January 2019

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International Journal of Speleology	48 (2)	191-202	Tampa, FL (USA)	May 2019

Available online at scholarcommons.usf.edu/ijs

International Journal of Speleology

Official Journal of Union Internationale de Spéléologie

Are microclimate conditions in El Malpais National Monument caves in New Mexico, USA suitable for *Pseudogymnoascus* growth?

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Abstract: White-nose syndrome (WNS) is a bat disease caused by the fungal pathogen *Pseudogymnoascus destructans*, which thrives in cold and very humid environments where bats frequently hibernate. Conidia of *Pseudogymnoascus* species are often documented on bats prior to the onset of WNS, but characterization of high-risk areas defined by microclimate cave conditions have been lacking. Investigating the occurrence of this fungal genus and appropriate environmental conditions to support *P. destructans* in southwestern U.S. caves is key to understanding the sites most likely to be impacted by WNS. Microclimate conditions in ten caves at El Malpais (ELMA) National Monument in New Mexico, USA were recorded using i-Button data loggers during the winters of 2011–2014 to assess appropriate environmental conditions (temperature and relative humidity) for *P. destructans* and other *Pseudogymnoascus* species. Optimal microclimate conditions for *P. destructans* and other psychrophilic fungi were found in all the caves with at least 50% of the caves identified as high-risk areas. *Pseudogymnoascus* species were detected in 70% of the caves using culturing methods and PCR, but no soil samples were positive for *P. destructans* using real-time PCR in soil and guano samples. *Pseudogymnoascus destructans* has a recognized range of appropriate temperatures and relative humidity for growth and cave microclimate can help define high-risk areas. This study offers resource managers guidance for establishing priority monitoring areas in their bat caves to determine which bat species are at higher risk.

Keywords: bats, cave microclimate, guano, *Pseudogymnoascus destructans*, white-nose syndrome

Received 10 February 2019; Revised 12 August 2019; Accepted 12 August 2019

Citation: Torres-Cruz T.J., Porras-Alfaro A., Caimi N.A., Nwabologu O., Strach E.W., Read K.J.H., Young J.M., Buecher D.C. and Northup D.E., 2019. Are microclimate conditions in El Malpais National Monument caves in New Mexico, USA suitable for *Pseudogymnoascus* growth? International Journal of Speleology, 48 (2), 191-202. Tampa, FL (USA) ISSN 0392-6672
https://doi.org/10.5038/1827-806X.48.2.2254

INTRODUCTION

White-nose syndrome (WNS) is an emergent fungal disease caused by *Pseudogymnoascus destructans* (Gargas et al., 2009; Minnis & Lindner, 2013). The disease was first documented in Howe Caverns (New York) on a few hibernating bats during the winter of 2006–2007 (Blehert et al., 2009). Subsequently, Lorch et al. (2011) and Warnecke et al. (2012) confirmed *P. destructans* as the causative agent of WNS. Since the first documented case, the disease has spread across eastern and midwestern North America. By 2019, it has been reported in bat hibernation sites in 33 U.S. states and seven Canadian provinces and the causative fungus has been found in three additional U.S. states (https://www.whitenosesyndrome.org/static-page/where-is-wns-now; Lorch et al., 2013). To date WNS has killed 5.5 to 6.7 million hibernating bats in the eastern half of North America (https://www.whitenosesyndrome.org/faq/how-many-bats-has-white-nose-syndrome-killed).

Pseudogymnoascus destructans is related to other psychrophilic (cold-adapted) fungi found in permafrost and grows between 3–16°C, showing optimal growth *in vitro* between 12.5–15.8°C (Verant et al., 2012). The fungus requires high relative humidity (>81%; Cryan et al., 2010; Foley, 2011; Marroquin et al., 2017) and has an upper critical temperature for growth between 19.0–19.8°C (Verant et al., 2012). Unfortunately, these conditions are also those preferred by many

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bat species in the family Vespertilionidae during winter hibernation (Flory et al., 2012). Cryan et al. (2013) suggested that temperature and relative humidity differences in cave environments could have major effects on which bat species are affected and the severity of the infection. Johnson et al. (2013) found that several psychrotolerant species of *Geomyces, Pseudogymnoascus,* and other fungi occurred on hibernating bats in caves in Illinois prior to the arrival of WNS. However, the distribution of these fungal genera at multiple sites within a cave and the characterization of specific conditions that can support *P. destructans* and other psychrophilic relatives have not yet been explored in southwestern U.S. caves (Vanderwolf et al., 2013a,b; Winter et al., 2017).

Scholars hypothesized that southwestern U.S. bats and their hibernacula would not be as severely impacted by WNS due to warmer regional temperatures and greater aridity in the region (Alves et al., 2014). Generally, cave temperatures are predicted to reflect mean annual surface temperature (MAST) of the surrounding area (Moore & Sullivan, 1997). However, area topography and cave passage geometry strongly affect cave microclimate and influence the location of suitable hibernation sites that may also provide appropriate conditions to support growth of *P. destructans* (Lewis, 1995; Perry, 2013). New Mexico caves that present appropriate conditions for bat hibernation are typically cold air traps, which are colder than the expected MAST and located in low points that collect runoff during storms (Buecher, 2011a). Basalt caves, such as those at El Malpais National Monument (ELMA; southwest of Grants, NM, USA), are generally situated in the bottom of canyons formed by the collapse of lava caves. These sites capture cold air and moisture during the winter months and the basalt insulates the passages from surface warming throughout the summer (Keszthelyi, 1995), making them cold and humid year-round. As a result, their internal temperatures are lower than predicted by MAST (ELMA MAST = 10.5°C), based on measurements taken outside ELMA 24. Thus, the surface topography combined with the geology and geometry of ELMA caves are different than the predicted environmental conditions for these caves, suggesting that they may be good habitats for *Pseudogymnoascus* spp.

Our goal was to measure microclimate conditions (temperature and relative humidity) within ten ELMA bat caves and determine areas with optimal microclimate conditions for the growth of *Pseudogymnoascus* species. We hypothesized that close relatives of *P. destructans* would be present in ELMA caves due to the ubiquitous distribution of this genus as saprophyte and psychrophilic fungi.

MATERIALS AND METHODS

Study site

El Malpais National Monument is managed by the National Park Service and is located in northwestern New Mexico (35°N 108°W, elevation ~2400 m.a.s.l.), just southwest of Interstate 40 and Grants, NM (Valentine-Darby et al., 2016). This monument preserves large tracts of land with some of the best geologic examples of volcanism in the U.S. There are many naturally formed lava caves at ELMA, from small single rooms to longer passages with multiple rooms. The vegetation in this high desert environment is a matrix of native short grass prairies, interspersed with cacti and herbaceous plants, and surrounded by pinyon-juniper, ponderosa pine, and other conifers. Because of the threat of WNS arriving in western caves, ELMA resource managers closed their caves to recreational caving on 6 December 2010, with a limited number of them reopened in 2013.

The ELMA caves chosen for microclimate logger deployment were selected for our study based on evidence of bat-use (e.g., bone material, guano, discarded moth wings and/or roosting bats) (Fig. 1). The basalt caves selected for this study had assorted passage geometry and a variety of microclimate conditions with the potential for providing temperatures

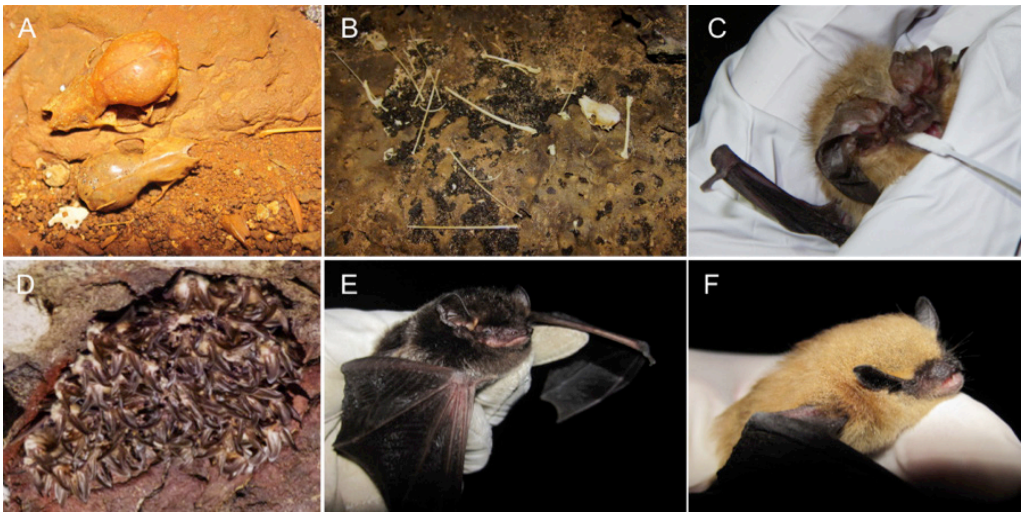

Fig. 1. Bat activity and main species found at El Malpais National Monument, New Mexico. A) Two bat skulls found in the back of ELMA 315 – differences in size and shape indicate that they belong to different bat species; B) Bat skeleton found in cave ELMA 12; C) Photo of sampling on a Townsend's big-eared bat *(Corynorhinus townsendii)*; D) Cluster of active Townsend's big-eared bats found in ELMA 62 in April 2012; E) Silver-haired bat *(Lasionycteris noctivagans)* found hibernating in ELMA 110 in March 2011; F) Small Myotis found near the back of ELMA 110 (forearm = 33 mm).

and relative humidity appropriate for multiple bat species (Tuttle & Stevenson, 1978; Perry, 2013). The microclimate study was conducted between January 2011 and April 2014. The original microclimate loggers were deployed on January 2011 into ten ELMA study caves. An initial winter census of ELMA cave bats was conducted on this trip to determine the bat species and numbers of bats that used the lava caves for hibernation. A few representative bats found during microclimate data logger maintenance were plucked from walls or crevices in the study caves just before or after hibernation for evaluation (Buecher, 2011b). Bats were identified to species, sex, reproductive status and age (when possible) using standard morphological characteristics (Hoffmeister, 1986). Standard measurements on bats were recorded, including forearm (mm) and body mass (g). Bats' wings, muzzle, ears and uropatagium were evaluated for any tissue damage (necrosis), lesions, scarring or skin mottling potentially caused by *P. destructans* (Reichard & Kunz, 2009; Cryan et al., 2010). Following evaluation, all bats were released on site.

Microclimate assessment

In order to reduce human impact and yet take multiple daily temperatures and relative humidity readings in the study caves, we deployed 26 Maxim (DS1923) iButton® Hygrochron temperature/humidity loggers with 8kb data-log memory (Table 1). An additional logger was deployed on the surface near ELMA 24 to measure mean area surface conditions. These loggers have sufficient memory for 4,096 16-bit readings and are programmable to sample at any rate (1 second to 273 hours), with a temperature resolution of 0.0625°C (11-bit) and a humidity resolution of 0.04% (12-bit) (https://www.maximintegrated.com/en.html). Willis et al. (2009) suggested that i-Buttons emit ultrasonic noise. However, prior to deployment we checked all loggers with three models of ultrasonic bat detectors and heard no noise from the equipment. Loggers were primarily placed in areas of the caves that showed evidence of seasonal bat presence. In addition, cave geometry conducive to higher relative humidity (i.e., not near entrances or skylights) and the complexity of passages in a given cave were also considered. Loggers were hung from wall projections, approximately two meters above the floor in inconspicuous places to prevent theft by human visitors. Loggers were not enclosed in any containers. The number of loggers per cave varied from one to four depending on the length and complexity of each cave (Table 1).

Loggers were programmed to sample every four hours at the highest resolution, allowing 2,048 measurements before the internal memory was filled (approximately 341 days). These loggers were chosen because they are small, could be deployed in areas where bats hibernate without negatively impacting the animals, and functioned an entire winter without additional maintenance. The loggers ran continuously between January 2011 and April 2014. Logger data were downloaded every April and in late October from 2011 through 2014. Only November – April data were analyzed to specifically assess microclimate conditions during the hibernation period for ELMA bats in our study caves (November-April), and are reported here. We used the statistical software package JMP 4.0 (JMP®, Version <x>, SAS Institute Inc., Cary, NC, 1989-2019) to analyze the logger data. For temperatures and relative humidity from each logger, we calculated: mean ± standard error, the 95% confidence interval, and tabulated the minimum and maximum temperatures and relative humidity (Table 1). To generate boxplots, microclimate data were read into the R statistical software package (R Core Team, 2015) as a tab delimited file. The boxplots were generated with ggplot2 (Wickham, 2009) using geom_boxplot and visualized within RStudio (RStudio Team, 2015). Studies reporting appropriate microclimate conditions for *P. destructans* are performed under stable conditions mainly in the laboratory, where temperature and relative humidity do not fluctuate significantly. Similar to other caves, microclimate in ELMA caves was variable. Therefore, we defined a set of conditions based on reported literature to determine caves at high-risk level for the introduction and active growth of *P. destructans*. At each logger site we established the criteria that a site must have appropriate temperatures (3–16°C) and relative

Table 1. Summary of microclimate data and bat species observed during cave visits to ten caves at El Malpais National Monument during the winters of 2011–2014 (November–April).

Cave name	Number loggers	Elevation / length (m)	MAST[4] (°C)	Mean temp. ±SE (°C)	Mean RH±SE (%)[5]	Conditions >50%[6]
ELMA 12[1]	4	2332/339	10.5	0.51 ± 0.04	89.6 ± 0.2	Yes
ELMA 24[1]	3	2292/1025	10.5	1.76 ± 0.08	94.7 ± 0.4	Yes
ELMA 54[1]	3	2220/360	10.5	3.17 ± 0.05	87.1 ± 0.2	Yes
ELMA 58[2]	1	2225/107	10.5	4.79 ± 0.08	57.6 ± 0.4	No
ELMA 62[1]	3	2230/610	10.5	3.10 ± 0.05	66.5 ± 0.2	No
ELMA 108[2]	2	2304/76	10.5	0.15 ± 0.06	69.0 ± 0.3	No
ELMA 110[3]	4	2286/1219	10.5	5.40 ± 0.04	67.4 ± 0.2	No
ELMA 144[2]	1	2220/80	10.5	5.95 ± 0.08	60.9 ± 0.4	No
ELMA 261[2]	2	2264/182	10.5	6.80 ± 0.06	99.5 ± 0.3	Yes
ELMA 315[2]	2	2256/91	10.5	4.73 ± 0.06	100 ± 0.3	Yes
Surface	1	2292/NA	10.5	10.5 ± 0.13	54.4 ± 0.4	No

[1]*Corynorhinus townsendii* is the primary bat species; [2]Usage by bats only in summer; [3]Other bat species use this site; [4]Mean annual surface temperature recorded at ELMA 24; [5]Relative humidity; [6]Seasonal (November-April) condition 50% of the time.

humidity (90–100% RH) ≥50% of the sampling period (November– April) to be considered at high risk for the growth of *P. destructans*, if introduced to the cave.

Sediment and guano sampling

We sampled sediments that consisted primarily of powdered basalt and bat guano at regular spatial intervals where availability of material allowed (2 to 12 samples/study cave), in the ten study caves during October and November 2010 (Table 2) under NPS collection permits ELMA-2011-SCI-0004, ELMA-2012-SCI-0001, ELMA-2013-SCI-0003, ELMA-2014-SCI-0008. Sampling areas were chosen where microclimate was appropriate for the fungi to survive (i.e., stable, higher relative humidity and appropriately cold temperatures) and/or areas that showed evidence of bat use by deposition of fresh guano. Approximately 25–40 cm^3 of soil or guano deposits were sampled at each site within a 0.3 m^2 area with sterile spoons and 50 cm^3 sterile Falcon tubes. Sterile sucrose lysis buffer (Giovannoni et al., 1990) was added to each tube on site to preserve the DNA present in the soil/guano samples, following procedures that we developed for other cave microbiological sampling studies (Northup et al., 2011; Lavoie et al., 2017). Following their return to the laboratory, samples were placed in a -80°C freezer until DNA extraction.

Table 2. Number of samples tested from each El Malpais National Monument study cave and number of samples positive for *Pseudogymnoascus* spp. in each cave.

Cave number	Number of soil samples	Number of soil samples positive for *Pseudogymnoascus* spp. (%)
ELMA 12	11	7(64)
ELMA 24	4	0
ELMA 54	9	5(56)
ELMA 58	4	0
ELMA 62	6	1(17)
ELMA 108	11	4(36)
ELMA 110	12	3(33)
ELMA 144	2	0
ELMA 261	9	7(77)
ELMA RGC	7	2(29)

Testing for presence of *Pseudogymnoascus* spp. in soil and guano

DNA was extracted from the soil/guano samples using the MoBio PowerSoil Kit DNA Isolation Kit (MoBio, Carlsbad, CA), following the manufacturer's protocol with the exception of using bead beating rather than vortexing. The extracted DNA was amplified by the polymerase chain reaction (PCR) using Promega's PCR Master Mix (2X; Promega, Madison, WI), 1% BSA, and primers designed specifically for the ribosomal RNA internal transcribed spacer region of *Pseudogymnoascus* spp. (Lorch et al., 2010). Our positive control for *P. destructans* was obtained from a direct wing punch from a bat infected with white-nose syndrome in Illinois, which we extracted and cloned to provide abundant positive control DNA (TW250, ITS region: KP902684, LSU: KP902702). The Lorch et al. (2010) primers were chosen since they effectively identify *Pseudogymnoascus* as a genus, but are not definitive for the presence of *P. destructans.* All samples that were positively identified as *Pseudogymnoascus* spp. using the Lorch et al. (2010) primers were then tested using the QuantiFast® Probe PCR Kit for real-time PCR (Qiagen, Venio, Netherlands), following the protocol in Muller et al. (2013). This test gives a definite yes or no for the presence of *P. destructans* in soil samples, defined as crossing the "threshold baseline within 40 cycles" (Muller et al., 2013).

Fungal isolation and identification

To culture fungi from guano/soils in Caves 12, 54, and 108 at ELMA in July and November 2014, we moistened sterile swabs with sterile water and rolled the swab in bat guano deposits, thus picking up several pieces of guano on the swab. The moistened sample swab was placed in a tube with 10 ml of sterile water and gently mixed. Serial dilutions were prepared (10^{-2} to 10^{-10}). One hundred microliters (0.1 ml) of dilutions (10^{-5} to 10^{-10}) were inoculated onto Sabouraud dextrose agar (SDA) (Lorch et al. 2013) supplemented with two antibiotics, streptomycin and tetracycline (50 mg/l), to inhibit bacterial growth. Two additional swabs were obtained in June 2014 by swabbing two surface-captured bats that had visible fungal colonies on their wings and inoculating the SDA plates described above directly with the swabs immediately after swabbing the bats. Inoculations were conducted on site under UNM IACUC 12-100835-MCC and NPS Protocol Number: IMR_ELMA_Northup_bats_2013.A2, and transported to the lab in a cooler. Cultures were incubated at 6°C, to target only psychrophilic fungi. Once cultures began to grow (1-4 weeks), isolates were screened for morphology and coloration indicative of possible *Pseudogymnoascus* spp. and candidates were sub-cultured on SDA.

Pure cultures were obtained on SDA and genomic DNA was extracted using the MoBio Ultraclean Microbial DNA Isolation Kit (QIAGEN, Germantown, MD) following the manufacturer's conditions. PCR was used to amplify the ITS1-5.8S-ITS2 nrRNA (ITS barcode) using primers ITS1 and ITS4 (White et al., 1990; Gardes & Bruns, 1993). Each 25 µl of PCR mixture contained 12.5 µl of PCR master mix (Promega, Madison, Wisconsin), 3 µl of 1% bovine serum albumin (BSA), 1 µl of each primer (5 µM), 5.5 µl of nuclease-free water and 2 µl of DNA. The

following PCR conditions were used: 95°C for 5 min; 30 cycles of 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 55 s; and a final extension at 72°C for 7 min. PCR products were checked using gel electrophoresis (1.2% agarose in Tris-acetate-EDTA buffer). Amplicons were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA) using the manufacturer's instructions, for later in-house sequencing with the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The reactions contained 3 µl of BigDye buffer, 0.5 µl BigDye, 0.5 µl primer, 2 µl H_2O, and 4 µl of template DNA. Sequencing in both directions was performed at the Molecular Biology Facility in the Biology Department at the University of New Mexico, using an AB3130 sequencer and the same PCR primers described previously. Sequences obtained were trimmed and edited using Sequencher (Gene Codes, Ann Arbor, MI). Closest relatives of isolates were determined using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST), UNITE (Kõljalg et al., 2013), and phylogenetic analysis (see below). Fungal sequences were deposited in GenBank under accession numbers KX610331–KX610369 and fungal isolates are housed in the Northup lab at the University of New Mexico.

Phylogenetic analysis

Sequences from Minnis & Lindner (2013) were used in addition to the ITS sequences generated in this study. Five sequences (KX610346, KX610343, KX610344, KX610345, and KX610354) from cultures (F282, F264, F268, F274, and F307, respectively) from Goat Cave, Carlsbad Caverns National Park under NPS collecting permit CAVE-2014-SCI-0013, collected in the same manner as described above, were included in the phylogenetic analysis due to their close relationship to the sequences in this study and *P. destructans*. Sequences were aligned using MUSCLE (Edgar, 2004) and trimmed using MEGA7 (Kumar et al., 2016). MEGA7 was used to find the best DNA model of evolution for the data set. Maximum-likelihood (ML) analysis was conducted under the Tamura-Nei substitution model. Bootstrap support was determined with 1000 replicates. Clades were considered significant and supported when the bootstrap was higher than 70%. *Pseudeurotium zonatum* was used as outgroup taxa, based on previous phylogenetic analyses (Sogonov et al., 2005; Wang et al., 2006a,b; Lorch et al., 2013; Minnis & Lindner, 2013).

RESULTS

Bats in ELMA caves

We observed 521 bats from five species (*Corynorhinus townsendii*, *Eptesicus fuscus*, *Lasionycteris noctivagans*, *Myotis ciliolabrum*, and *M. thysanodes*); with *C. townsendii* as the most commonly encountered bat species (Table 1). We found bats in deep torpor between January 2011 and April 2014, during nine visits into six of the ten ELMA caves (Fig. 1). Although the remaining four study caves showed evidence of bat-use, bats were not observed during our visits. Because ELMA caves 58, 62, 108, 110, and 144 had sizeable guano piles and discarded moth wings, these sites are more indicative of summer roosts. Although we only analyzed cave microclimate over the hibernation period (Table 1), we recorded temperature and relative humidity throughout the year during our study. The small SEs noted in Table 1 reveal that the year-to-year variation was small. Although our summer microclimate data were not analyzed, recent work suggests that summer bat roosts may also be implicated in the presence and possible spread of *P. destructans* spores (Carpenter et al., 2016; Ballmann et al., 2017).

Two surface caught bats had visible fungal colonies on their wings that were cultured as noted in the Methods (Fig. 4; Table S1). However, none of the observed cave-roosting bats showed any evidence of tissue damage (necrosis), lesions, scarring or skin mottling that are attributed to infection by *P. destructans* (Reichard & Kunz, 2009; Cryan et al., 2010). Most of the bats were roosting as solitary individuals, with only a few clusters of two or more bats (Fig. 1D). This lack of large clusters of hibernating bats differs from observations of the bat species most severely impacted by WNS in eastern U.S. hibernacula. Some eastern *Myotis* spp. (e.g., little brown bats) typically cluster in groups of 100–1000 animals on a cave ceiling (Thomson, 1982). However, these behaviors were not observed in the bats hibernating in ELMA caves.

Micrometeorological studies

I-Button loggers sampled temperature and relative humidity every four hours in ten study caves during the winter months of 2011–2014 (November-April), resulting in >19,440 temperature and humidity readings. A few i-Button data loggers malfunctioned after they were deployed in areas with near 100% relative humidity. A similar observation was documented by Vanderwolf et al. (2012), who found that Hobos are more reliable. Box-plots of readings for ten ELMA caves were generated for temperature and humidity (Fig. 2) and revealed diverse conditions from cave to cave. ELMA 12 showed the highest variability in temperature with minimum temperatures of -13.2°C and maximum temperatures of 4.2°C (Fig. 2), possibly due to its passage geometry, with an entrance at each end of the cave. Median temperature and relative humidity were higher for ELMA caves 54, 261, and 315 than for caves 12, 24, and 62 (Fig. 2).

Temperature and relative humidity varied greatly between caves and between sites within caves (summary by cave presented in Table 1). This variabilityin temperature and relative humidity (Kurta, 2014), makes it difficult to predict suitable conditions for the active growth and survival of *P. destructans* (e.g., Marroquin et al., 2017; Verant et al., 2012). Since previous studies have focused on *in vitro* experiments for *P. destructans* and not long-term evaluations in caves, we established high-risk criteria for caves that maintained appropriate temperatures (3–16°C) and relative humidity (90–100% RH) ≥50% during hibernation periods in ELMA caves (Table 1).

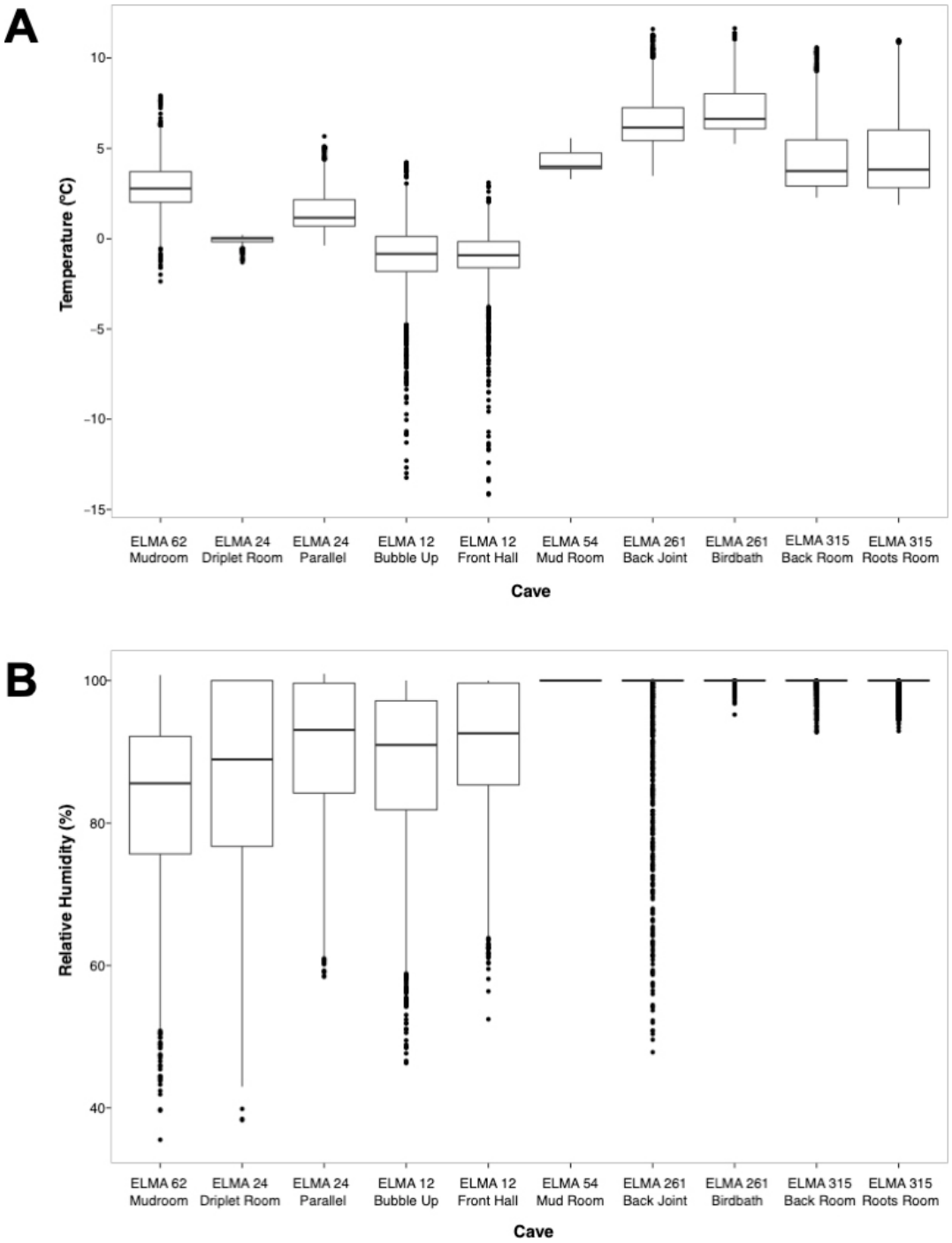

Fig. 2. Microclimate conditions in one or two locations (e.g. ELMA 12, Bubble Up, and ELMA 12, Front Hall) in six of the ten caves at El Malpais National Monument during the winters of 2011–2014 (November–April). A) Temperature; B) Relative Humidity.

Given those criteria, temperature and relative humidity conditions were appropriate to sustain *P. destructans* growth during at least half of all 4-hr periods in eight of the ten caves during winter 2011–2014.

Scatter plots for each logger were used to show how the data clustered through the winter and to better understand micrometeorological patterns in each cave (examples in Figs. 3B–C and S1B). These scatter plots illustrate which sites have microclimate conditions appropriate for the optimal growth of *P. destructans* based on Blehert et al. (2009). Environmental conditions inside of the same cave can vary, showing sites where microclimate is conducive to WNS >50% of the time (Fig. 3B) and sites where microclimate is not conducive to WNS >50% (Fig. 3C).

Our data illustrate that some sites had very stable microclimate conditions ideal to sustain the active growth of *P. destructans*, whereas sites located in areas with higher airflow resulted in more variable conditions. Because loggers were typically deployed in areas where there were indications of bat-use, further research in caves with larger bat hibernacula could help characterize microclimate conditions required by different bat species.

Presence of *Pseudogymnoascus destructans* in ELMA caves

Two to twelve soil/guano samples were collected from each of the ten ELMA study caves, depending on the size of the cave (Table 2). Additional samples were taken in some caves in which microclimate data

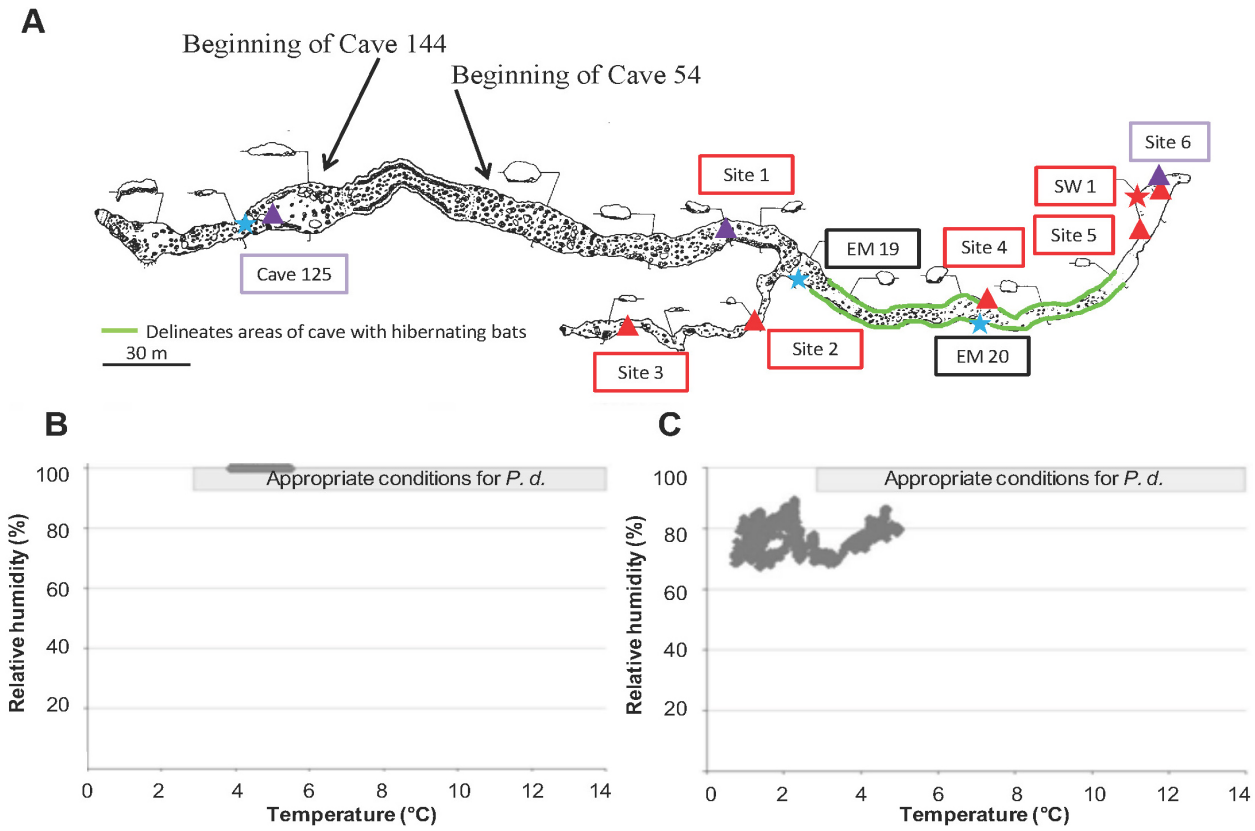

Fig. 3. Map and microclimate data for ELMA144/ELMA54 (formed along the same basalt trench). A) Map of ELMA144/54 showing data logger and soil sampling. Stars indicate data logger position, ★ (blue star) Data-logger where climate is not conducive to WNS >50% of the time, ★ (red star) Data-logger where climate is conducive to WNS >50% of the time. Triangles indicate soil sampling locations, ▲ (purple triangle) Soil sample with no indication of Pseudogymnoascus spp., ▲ (red triangle) Soil sample with presence of Pseudogymnoascus spp.; Site names in red boxes tested positive for Pseudogymnoascus spp. B) Microclimate scatter plot for ELMA 54 – Logger SW1 (where climate is conducive to WNS >50% of the season) for November 2011-April 2012. C). Microclimate scatter plot for ELMA 54 – Logger EM20 (where climate is not conducive to WNS >50% of the time) for November 2011-April 2012. Map source: El Malpais National Monument Personnel—personal communication.

suggested conditions suitable for the growth of *P. destructans*. Overall, 70% of the caves were positive for *Pseudogymnoascus* spp. (Table 2) using the Lorch ITS primers (Lorch et al., 2010) with a range of 17–77% positive soil samples per cave. However, all ten caves were negative for the presence of *P. destructans* when tested with the more specific real-time PCR test developed by Muller et al. (2013).

Despite ample evidence of bat activity (i.e., bat guano and insect debris) in caves 144 and 58, we detected no *Pseudogymnoascus* spp. We never observed bats hibernating in these short caves, suggesting only summer use by bats. However, Cave 24 is a significant bat hibernaculum that has areas with conditions suitable for the growth of *P. destructans*. Despite this, no *Pseudogymnoascus* spp. were detected. We hypothesized that fungal distribution, can be patchy and negative results may not be indicative of the absence of *Pseudogymnoascus* spp., but a simple sampling artifact if fungal abundance is low. Some caves, such as Cave 108, have limited bat activity, but contain small areas with more appropriate microclimate habitat for *P. destructans*. However, Cave 108 loggers did not reflect ideal growth conditions. This could be a result of the data loggers being placed high on the wall at the level where bats would be roosting, but not at soil level where samples were taken. Other caves, such as Caves 261 and 315, contain ideal growth conditions for *P. destructans*, but we rarely observed bats or bat evidence. Cave 54 contained ideal growth conditions for *P. destructans* and the largest number of hibernating bats. It was also the only site with several genetic clone sequences (ITS region) that were positive for *Pseudogymnoascus* spp. However, the samples were negative when tested with the more sensitive real-time PCR test developed by Muller et al. (2013).

Pseudogymnoascus isolates in ELMA caves

A total of 34 *Pseudogymnoascus* spp. isolates were obtained during this study from Cave 12 (Fig. S1; Table S1), Cave 54 (Fig. 3A; Table S1), and from two surface-caught bats with visible fungal colonies on their wings (Fig. 4; Table S1). Isolate sequences clustered with the *Pseudogymnoascus* clade using a maximum likelihood analysis (Fig. 4), but the resolution of the ITS rRNA region do not allow identification to the species level.

DISCUSSION

In this study, we measured microclimate conditions in ten ELMA caves to determine whether microclimate conditions suitable for *P. destructans* growth were present. We evaluated potential niches for *P. destructans* and determined the presence of *Pseudogymnoascus* species (Tables 1–2; Figs. 2–4). We did not detect *P. destructans*, the causal agent of WNS, in any of the ELMA study caves at the time of sampling and no evidence of WNS was detected on

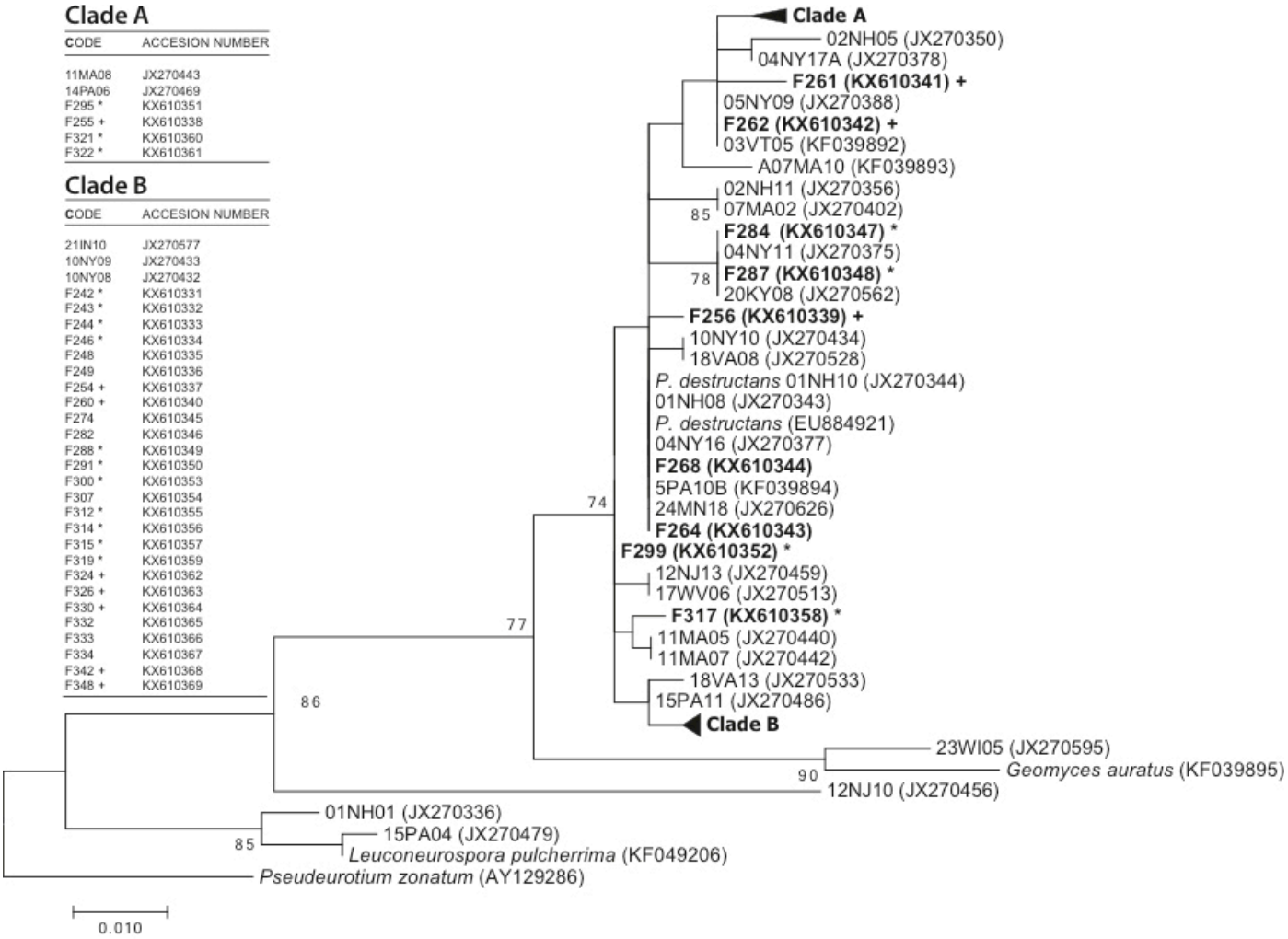

Fig. 4. Maximum Likelihood tree of *Pseudogymnoascus* spp. isolates. Taxa in bold represent cultures that were isolated in this study. Bootstrap values higher than 70% are shown next to the branches. Branch lengths represent the number of substitutions per site. *Pseudeurotium zonatum* was used as outgroup. Sequences marked with an asterisk (*) were recovered from cave ELMA54 and those marked with a plus sign (+) were isolated from cave ELMA12, the two caves from which cultured isolates were successfully obtained.

bats. Despite a common hypothesis that the hot and arid southwestern U.S. caves do not possess conditions conducive for the survival of *P. destructans* (Alves et al., 2014), our observations on temperature and relative humidity at some of ELMA's caves reveal that they certainly provide adequate, if not favorable habitat conditions for *P. destructans* to grow. Our microclimate results will help define areas at high risk in these caves that should be targeted for monitoring for the arrival of *P. destructans*.

ELMA caves provide suitable habitat conditions for *P. destructans*

Many of the basalt caves located in El Malpais National Monument are cold and humid, not reflecting the natural mean annual surface temperature of the region, nor the expected relative humidity (Table 1). Evaluation of cave microclimate in the majority of sites sampled confirmed that the topography and geology of ELMA caves provide the temperature and relative humidity conditions suitable for the growth of *P. destructans* and other close relatives in the genus. In eight of the ten caves monitored, conditions fall within the temperature range for the growth of *P. destructans* established in laboratory studies, for at least a portion of the winter season and 50% of the caves present high-risk conditions (Table 1). Although these *P. destructans* criteria may not accurately represent what will occur in ELMA caves, they give a guide for establishing caves in need of more careful monitoring. Other criteria, such as bat species potential susceptibility to WNS, will also need to be taken into consideration.

Although *C. townsendii* is the primary bat species found in ELMA caves, that genus has never been found with WNS in the eastern US. The same is true for *Lasionycteris noctivagans*, although both species have been documented with *P. destructans* spores (Bernard et al., 2015). Molecular detection of *P. destructans* spores on Rafinesque's big-eared bats in caves with dying bats of other species suggests that a lack of exposure is not the reason they do not get WNS. It also appears that *Eptesicus fuscus* has some tolerance to WNS (Frank et al., 2014), *Myotis ciliolabrum* has been found with *P. destructans* but not WNS, and the status of *Myotis thysanodes* is unknown. Therefore, susceptibility of bats to WNS cannot be predicted by microclimate alone, as bats roosting in the same microclimate conditions can have radically different outcomes to *P. destructans* infection (Davy et al., 2017; Moore et al., 2017). One aspect to consider is the potential role of the microbiome living on western bat species. Winter et al. (2017) found that microorganisms found across 13 different bat species

may have the potential to influence their host's health and to provide defenses against invading pathogens. Hathaway et al. (personal communication) have shown that *C. townsendii* have a very diverse bacterial microbiota, which may contribute to their apparent resistance to the disease.

Marroquin et al. (2017) determined optimum relative humidity levels for the vegetative growth and sporulation (i.e., conidia formation) of *P. destructans* (isolate MYA-4855) when growing at optimal temperatures (13°C). Their study revealed that both mycelial growth and conidiation was higher with increased relative humidity three weeks post-inoculation, with an optimal effect on growth at 81.5% relative humidity (tested 70.5–96.5%). Mycelial growth is impeded at relative humidity lower than 70% at 13°C, yet it does not impede conidia production, which can act as propagules for transmission. Verant et al. (2018) noted that more stable temperatures were associated with higher abundance and probability of detection of *P. destructans*. Thus, our criteria to define caves in which bats may be at high risk from *P. destructans* fungal growth may be highly conservative, assuming that *P. destructans* produce conidia that could survive and remain viable in ELMA caves when appropriate growth conditions are not met.

Bat behavior as a potential advantage in the prevention of WNS dispersal

None of the five bat species found hibernating in ELMA caves clustered in aggregations during the winter. In the eastern US some gregarious bat species have been severely impacted by WNS, implying that clustering behavior facilitates the transmission of spores (Langwig et al., 2012). However, not all gregarious bat species have been impacted (e.g., *M. grisescens*) and bats that roost as individuals have been affected by WNS (e.g., *Perimyotis subflavous*). Therefore, it is unknown if ELMA bats will gain an advantage by roosting more as solitary individuals. It is possible this low-density hibernation behavior may reduce the spread of spores if *P. destructans* is introduced into ELMA caves and elsewhere in the West. The quantification of known hibernacula microclimate conditions and clustering behavior during hibernation before WNS arrives in New Mexico may help estimate which bats species are at greatest risk from *P. destructans*. Little information is available about survival rates and active growth of *P. destructans* across different cave microclimates and seasons (Foley et al., 2011; Lorch et al., 2012; Verant et al., 2018). Understanding this is critical for knowing which sites and bat species will be at greatest risk from *P. destructans*. Furthermore, this knowledge can be applied to targeting our monitoring efforts in the southwestern U.S.

Much remains to be learned about the biology and distribution of *Pseudogymnascus* and other *Geomyces* spp. (Reeder et al., 2017; Verant et al., 2018). Hayes (2012) observed these fungi globally in both soils and marine environments and they are often known as psychrophilic (Shuey et al., 2014) and keratinophilic (degraders of keratin). Hayes (2012) also reported them in a wealth of cold environments, *P. panorum* (formerly *G. panorum)* being a common inhabitant in temperatures of -9 to -11°C. *Geomyces* and *Pseudogymnoascus* spp. are well adapted to cold environments with low availability of liquid water. For example, *P. panorum* has been found associated with the paint pigments of the famous paintings of Lascaux Cave in France (Bastian et al., 2009).

Wilson et al. (2017) compared the competitive ability between non-pathogenic *Pseudogymnoascus* spp. isolates and *P. destructans* to determine adaptations for survival in sediments of hibernacula; showing that non-pathogenic *Pseudogymnoascus* spp. isolates can grow faster, while also using a broader range of substrates with higher efficiency, which could explain the presence of abundant *Pseudogymnoascus* isolates obtained in this study. Additionally, Palmer et al. (2018) demonstrated that *P. destructans*, in comparison to its close relatives, has lost the key enzyme UVE1 that assists in DNA repair due to UV damage and has a great reduction in its carbohydrate utilizing enzymes. Thus, the finding of several *Pseudogymnoascus* spp. in ELMA caves with cold temperatures is not unexpected (Lorch et al., 2012). Reynolds et al. (2015) used cultivation and mathematical modeling to establish that *P. destructans have the capacity to survive* in the environment once introduced and can persist for decades. Our microclimate data and these studies suggest that once introduced to ELMA caves, *P. destructans* could survive and potentially infect bats in this area.

Fortunately, we did not detect *P. destructans* in the ten ELMA caves during the period of this study. The occurrence patterns of other *Pseudogymnoascus* spp. and the microclimate data will facilitate the selection of high-risk sites for monitoring the arrival of *P. destructans.* Some of the ELMA caves are open to the public during summer months. Because there is evidence that humans can be a vehicle of transmission of *P. destructans* spores (Ballmann et al., 2017), and because there is a misconception among some that southwestern U.S. caves are warm and dry, our results support continued monitoring in ELMA caves with human visitation.

ACKNOWLEDGEMENTS

The authors thank El Malpais National Monument (Kayci Cook, Dana Sullivan, Baker Holden, David Hays, and Rob Bastik) for the scientific collecting permits for this study and access to the study caves. The authors also thank Dr. Ara Winter for his help with the microclimate analyses. We acknowledge the funding provided by The Colorado Plateau Cooperative Ecosystem Study Unit (CPCESU) and El Malpais National Monument. We appreciate the help of Drs. Judy Bischoff and Todd Chaudhry, Research Coordinators, CPCESU and grant support staff at the University of New Mexico. We are grateful for the funding from the Cave Conservancy Foundation for the fungal cultivation that allowed us to identify the close relatives of *P. destructans.* We acknowledge technical support from the Molecular Biology Facility, which was supported by NIH grant number

P20RR018754. Research reported in this publication was supported in part by the National Institute of General Medical Sciences of the National Institutes of Health under award number P30 GM110907. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We thank the New Mexico Game and Fish Department for scientific collecting permit for handling New Mexico bats. We thank Dr. Ernie Valdez (USGS) for identification of bat skulls found in five of the ELMA study caves. Dr. Valdez also conducted surface netting, where we found two individual free-flying bats with fungal growth. Kenneth Ingham and Bob Buecher assisted with photo-documentation. We thank the two reviewers for their suggestions, which improved the manuscript.

This material is based upon work supported by the National Science Foundation (APA). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

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