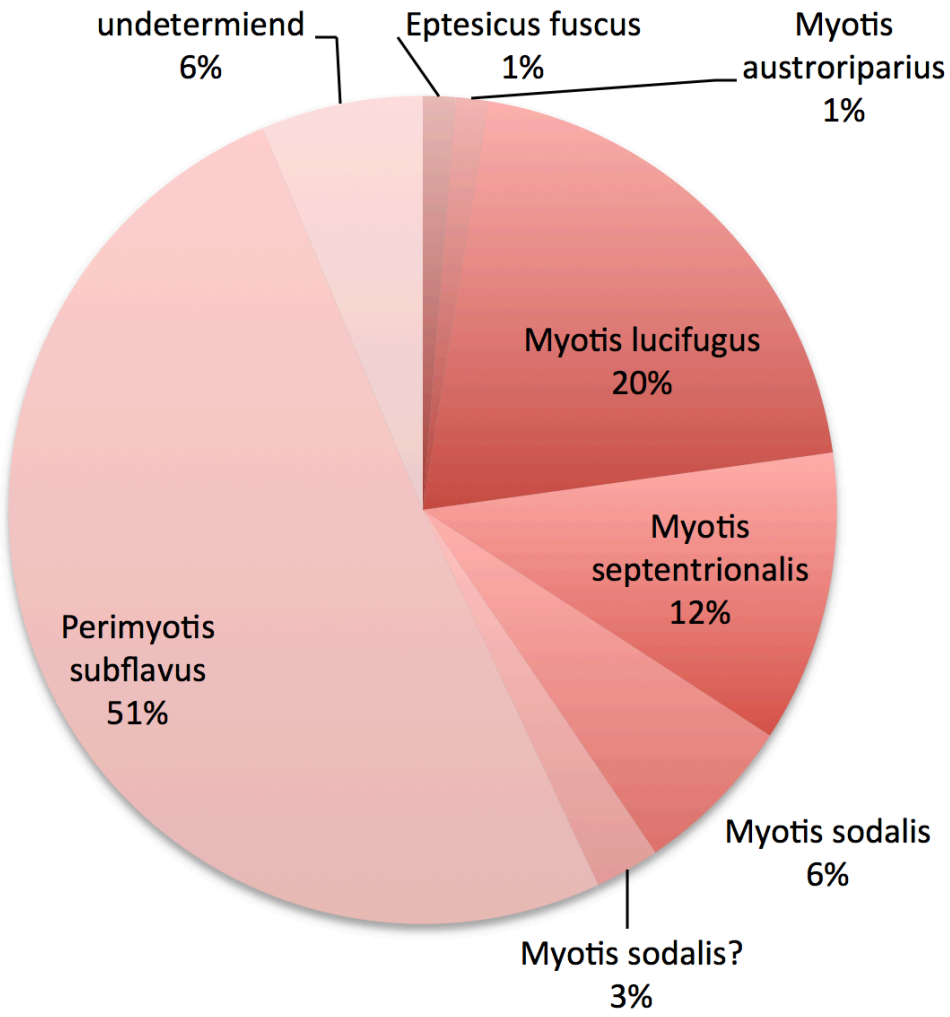

Figure 2. Summary of species of bats from which we collected samples in the winters of 2011-2012 and 2012-2013 in Illinois hibernacula.

Temperature, humidity and light readings spanned a broad range, with most bats being found in complete darkness, at locations where humidity exceeded 60% (**Figure 4**), where air temperatures were between 4 and 14 °C (**Figure 5**), where soil temperatures were between 4.1 and 13.3 °C (**Figure 6**), and where roost substrate temperatures were between 5.4 and 13.3 °C (**Figure 7**).

We documented the arrival of WNS into Illinois during our second winter (2012-2013) of fieldwork, when the disease was recorded from sites in Monroe, La Salle, Hardin, and Pope counties.

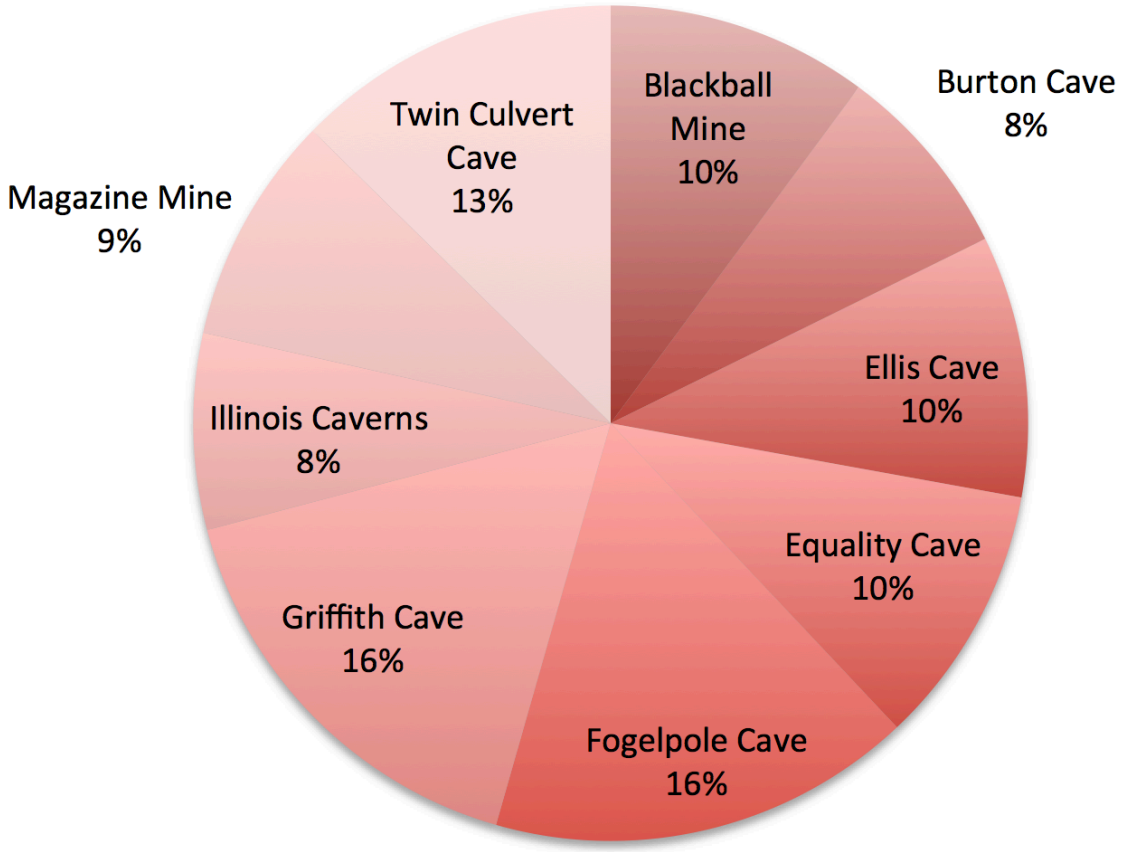

Figure 3. Summary of number of bats from which we collected samples in the winters of 2011-2012 and 2012-2013 in Illinois, as a proportion of total bats in each hibernaculum.

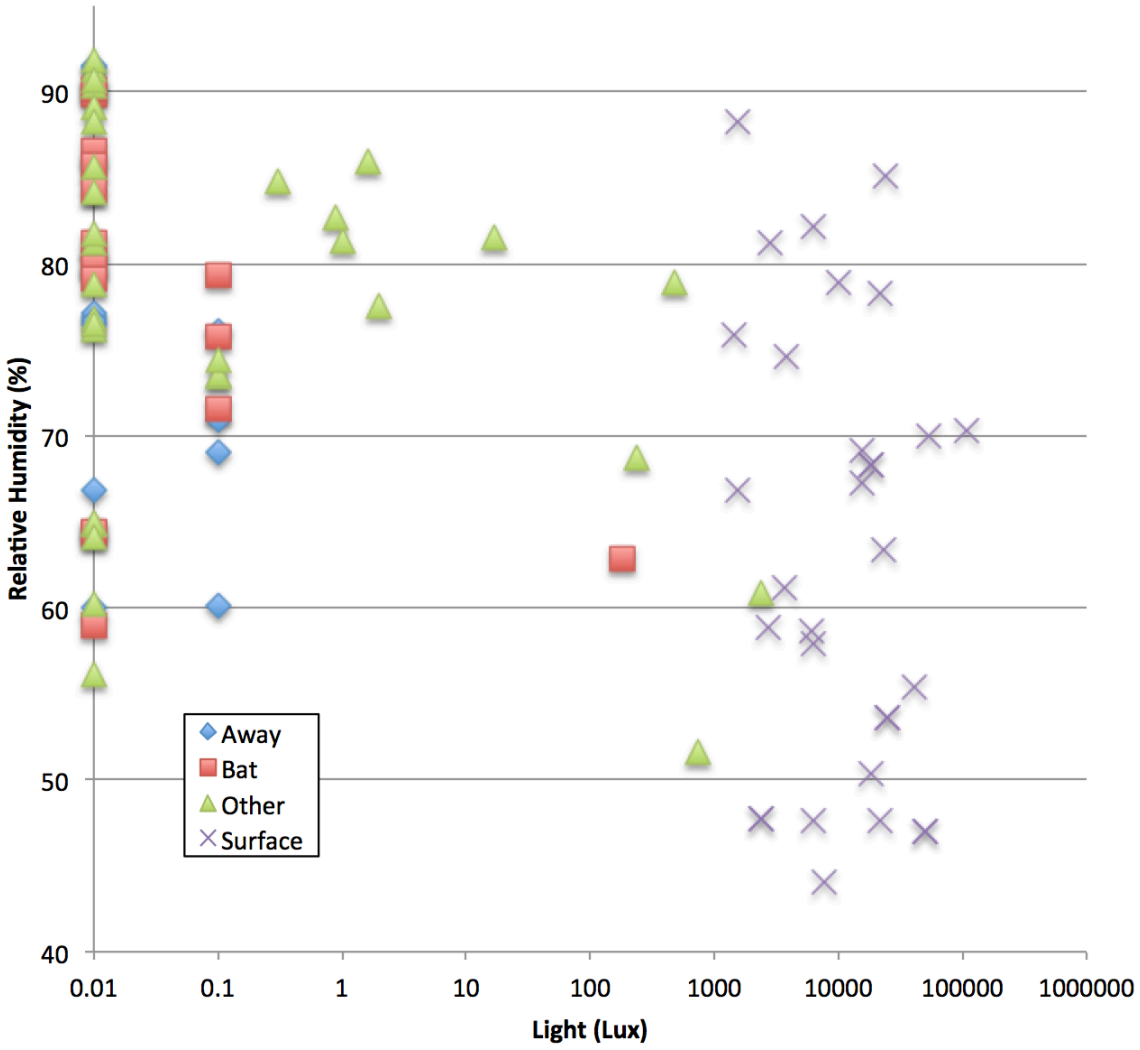

Figure 4. Humidity and light at locations were sample data were collected in association with WNS research at Illinois hibernacula in the winters of 2011-2012 and 2012-2013. Symbols correspond to different sample types (see text). The complete absence of light (0 lux) is plotted as 0.01 lux, as 0 cannot be plotted on a $\log_{10}$ scale.

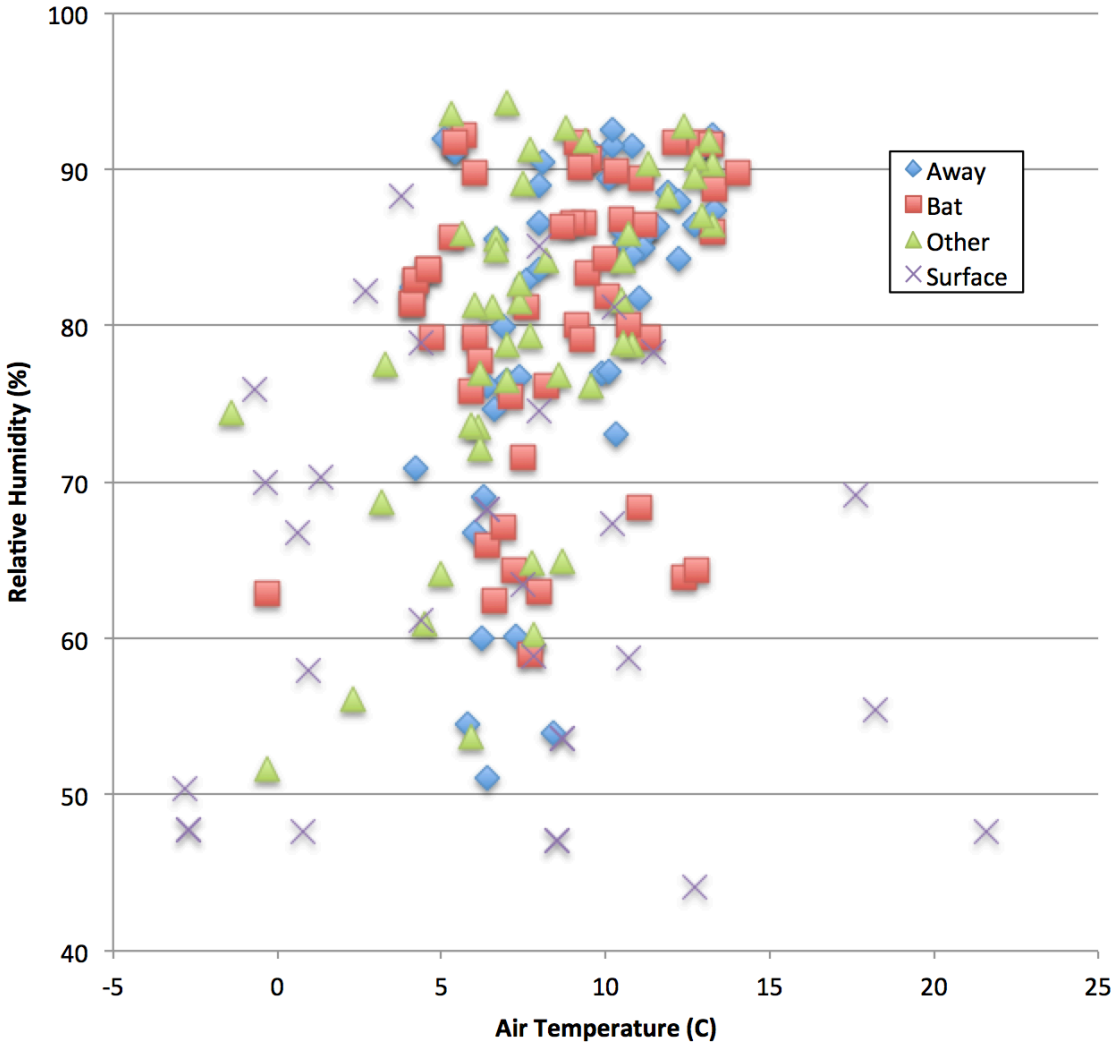

Figure 5. Humidity and air temperature at locations were sample data were collected in association with WNS research at Illinois hibernacula in the winters of 2011-2012 and 2012-2013. Symbols correspond to different sample types (see text).

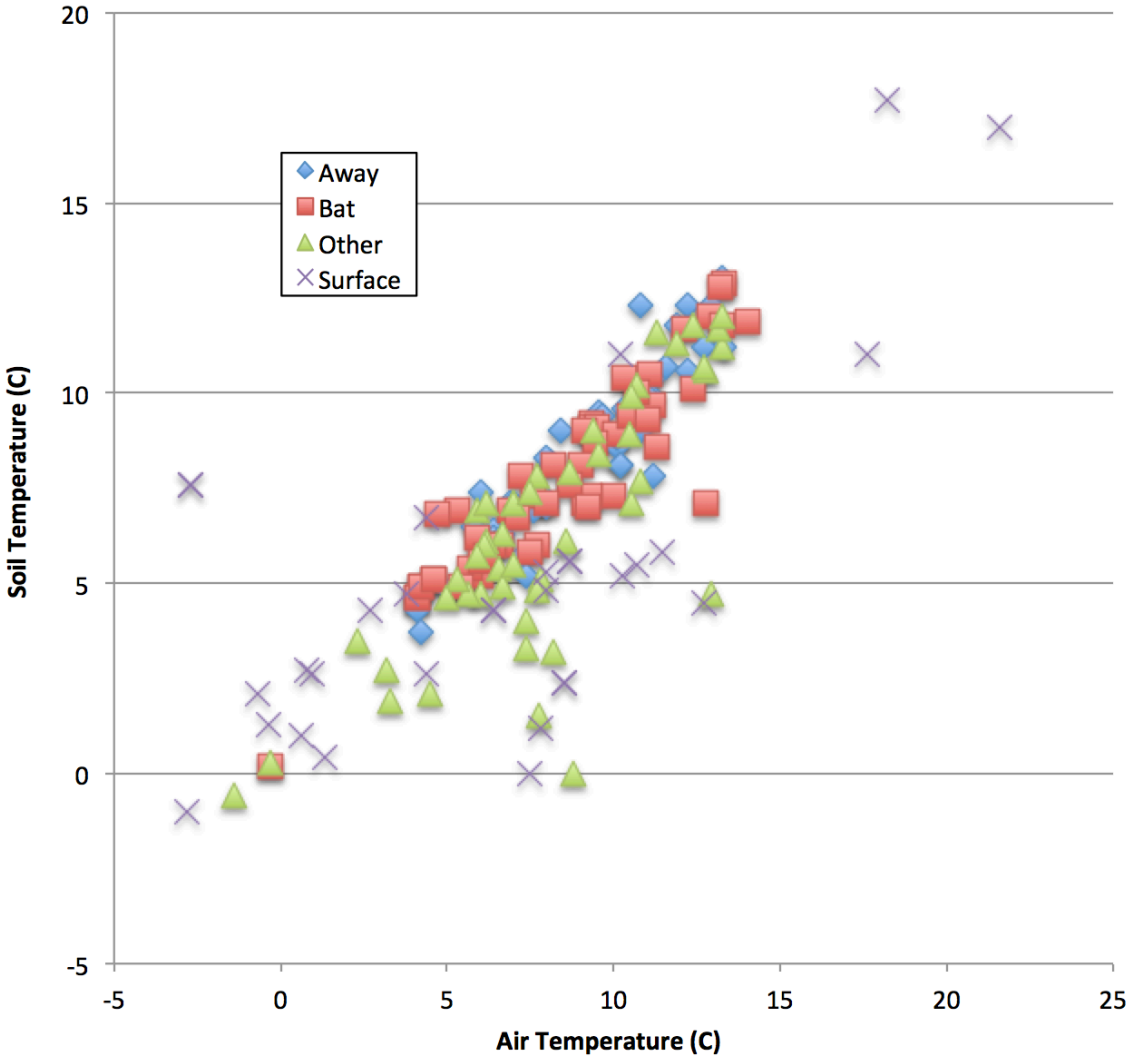

Figure 6. Soil and air temperatures at locations were sample data were collected in association with WNS research at Illinois hibernacula in the winters of 2011-2012 and 2012-2013. Symbols correspond to different sample types (see text).

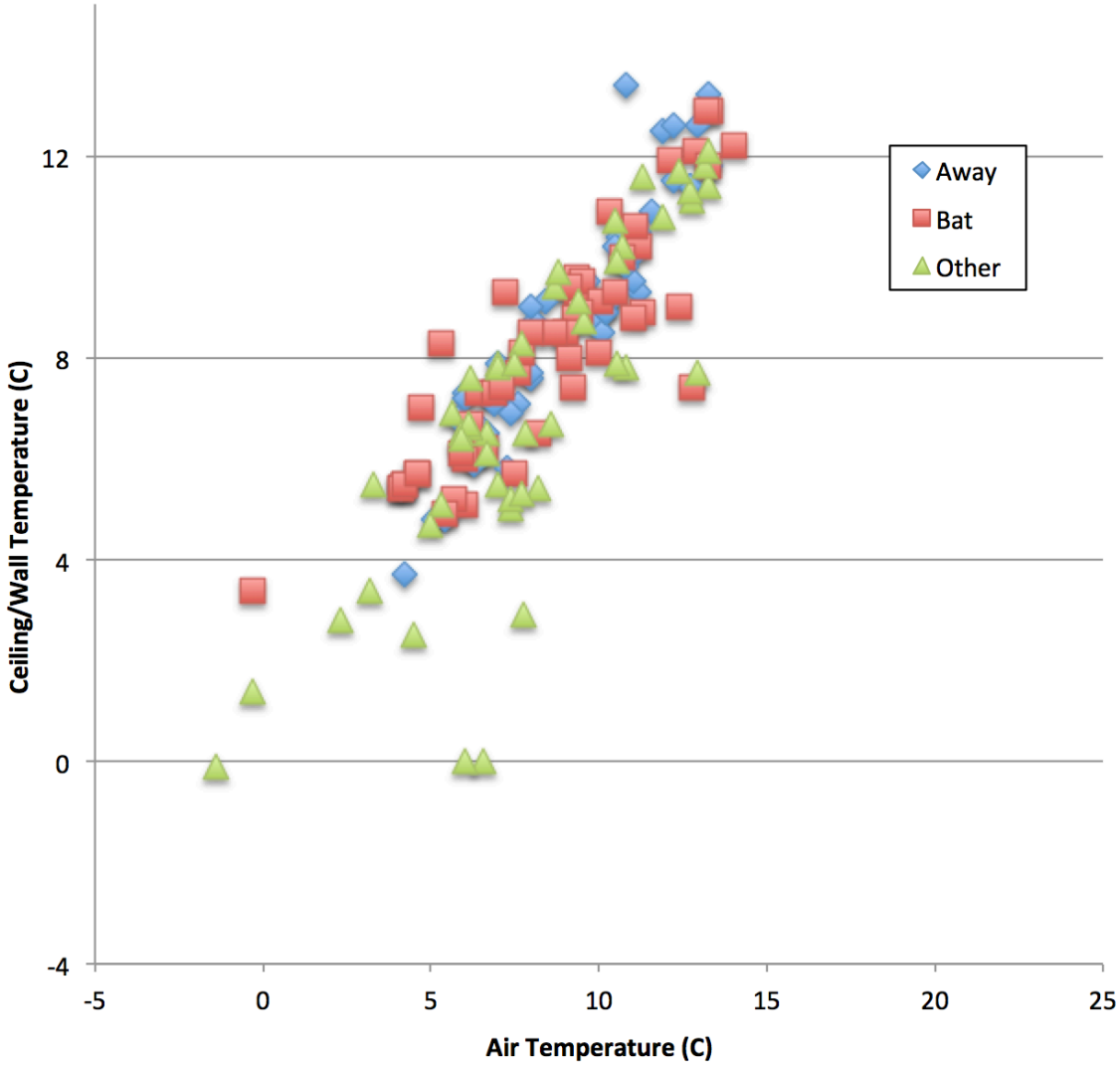

Figure 7. Ceiling/wall temperature and air temperatures at locations were sample data were collected in association with WNS research at Illinois hibernacula in the winters of 2011-2012 and 2012-2013. Symbols correspond to different sample types (see text).

Laboratory Analyses

Fungal Isolates and ITS Sequences

We successfully obtained 1,460 isolates in 2012. Of these, 566 were sequenced and 447 (79%) were successful. In 2013, we obtained 713 isolates and sequenced 599 of which 491 (82%) were successful. Since many of the isolates from 2012 were duplicates we decided to focus our efforts on isolating unique taxa in 2013. Although the number of isolates obtained in 2013 was about half of 2012, the number of successful sequences was similar. In addition, 3 and 109 isolates in 2012 and 2013, respectively, were identified by Dr. Alberto M. Stchigel based on morphology. All air, soil and swab controls were negative except for two putative fungal contaminates (*Oidiodendron* sp. and *Trichosporon* sp.) isolated from control swabs from Burton in 2012. There were 1,501 isolates that were successfully identified representing ~250 species. The number of isolates per cave/mine is shown for 2012 (Fig. 8) and 2013 (Fig. 9). Fungal isolates were from 31 orders (Table 3) and several genera were routinely isolated from most caves (Table 4). The length for the ITS region ranged from 332–961 and averaged 588 nucleotides (Fig. 10). Except in one isolate, members of *Pseudogymnoascus* always contained an intron in the ITS1 region and, thus, their ITS sequences ranged from 787–961 and averaged 882 nucleotides.

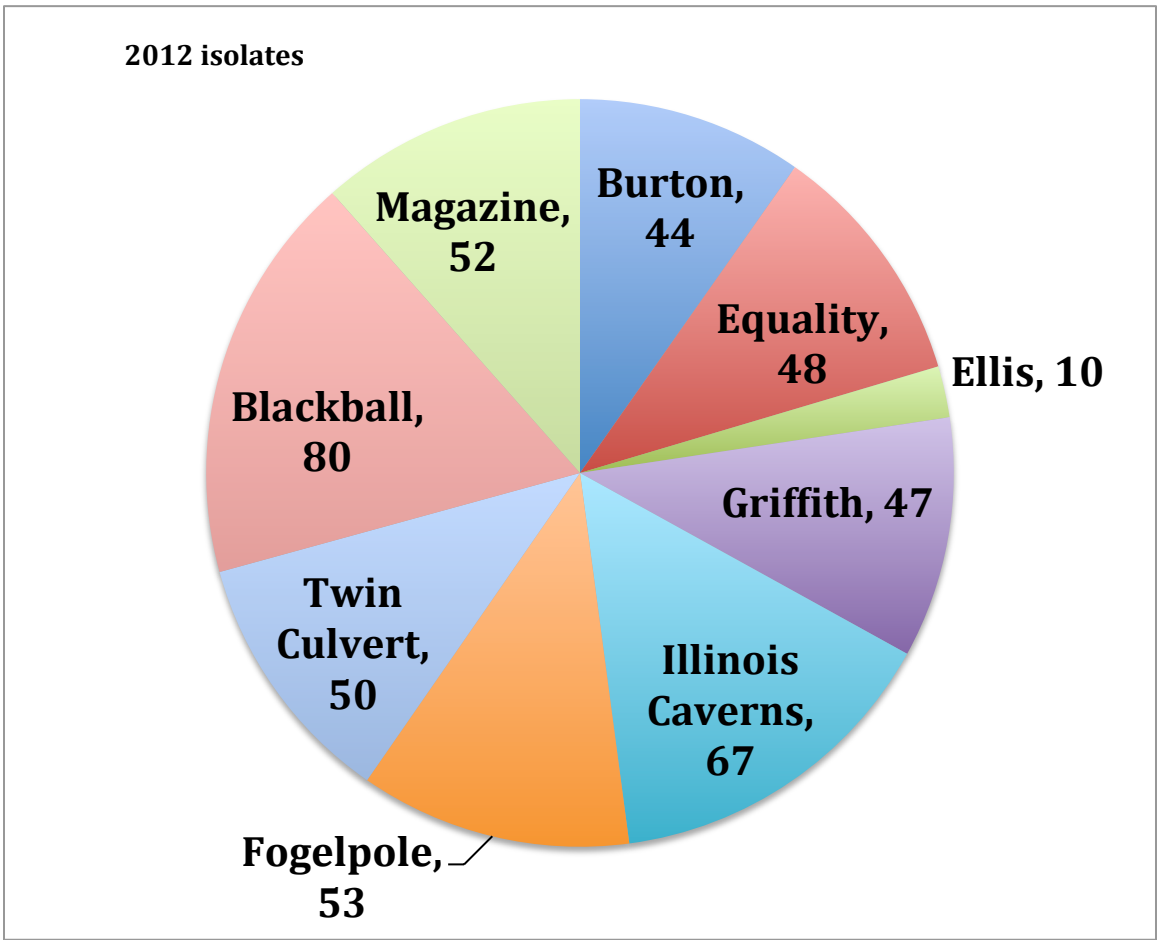

Figure 8. Number of isolates obtained from each location in 2012.

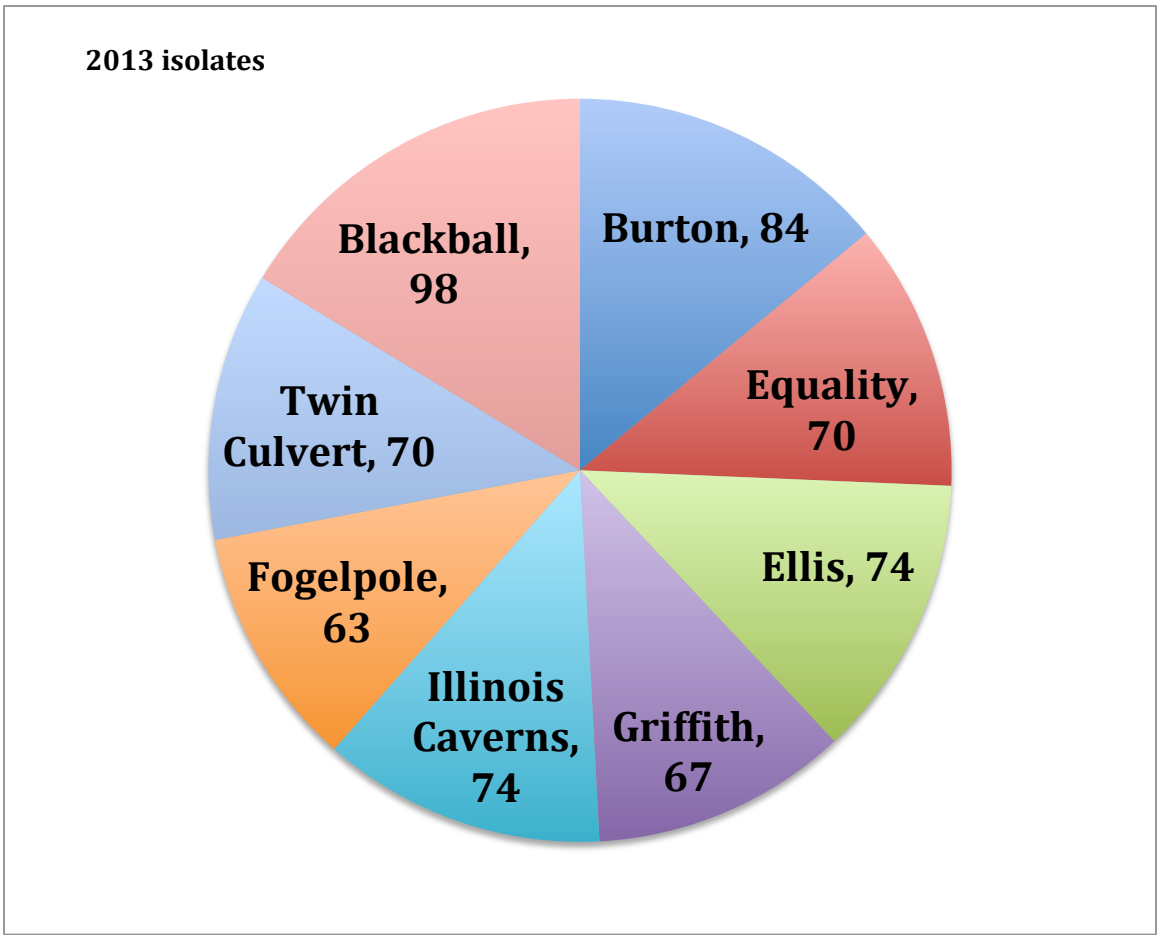

Figure 9. Number of isolates obtained from each location in 2013.

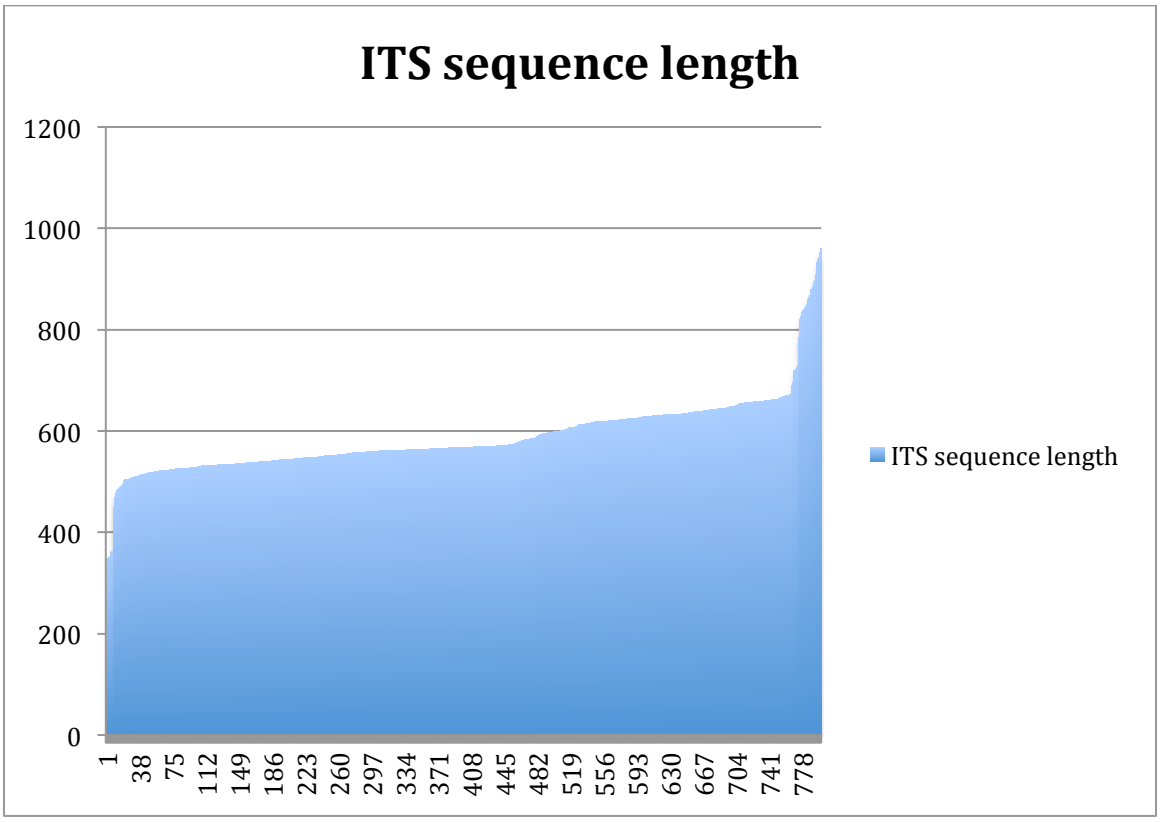

Figure 10. Sequence length variation in the ITS region for 799 sequences. Longer sequences displayed by *Pseudogymnoascus* spp. are shown along the right.

Pd Discovered

Pd was discovered for the first time in Illinois in 2013 in five locations (Blackball, Ellis, Fogelpole, Griffith, and Illinois Caverns). The fungus was isolated from bat swabs from Blackball and Illinois Caverns, a bat punch from Fogelpole, from bat guano from Griffith, and from a ceiling/wall swab at the hibernaculum from Ellis. A phylogenetic tree showing the evolutionary relationships of these five isolates along with the other 20 isolates of *Pseudogymnoascus* is shown in Figure 11.

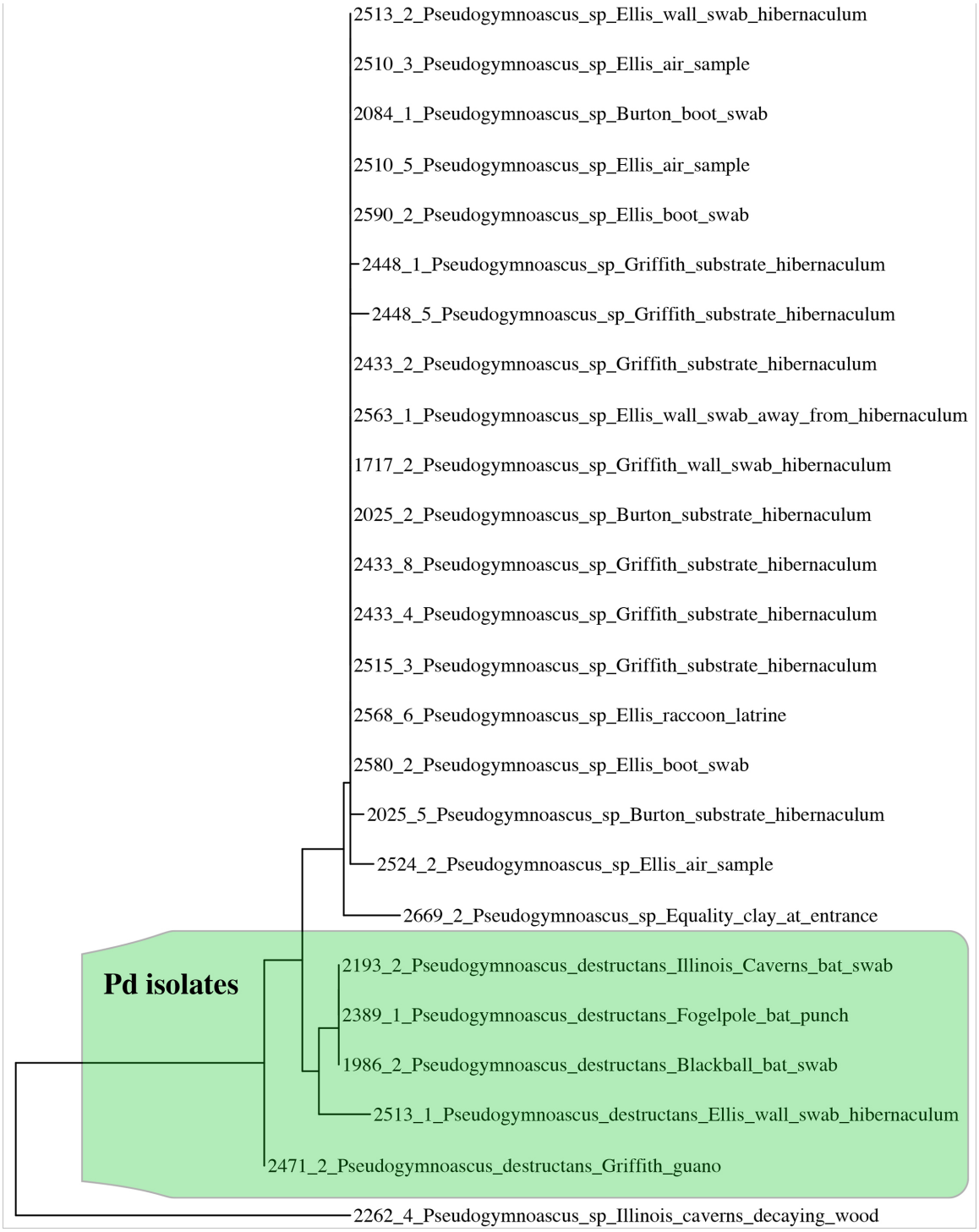

Figure 11. Maximum likelihood analysis of 25 ITS sequences of *Pseudogymnoascus* species from Illinois including five isolates of Pd. Taxon names include isolate number, cave/mine and location within cave/mine from where the sample was isolated.

Table 3. Fungal orders isolated from Illinois caves.

2012	2013
Agaricales	Acrospermales
Capnodiales	Agaricales
Coniochaetales	Capnodiales
Eurotiales	Cystofilobasidiales
Hypocreales	Diaporthales
Incertae sedis	Dothideales
Leucosporidiales	Eurotiales
Mortierellales	Helotiales
Mucorales	Hypocreales
Onygenales	Incertae sedis
Ophiostomatales	Lulworthiales
Pleosporales	Microascales
Polyporales	Mortierellales
Russulales	Mucorales
Saccharomycetales	Onygenales
Sordariales	Ophiostomatales
Tremellales	Peronosporales
Xylariales	Pezizales
	Pleosporales
	Polyporales
	Saccharomycetales
	Sordariales
	Sporidiobolales
	Thelebolales
	Tremellales
	Ustilaginales
	Venturiales
	Xylariales

Table 4. Common Illinois cave-associated fungal genera.

2012	2013
Chaetomium	Chaetomium
Cladosporium	Cladosporium
Geomyces	Doratomyces
Helicostylum	Fusarium
Mortierella	Geomyces
Mucor	Mortierella
Penicillium	Mucor
Pestalotiopsis	Oidiodendron
Trichosporon	Paecilomyces
	Penicillium
	Phoma
	Pseudogymnoascus
	Trichosporon

Microbial community composition

Within each cave, microbial community composition varied significantly between soils, ceiling/walls, and bat fur (permutational multivariate analysis of variance (perMANOVA) pseudo-F = 2.34, $p < 0.001$). However, microbial communities on the wings of hibernating bats were much more similar to those of cave walls/ceilings (i.e. bat roosts) than they were to soil communities (Figure 12).

Altogether, we generated over 40 million bacterial DNA sequences from cave soils. For most caves, the average per-sample richness of bacterial OTUs varied from 1419 OTUs per sample to 2018 OTUs per sample, except for Ellis Cave, which was unusually species-poor (Figure 13). Cave soil community composition varied significantly across caves (Figure 14, perMANOVA pseudo-F = 3.64, $p < 0.001$), years (Figure 14, perMANOVA pseudo-F = 1.88, $p = 0.005$), and locations within caves (Figure 15, perMANOVA pseudo-F = 3.48, $p < 0.001$). There was also a significant cave-by-year interaction (perMANOVA pseudo-F = 2.00, $p < 0.001$) and cave-by-location interaction (perMANOVA pseudo-F = 1.67, $p < 0.001$). Some of these interactions may relate to the arrival of *P. destructans*. For example, we detected *P. destructans* in Blackball mine for the first time in 2013. While the bacterial communities at the entrance of Blackball mine changed very little

from 2012 to 2013 (Figures 15-16), we found substantial change in the soil communities of interior cave locations (Figure 15). In these locations, the arrival of *P. destructans* coincided with an increase in the proportion of Proteobacteria and Acidobacteria, largely at the expense of Actinobacteria, Bacteroidetes, and Firmicutes (Figure 16). In contrast, in 2013 Actinobacteria and Firmicutes increased their relative abundances in the hibernaculum of Equality Cave, where *P. destructans* was absent (Figure 17).

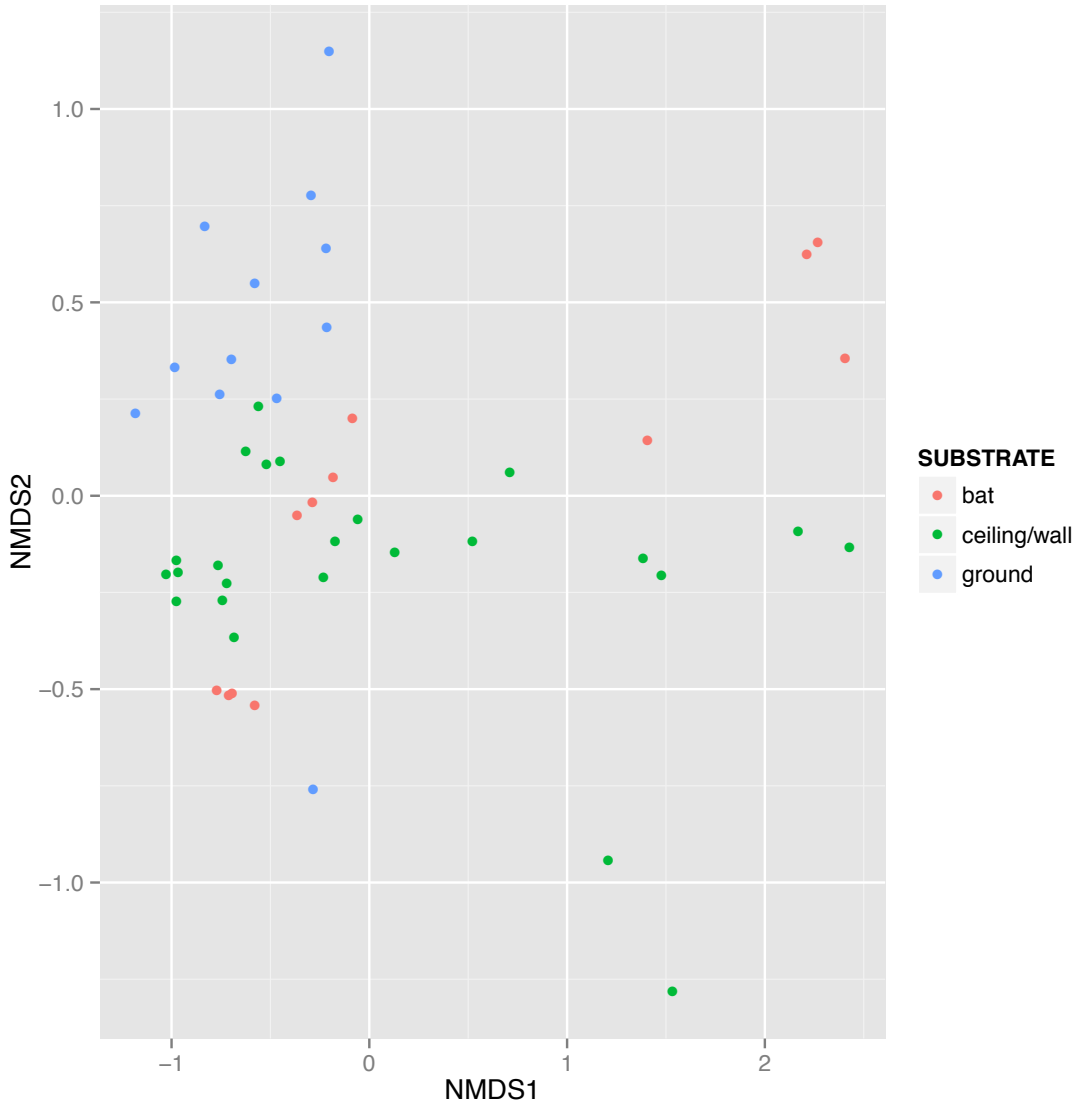

Figure 12. Nonmetric multidimensional scaling plot showing the variation of microbial communities by substrate type. Community data are from ARISA profiling of samples collected in Illinois Caverns in 2012.

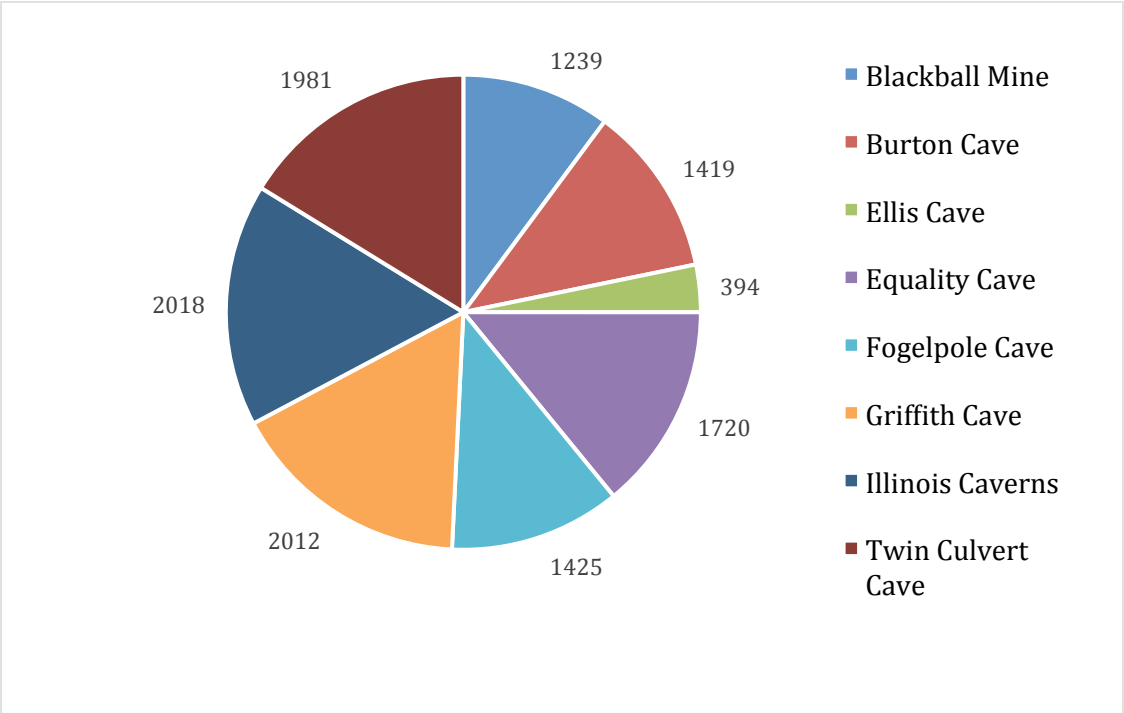

Figure 13. Average per sample richness of bacterial OTUs (operational taxonomic units) in each study cave. Data are based on high throughput DNA sequencing of cave soil samples obtained in 2012 and 2013.

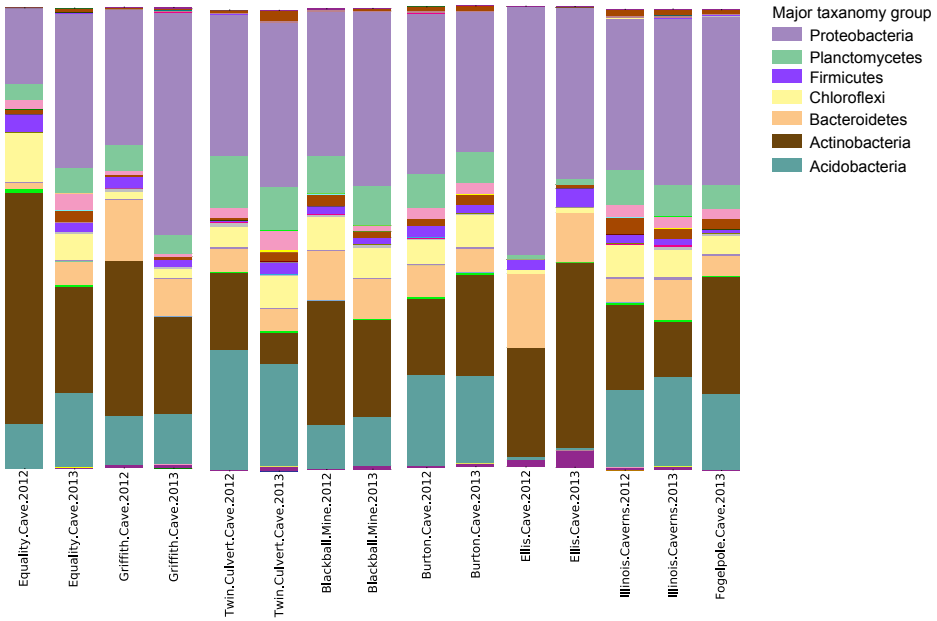

Figure 14. Phylum-level composition of bacterial communities varies between caves and across years. Data are based on high throughput DNA sequencing of cave soil samples obtained in 2012 and 2013.

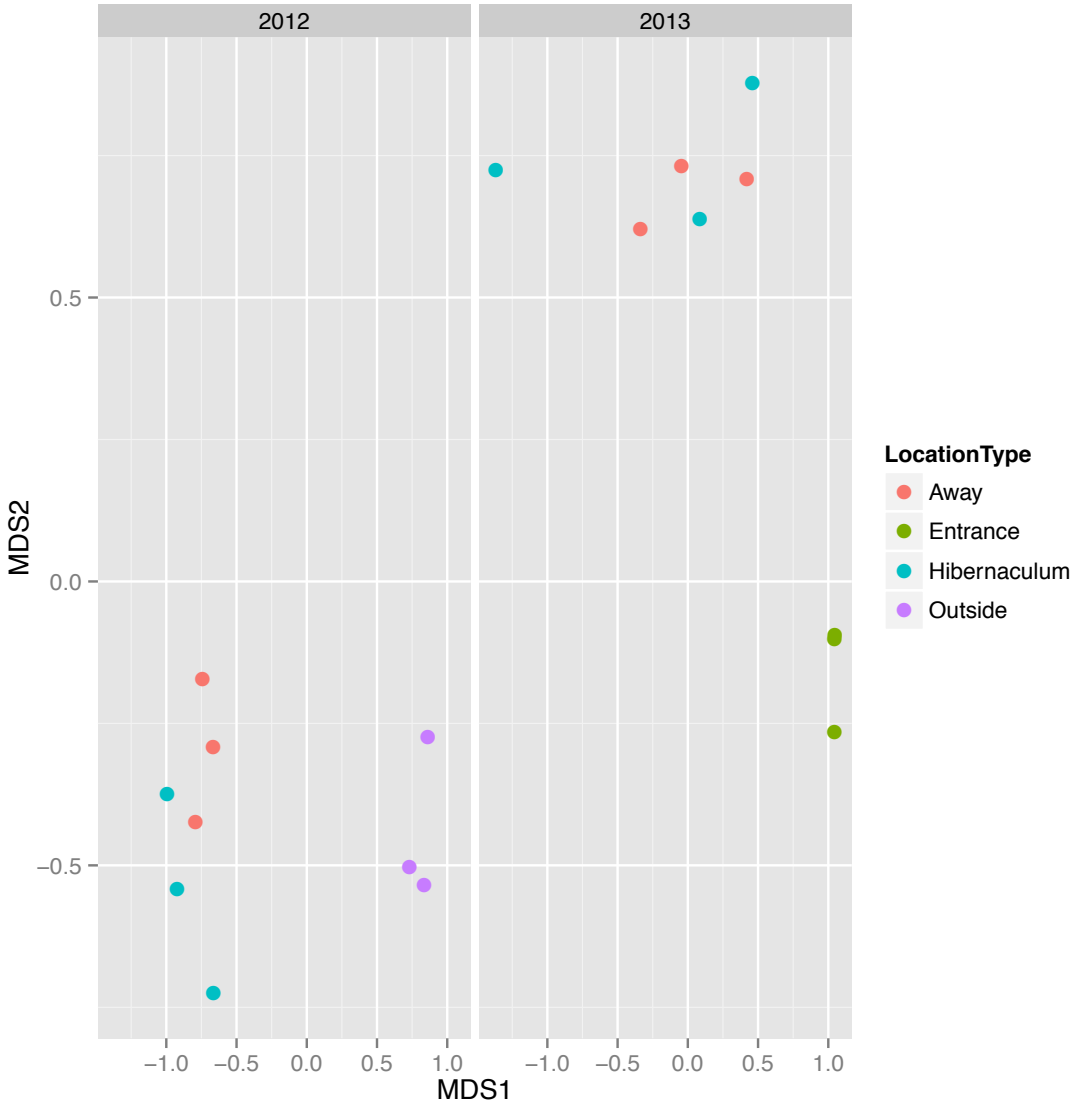

Figure 15. Nonmetric multidimensional scaling plot showing that bacterial community composition varies by location within cave and across years. While the composition of communities near the cave entrance remains relatively constant across years, the composition of interior locations changed substantially between 2012 and 2013, coincident with the arrival of *P. destructans*. Data are based on high throughput DNA sequencing of cave soil samples obtained in Blackball Mine in 2012 and 2013.

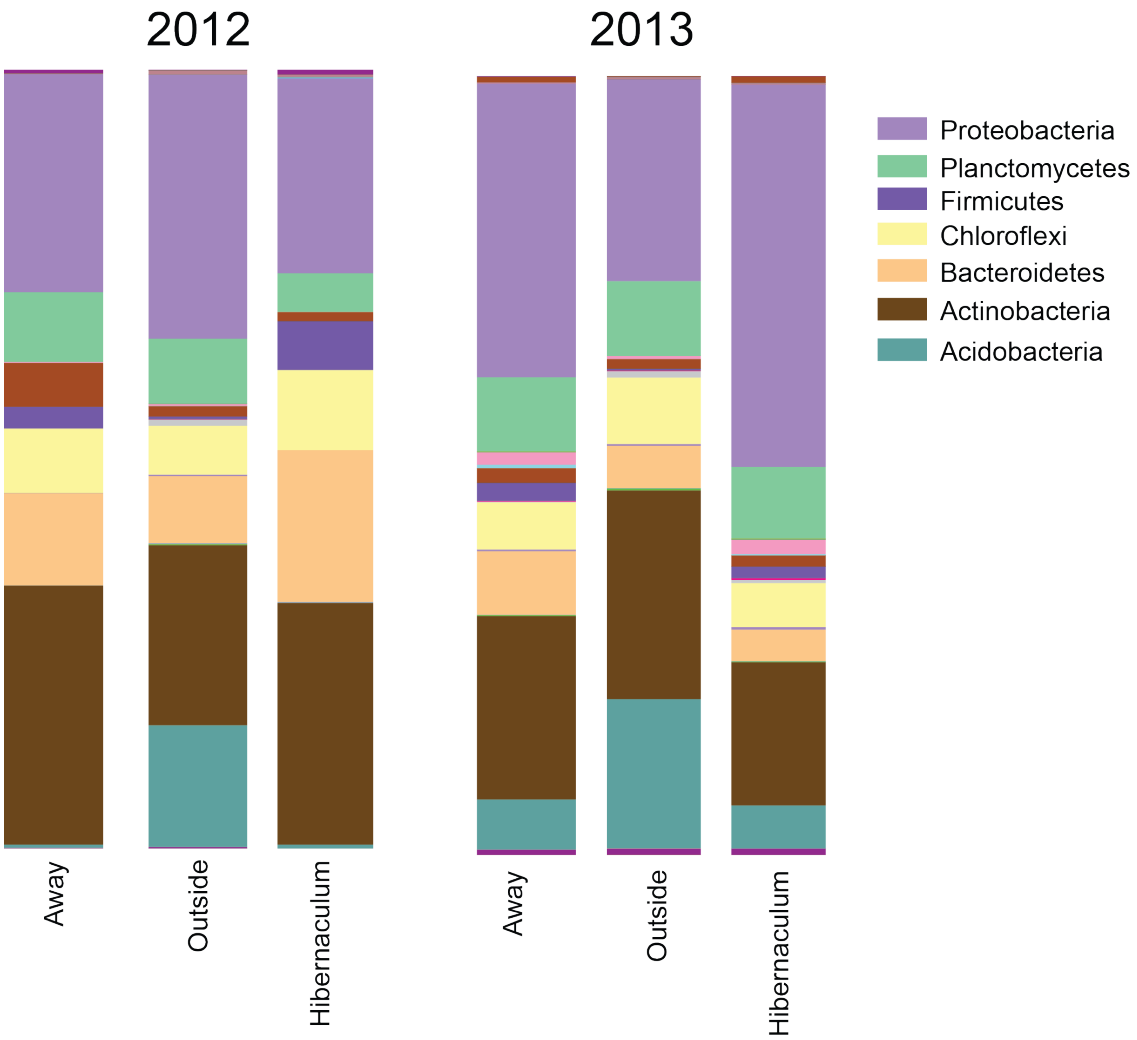

Figure 16. Phylum-level composition of bacterial communities varies between cave locations and across years. Data are based on high throughput DNA sequencing of cave soil samples obtained in Blackball Mine in 2012 and 2013 (*P. destructans* was detected in this cave in 2013).

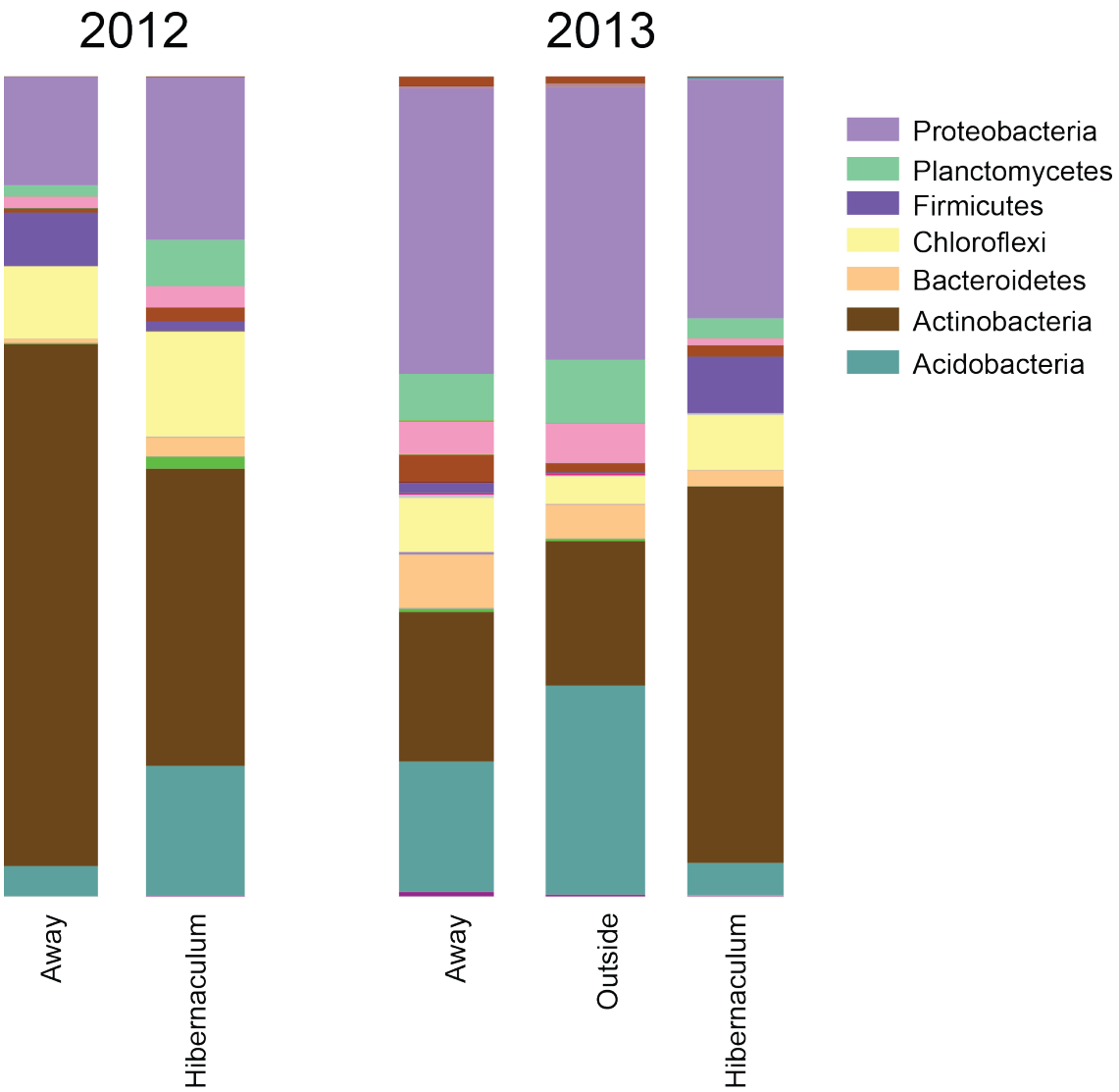

Figure 17. Phylum-level composition of bacterial communities varies between cave locations and across years. Data are based on high throughput DNA sequencing of cave soil samples obtained in Equality Cave in 2012 and 2013 (no *P. destructans* in either year).

Conclusions

Our research group documented the first detection of white-nose syndrome in Illinois bats and the first appearance of its causative agent, the fungus *Pseudogymnoascus destructans*, in Illinois caves. We obtained *P. destructans* isolates from bat swab and wing punch samples in Blackball Mine, Fogelpole Cave, and Illinois Caverns in 2013 (Figure 11). Importantly, we also obtained an isolate from a cave wall swab in Ellis Cave (Figure 11), a cave that had unusually species-poor microbial communities (Figure 13). We found that microbial communities on bat fur most closely resemble those of the surfaces on which they roost (Figure 12), suggesting that exchange of microbes between bats and cave surfaces is a potential avenue of spread for *P. destructans*. In addition, we documented changes in soil microbial communities that coincided with the arrival of *P. destructans* in several of our caves in 2013. These changes provide clues about the microbial ecology of *P. destructans* establishment in cave communities, and they may reveal antagonistic interactions that can be used to combat this fungus.

Acknowledgements

We thank Joseph A. Kath (Endangered Species Manager/Bat Specialist, Illinois Department of Natural Resources) for his commitment to this project, invaluable assistance with fieldwork, and coordination with landowners and agency personnel. Rod D. McClanahan (Wildlife Biologist, Shawnee National Forest) coordinated and assisted with site visits on lands managed by the USDA Forest Service, and was a valued member of the field team at these sites. Dr. Adam W. Stern (Veterinary Diagnostic Laboratory, UIUC College of Veterinary Medicine) was a vital member of our team, providing histological examination during necropsy and confirmation of WNS in the laboratory. Dr. Timothy Carter (Department of Biology, Ball State University) and Dr. Andrea Porras-Alfaro (Department of Biological Sciences, Western Illinois University) provided useful insights on approaches and techniques including field sampling and decontamination protocols. We also thank Rich Geboy (Midwest Regional WNS Coordinator, US Fish & Wildlife Service) and Jody Millar (Rock Island Field Office, Ecological Services, US Fish & Wildlife Service), for their contributions to our study design/approach and for shepherding our proposal & permits through the maze.

For assistance with fieldwork, we thank Dan Kirk (IDNR District Heritage Biologist), Stefanie Fitzsimons (IDNR Intern working with Dan Kirk), Dean Corgiat (IDNR District Heritage Biologist), Ray Geroff (intern for Angella Moorehouse, INPC Area 4 Natural Areas Preservation Specialist), Rod McClanahan (USFS Wildlife Biologist), Nick Hausen (WSIL-TV 3), Gary Slusher (Unimin Corporation).

Appendix 1. Field forms. A two-page form was used to record all data from each site visit. A 3 digit numbering system was assigned for individual bats, and a four digit numbering system was applied to samples.

WNS IL Field Data Page 1 of 2

Site_____Date_____
Lat/Long_____epe____Time IN____
Field Crew_____

Surface Meters: Light [ ] RH [ ] Air [ ] Ground [ ]

Bat 1 Species [ ] Sex [ ] Weight(g) [ ] Lesions? y / n Fungal Growth? y / n
[ ] Swab1-Right [ ] Swab3-Left [ ] Notes [ ]
Swab2-Right [ ] Swab4-Left [ ]
Punch5-Right [ ] Punch6-Left [ ] **Petri:** Air-7 [ ]
Ceiling/Wall: Swab-8 [ ] Swab-9 [ ] Swab-10 [ ] Swab-11 [ ]
Substrate: Soil-12 [ ] Soil-13 [ ] WhirlPack-14 [ ]
Meters: Light [ ] RH [ ] Air [ ] Ceiling/Wall [ ] Ground [ ]

Bat 2 Species [ ] Sex [ ] Weight(g) [ ] Lesions? y / n Fungal Growth? y / n
[ ] Swab15-Right [ ] Swab17-Left [ ] Notes [ ]
Swab16-Right [ ] Swab18-Left [ ]
Punch19-Right [ ] Punch20-Left [ ] **Petri:** Air-21 [ ]
Ceiling/Wall: Swab-22 [ ] Swab-23 [ ] Swab-24 [ ] Swab-25 [ ]
Substrate: Soil-26 [ ] Soil-27 [ ] WhirlPack-28 [ ]
Meters: Light [ ] RH [ ] Air [ ] Ceiling/Wall [ ] Ground [ ]

Bat 3 Species [ ] Sex [ ] Weight(g) [ ] Lesions? y / n Fungal Growth? y / n
[ ] Swab29-Right [ ] Swab31-Left [ ] Notes [ ]
Swab30-Right [ ] Swab32-Left [ ]
Punch33-Right [ ] Punch34-Left [ ] **Petri:** Air-CONTROL -35 [ ]
Ceiling/Wall: Swab-36 [ ] Swab-37 [ ] Swab-38 [ ] Swab-39 [ ]
Substrate: Soil-40 [ ] Soil-41 [ ] WhirlPack-42 [ ]
Meters: Light [ ] RH [ ] Air [ ] Ceiling/Wall [ ] Ground [ ]

Away From Bats 4 Notes [ ]
Ceiling/Wall: Swab-43 [ ] Swab-44 [ ] Swab-45 [ ] Swab-46 [ ]
Substrate: Soil-47 [ ] Soil-48 [ ] WhirlPack-49 [ ]
Meters: Light [ ] RH [ ] Air [ ] Ceiling/Wall [ ] Ground [ ]

Away From Bats 5 Notes [ ]
Ceiling/Wall: Swab-50 [ ] Swab-51 [ ] Swab-52 [ ] Swab-53 [ ]
Substrate: Soil-54 [ ] Soil-55 [ ] WhirlPack-56 [ ]
Meters: Light [ ] RH [ ] Air [ ] Ceiling/Wall [ ] Ground [ ]

Away From Bats 6 Notes [ ]
Ceiling/Wall: Swab-57 [ ] Swab-58 [ ] Swab-59 [ ] Swab-60 [ ]
Substrate: Soil-61 [ ] Soil-62 [ ] WhirlPack-63 [ ]
Meters: Light [ ] RH [ ] Air [ ] Ceiling/Wall [ ] Ground [ ]

WNS IL Field Data Page 2 of 2

Site_____Date_____

Other Subtrate 7 Description

Substrate: Soil-64 Soil-65 WhirlPack-66

Meters: Light RH Air Ceiling/Wall Ground

Other Subtrate 8 Description

Substrate: Soil-67 Soil-68 WhirlPack-69

Meters: Light RH Air Ceiling/Wall Ground

Other Subtrate 9 Description

Substrate: Soil-70 Soil-71 WhirlPack-72

Meters: Light RH Air Ceiling/Wall Ground

Other Dead & Dying (Euthanized) Bats-as samples:

to Vet-Med: Bat-10 Bat-11 Bat-12

Controls 13:

Soil-73 Soil-74 Swab-75 Swab-76

Entrance as Leaving, Boot Swabs:

Boot 14: Swab-77 Swab-78 Swab-79 Swab-80

Boot 15: Swab-81 Swab-82 Swab-83 Swab-84

Boot 16: Swab-85 Swab-86 Swab-87 Swab-88

Entrance as Leaving, Soil:

Soil 17: Soil-89 Soil-90 WhirlPack-91

Soil 18: Soil-92 Soil-93

Soil 19: Soil-94 Soil-95

Meters: Light RH Air Ground

Time OUT_____Notes_____

Appendix 2. Publications, presentations, and student training as a part of our study of WNS.

Publications:

Raudabaugh, D.B. and A.N. Miller. 2013. Nutritional capability of and substrate suitability forPseudogymnoascus destructans, the causal agent of bat White-Nose Syndrome. PLoS ONE 8(10): e78300. DOI: 10.1371/journal.pone.0078300

Johnson, L.J.A.N., A.N. Miller, R.A. McCleery, R. McClanahan, J.A. Kath, S. Lueschow, and A. Porres-Alfaro. 2013. Psychrophilic and psychrotolerant fungi on bats: Geomyces a common fungus on bat wings prior to the arrival of White Nose Syndrome. Applied and Environmental Microbiology 71: 5465–5471. DOI: 10.1128/AEM.01429-13

Presentations:

Raudabaugh, D.B. and A.N. Miller. 2014. Biotic and abiotic factors influencing the environmental persistence and growth of Pseudogymnoascus destructans. Abstracts of the Mycological Society of America Meeting. Published online. (Abstr.) Symposium talk Raudabaugh, D.B. and A.N. Miller. 2014. Biotic and abiotic factors influencing the environmental persistence and growth of Pseudogymnoascus destructans. Midwest Bat Working Group Meeting, Indiana State University, April 3, 2014.

Merritt, J.F., S.J. Taylor, E.J. Heske, J.A. Kath, R.D. McClanahan, A.N. Miller, D.B. Raudabaugh, A. Stern, N. Mateus-Pinilla, A.C. Yannarell, J. Foster, and W. Frick. 2014. Detection of WNS during initial invasion: agreement among survey methods. Midwest Bat Working Group Meeting, Indiana State University, April 3, 2014.

Raudabaugh, D.B., D.L. Lindner, A. Porras-Alfaro, and A.N. Miller. 2013. Nutritional capability and substrate suitability of psychrophilic species of Geomyces from the United States. page 119 in APS-MSA Joint Meeting, AUG 10-14, 2013 (abstract published JUN 2013). Phytopathology, 103(6).

Taylor, S.J., J.A. Kath, E.J. Heske (presenter), R.D. McClanahan, A.N. Miller, A. Stern, N. Mateus-Pinilla, A.C. Yannarell, D.B. Raudabaugh, and H.M. Lin. 2013. White-Nose Syndrome Confirmed in Illinois. Poster presentation. Annual meeting of the Illinois Chapter of The Wildlife Society, April 14-16, 2013, in Peoria, Illinois.

Johnson, L.J.A.N., A.N. Miller, R.A. McCleery, R. McClanahan, J.A. Kath, and A. Porras-Alfaro. Healthy bats harbor diverse Geomyces strains closely related to Geomyces desctructans, a recent bat pathogen. Central States Universities Inc. Conference, Argonne National Laboratory, October 26, 2012.

Taylor, Steven J., A.N. Miller, A.C. Yannarell, J.F. Merritt, N. Mateus-Pinilla, E.J. Heske, V.P. Hustad, H.M. Lin, J.A. Kath, and R.D. McClanahan. 2012. White-nose syndrome and Illinois bat hibernacula. Abstracts of the Mycological Society of America Meeting. p. 47. (Abstr.)

Kuldeep Singh (presenter), Nohra Mateus-Pinilla, Zoltan Demeter, Carol Maddox, Andrew N. Miller, Joseph A. Kath, Joseph F. Merritt, Edward J. Heske, Steve J. Taylor, Anthony C. Yannarell. 2012. Histology and fungal microscopic morphology in 4 Illinois bats. Poster Presentation. 2012 annual meeting Illinois Chapter of The Wildlife Society. (15-17 April 2012, Makanda, Ilinois).

Merritt, J.F., J.A. Kath, E.J. Heske (presenter), A.N. Miller, A.C. Yannarell, N. Mateus-Pinilla, V.P. Hustad, H.M. Lin, R.D. McClanahan, and S.J. Taylor. 2012. Current status of White-nose Syndrome research at Illinois bat hibernacula. Poster Presentation. 2012 annual meeting Illinois Chapter of The Wildlife Society. (15-17 April 2012, Makanda, Ilinois).

Merritt, J.F. (presenter), J.A. Kath, E.J. Heske, A.N. Miller, A.C. Yannarell, N. Mateus-Pinilla, V.P. Hustad, H.M. Lin, R.D. McClanahan, and S.J. Taylor. 2012. Current status of White-nose Syndrome research at Illinois bat hibernacula. Poster Presentation. 2012 Midwest Bat Working Group Meeting. (5-6 April 2012, Terra Haute, Indiana).

Students trained:

Dan Raudabaugh, M.S. student, University of Illinois Urbana-Champaign, Champaign, IL, 2012-2014 (fully funded on a Research Assistantship for MS)

Gretchen Anchor, independent research project, University of Illinois Urbana-Champaign, Champaign, IL, 2013-present.

Abraham Matlak, independent research project, University of Illinois Urbana-Champaign, Champaign, IL, 2013-present.

Shivali Patel, I-STEM high school technical assistant, University of Illinois Urbana-Champaign, Champaign, IL, May-July 2012.

Stephanie Nguyen, I-STEM high school technical assistant, University of Illinois Urbana-Champaign, Champaign, IL, May-July 2012.

Huei-Ming “Derrick” Lin, M.S. student, University of Illinois Urbana-Champaign, Champaign, IL, 2009-2012 (partially funded on a Research Assistantship).

Alyssa Beck, Ph.D. student, University of Illinois at Urbana-Champaign, Champaign, IL, 2012-present (partially funded on a Research Assistantship).

Scott Rysz, independent research project, University of Illinois at Urbana-Champaign, 2013.