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Psychrophilic and Psychrotolerant Fungi on Bats and the Presence of *Geomyces* spp. on Bat Wings Prior to the Arrival of White Nose Syndrome

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Since 2006, *Geomyces destructans*, the causative agent of white nose syndrome (WNS), has killed over 5.7 million bats in North America. The current hypothesis suggests that this novel fungus is an invasive species from Europe, but little is known about the diversity within the genus *Geomyces* and its distribution on bats in the United States. We documented the psychrophilic and psychrotolerant fungal flora of hibernating bats prior to the arrival of WNS using culture-based techniques. A total of 149 cultures, which were obtained from 30 bats in five bat hibernacula located in four caves and one mine, were sequenced for the entire internal transcribed spacer (ITS) nuclear ribosomal DNA (nrDNA) region. Approximately 53 operational taxonomic units (OTUs) at 97% similarity were recovered from bat wings, with the community dominated by fungi within the genera *Cladosporium*, *Fusarium*, *Geomyces*, *Mortierella*, *Penicillium*, and *Trichosporon*. Eleven *Geomyces* isolates were obtained and placed in at least seven distinct *Geomyces* clades based on maximum-likelihood phylogenetic analyses. Temperature experiments revealed that all *Geomyces* strains isolated are psychrotolerant, unlike *G. destructans*, which is a true psychrophile. Our results confirm that a large diversity of fungi, including several *Geomyces* isolates, occurs on bats prior to the arrival of WNS. Most of these isolates were obtained from damaged wings. Additional studies need to be conducted to determine potential ecological roles of these abundant *Geomyces* strains isolated from bats.

Large numbers of bats started dying in and around their winter hibernacula after the detection of white nose syndrome (WNS) in Albany, NY (1). The dead bats were characterized by wing lesions and mycelial growth on the bat's muzzle and body (2–4), and accordingly, this new, mysterious disease was named white nose syndrome (5). In subsequent years, WNS has devastated bat communities, spreading rapidly from Albany to 24 U.S. states and five Canadian provinces and resulting in the death of over 5.7 million bats. Once a hibernaculum becomes infected with the disease, bat mortality rates have ranged from 30 to 99% (5–8). In North America, multiple bat species have been affected, including *Eptesicus fuscus*, *Myotis leibii*, *Myotis lucifugus*, *Myotis septentrionalis*, *Myotis sodalis*, and *Perimyotis subflavus*, with no species showing complete immunity (4).

Bats are fundamental to ecosystems, and their abilities to suppress insect populations, pollinate crops, and disperse seeds are vital to the agricultural industry (9–11). Therefore, the spread of WNS jeopardizes agricultural production and ecosystem integrity, and it has become a major threat to once-common bat species in the United States (6, 12).

WNS is caused by a novel fungal species, *Geomyces destructans* (13, 14), but the origin of WNS is still uncertain. *Geomyces destructans* strains have been found on European bats without causing the infection or mortality levels seen in North American bats. Lorch et al. (15) showed a correlation between the distribution of *G. destructans* and WNS-positive sites in North America, and it has been suggested that this pathogen is likely of European origin and novel to North American bats (16–19).

Geomyces species are cosmopolitan and have been isolated from cave deposits, bats, and soils of cold climates such as Antarctic soils, Artic cryopegs, submarine soils, and Canadian *Sphagnum* bogs (15, 20–25). *Geomyces destructans* is considered a true psychrophile, with a growth temperature ranging from 3 to 20°C and no growth occurring at 24°C or higher (13, 26, 27). Optimal growth rates of other species of *Geomyces* are unknown. Apart from *G. destructans*, within this genus only *G. pannorum* is known to be pathogenic to mammals (28, 29).

With the high mortality caused by WNS in North America, much focus has been placed on *G. destructans*, but current literature is lacking a detailed analysis regarding the composition of psychrophilic fungal communities on healthy bats, including other potential *Geomyces* species present in bat hibernacula prior to the arrival of WNS. This is of vital importance because bats may act as reservoirs of fungi that are opportunistic pathogens or biological control agents that may have a role in the spread or control of WNS (25). Previous studies documenting fungal diversity in caves have focused mostly on cave soil and bat guano (15, 24, 30, 31) and rarely on the composition of mycobiomes on bats (32, 33). The main goal of this study was to document the psychrophilic fungal community on bats prior to the arrival of WNS.

TABLE 1 Average number of pure cultures isolated from wings by hibernaculum

Bat species	Hibernaculum	Avg (SD) no. of fungal morphotypes	No. of bats
M. sodalis	Burton Cave, IL	15	1[a]
P. subflavus	Twin Culvert Cave, IL	4.83 (2.04)	6
	Burton Cave, IL	7.25 (4.57)	4
	Mesmore Cave, IN[b]	2.2 (1.64)	5
M. septentrionalis	Siloam Spring State Park, IL	9.75 (1.49)	8
	Magazine Mine, IL	5.17 (4.92)	6
Total			30

[a] Only one bat was recovered for this particular species.
[b] Mesmore Cave was positive for WNS.

Growth rates of isolated *Geomyces* strains were also assessed through an experimental temperature study.

MATERIALS AND METHODS

Geomyces isolation methods were optimized in the laboratory before field sampling from 2010 to 2011. We sampled 30 bats from five hibernacula in Illinois and Indiana (Table 1). Illinois was an ideal location to study psychrophilic mycobiota associated with bats since the state was free of WNS until February 2013. Caves were monitored for 2 years for the presence of *G. destructans* to ensure that the samples were obtained from WNS-free bats. In addition to the isolation of psychrophilic fungi, we sampled 110 bats using the tape lift method, in which sticky fungal tape was pressed against the bat wings and nose to collect spores. The tape was then attached to a microscope slide and later observed under the microscope (http://www.nwhc.usgs.gov/disease_information/white-nose_syndrome/USGS_NWHC_Bat_WNS_submission_protocol.pdf). We also used direct PCR amplification on 74 wing biopsy specimens with *Geomyces*-specific primers as described by Lorch et al. (34) (PCR conditions are described below). All samples from Illinois were negative for *G. destructans*, and bat mortality associated with WNS was not detected in caves during sampling (data not shown).

Sample collection: wing swab sampling. A total of 25 bats were sampled from Burton Cave (BC), Magazine Mine (MM), Twin Culvert Cave (TC), and Siloam Spring State Park (SSSP) within Illinois during April and May 2010 (Table 1). An additional five *P. subflavus* wing swab samples were also obtained from Mesmore Cave, IN, during June 2011 (Table 1). WNS was confirmed in Indiana in 2011, but during sampling, WNS was suspected but not reported in this cave.

Bats were captured using a harp trap (collection permits TE06797A-0 USFW and 10-30S IDNR), and each bat was separately placed in a brown paper bag for temporary storage. Bats were kept in paper bags (with care taken not to exceed 30 min) until they were identified to species, sexed, measured (wing forearm), weighed, and observed for wing damage and given a wing damage index (WDI) score (3). Some of the bats showed a WDI of ≤2. Bats were next held delicately (while latex gloves were worn), and one of the wings was spread open. A 10-cm^2 section of bat wing was swabbed using a 15-cm sterile cotton swab (Pur-Wraps, Guilford, ME) dipped into sterile saline solution (0.9% NaCl solution). Caution was taken to avoid cross contamination by wearing different latex gloves for each bat sampled. Bats were then released after swabbing.

The inoculated cotton swab was swabbed immediately onto a potato dextrose agar (PDA) petri plate (100 by 15 mm) containing both 50 μg/ml of streptomycin and 50 μg/ml of tetracycline to deter bacterial contamination. The petri plate was then wrapped with Parafilm M (Pechiney Plastic Packaging Company, Chicago, IL) and stored on ice. For negative controls, a sterile PDA plate containing antibiotics (streptomycin and

tetracycline, 50 μg/ml each) was swabbed in the field with a sterile cotton swab that was dipped in 0.9% sterile saline solution, and then the plate was wrapped with Parafilm M. PDA medium was used because *G. destructans* did not show significant differences in growth on PDA, malt extract agar, or Sabouraud dextrose agar (SDA) (data not shown). Samples were incubated at 6°C for further analysis.

Petri plates from wing swabbed samples were observed every 3 days for the first 3 weeks and monitored for new fungal growth for at least 3 months. Unique morphospecies were isolated from each petri plate, and new isolates were transferred to PDA plates (60 by 15 mm). All cultures were stored at 6°C. Pure fungal cultures were stored in 1.5-ml microcentrifuge tubes containing 1 ml of sterile mineral oil and kept at 6°C for long-term storage. Duplicate cultures are also stored at the Center for Forest Mycology Research (CFMR) in Madison, WI.

DNA extraction, PCR, and sequencing. Fungal mycelium was collected from pure cultures isolated from wing swabs, and DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI). PCR was performed using the internal transcribed spacer (ITS) nuclear ribosomal DNA (nrDNA) fungus-specific primers ITS1F and ITS4 (35, 36). Each 25-μl PCR mixture contained 1 μl of each primer (5 μM), 6.5 μl of nuclease-free water, 12.5 μl of PCR Master Mix (Promega, Madison, WI), 3 μl of 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO), and 1 μl of DNA for each sample. PCR was run under the following conditions: 95°C for 5 min; 30 cycles of 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s; and a final extension at 72°C for 7 min. Quantification of DNA was done using gel electrophoresis (1% agarose in Tris-acetate-EDTA buffer) (37).

PCR products were cleaned with ExoSAP-IT (Affymetrix, Cleveland, OH) following the manufacturer's specifications. Sequencing was done using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing primers were the same as PCR primers, and samples were sequenced in both directions at the Biology Department at the University of New Mexico. Closest relatives of fungal isolates were determined using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST) and phylogenetic analysis (see below).

Fungal isolates that were identified within the *Geomyces* genus using BLAST analysis were also amplified using large-subunit (LSU) primers (LR0R and LR3) (38) and *MCM7* (a gene encoding minichromosome maintenance protein) primers MCM7-709 and MCM7-1348rev (39). PCR specifications were kept the same for ITS and LSU reactions. PCR specifications for *MCM7* were as follows: 94°C for 5 min; 40 cycles of 94°C for 45 s, annealing at 50°C for 50 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min.

Contigs of forward and reverse sequences were edited using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI). Analyses of operational taxonomic units (OTUs) were defined in Sequencher at 97% similarity with 40% overlap (40).

Phylogenetic analyses. In addition to the ITS sequences generated during this study, numerous other sequences from various *Geomyces* species were included in the analyses (13, 15, 22–24, 41–56). The majority of *Geomyces* sequences included in the tree were derived from taxa found in soil under bat hibernacula in the northeastern United States (15). Other sequences were from isolates from Antarctic mosses, Arctic cryopegs, Arctic soil, Austria, Bulgaria, China, and marine deposits.

ITS sequences were aligned using MUSCLE (multiple comparison by log expectation) (57). Gblocks 0.91b (58) was used to delimit and exclude ambiguous regions under the following parameters: the minimum length of a block was set to two, and allowed gap positions were set to half. Thirty-nine ambiguous regions consisting of 149 nucleotides (nt) were excluded by Gblocks. The final alignment consisted of 210 sequences and was 628 nt in length. The Akaike information criterion (AIC) (59) implemented in jModelTest 2.1.1 (60) was used to select the best model of evolution for the ITS data set. Maximum-likelihood (ML) analyses were conducted under the general time reversible (GTR) substitution model with six rate classes and invariable sites optimized. An unrooted BioNJ

TABLE 2 Numbers of fungal phyla isolated from various bat species collected in hibernacula from Illinois during spring 2010

Bat species	Hibernaculum	% of sequences (no. of isolates)		
		Ascomycota	Basidiomycota	Zygomycota
M. sodalis	Burton Cave	8 (8)	14 (3)	10 (2)
P. subflavus	Twin Culvert Cave	12 (13)	10 (2)	45 (9)
	Burton Cave	18 (19)	14 (3)	20 (4)
M. septentrionalis	Siloam Spring State Park	45 (48)	62 (13)	15 (3)
	Magazine Mine	17 (18)	0	10 (2)
Total no. of isolates		106	21	20

starting tree was constructed, and the best of nearest-neighbor interchange (NNI) and subtree pruning and regrafting (SPR) tree improvement was implemented during the heuristic search. Nonparametric bootstrap support (61) (BS) was determined with 10,000 replicates. Clades were considered significant and highly supported when BS ≥70% (62).

Bayesian inference employing a Markov chain Monte Carlo (MCMC) algorithm was performed using MrBayes v. 3.1.2 (63) on the CIPRES Science Gateway Teragrid (64) as an additional means of branch support. The GTR+I+G model with six rate classes was employed. Four independent chains were run for 100 million generations and sampled every 1,000th generation to ensure that trees were not trapped in local optima. Tracer v. 1.5 (65) was used to visualize stationarity and to estimate burn-in, which was determined to be the first 30,000,000 generations (= first 30,000 trees). The consensus trees were visualized in PAUP 4.0b10 (66). Clades with a Bayesian posterior probability (BPP) of ≥95% were considered significant and highly supported (67).

Evaluation of growth rates of *Geomyces* isolates. To further characterize *Geomyces* isolates, growth rates were evaluated on SDA medium for 4 weeks. Three replicates per isolate were incubated at 6°C and 25°C. The diameter of each colony was measured weekly. Results reported are expressed as means ± standard errors.

Nucleotide sequence accession numbers. Sequences were deposited in GenBank under accession numbers KF212195 to KF212335 for ITS, KF212336 to KF212357 for LSU, and KF212358 to KF212373 for *MCM7* primers.

RESULTS

A total of 182 fungal isolates were cultured from 30 bats in all five hibernacula, and 149 isolates were successfully sequenced using ITS primers. Five of the isolates did not amplify with ITS primers, and the remaining 28 cultures were discarded because they showed signs of contamination. WNS free bats from Illinois showed a more diverse fungal community in comparison with WNS positive bats from Indiana (Table 1; see Fig. S1 in the supplemental material). Fungal communities on bats in the WNS-positive bat hibernacula in Indiana were dominated by *G. destructans*. Plates from Illinois had an average of 7.28 ± 4.04 (standard deviation [SD]) fungal morphotypes, compared to 2.2 ± 1.64 for those from Indiana (Table 1) (Mann-Whitney U test, $P < 0.05$).

All cultures were initially sequenced using ITS primers, but five cultures in the phylum Zygomycota could not be amplified using ITS primers (ITS1F, ITS1, and ITS4), so they were later sequenced using LSU primers. At 97% similarity 53 OTUs were identified. Ascomycota was the dominant phylum with 73% of the sequences, followed by Basidiomycota (14%) and Zygomycota (13%) (Table 2). At least eight classes and 42 genera of fungi were found based on closest fully identified species from BLAST matches (Table 3).

TABLE 3 Percentages of fungi isolated from bats based on preliminary taxonomy from closest BLAST searches

Class, order, and genus	% (no.) of isolates
Sordariomycetes	23 (34)
Hypocreales	11 (16)
Fusarium	4.8 (7)
Hypocrea	2.0 (3)
Paecilomyces	1.4 (2)
Acremonium	0.68 (1)
Gibberella	0.68 (1)
Hypomyces	0.68 (1)
Verticillium	0.68 (1)
Sordariales	4.1 (6)
Chaetomium	2.0 (3)
Trichocladium	2.0 (3)
Xylariales	2.7 (4)
Pestalotiopsis	2.0 (3)
Seiridium	0.68 (1)
Incertae sedis	1.4 (2)
Plectosphaerella	1.4 (2)
Microascales	1.4 (2)
Doratomyces	0.68 (1)
Kernia	0.68 (1)
Ophiostomatales	1.4 (2)
Ophiostoma	0.68 (1)
Sporothrix	0.68 (1)
Sordariomycetidae incertae sedis	1.4 (2)
Arthrinium	1.4 (2)
Dothideomycetes	20 (30)
Capnodiales	12 (17)
Cladosporium	9.5 (14)
Davidiella	2.0 (3)
Pleosporales	7.5 (11)
Phaeosphaeria	2.0 (3)
Alternaria	1.4 (2)
Phoma	1.4 (2)
Arthopyreniaceae sp.	0.68 (1)
Leptosphaeria	0.68 (1)
Leptosphaerulina	0.68 (1)
Paraphoma	0.68 (1)
Dothideales	1.4 (2)
Aureobasidium	1.4 (2)
Leotiomycetes	16 (23)
Helotiales	14 (20)
Geomyces	8.8 (13)
Oidiodendron	2.0 (3)
Phialocephala	1.4 (2)
Uncultured *Oidiodendron*	1.4 (2)
Incertae sedis	0.68 (1)
Leotiomycetes sp.	0.68 (1)
Leotiomycetes incertae sedis	0.68 (1)
Uncultured *Leotiomycetes* clone	0.68 (1)
Thelebolales	0.68 (1)
Thelebolus	0.68 (1)
Incertae sedis	14 (20)
Mortierellales	8.8 (13)
Mortierella	8.8 (13)
Mucorales	4.8 (7)
Helicostylum	2.0 (3)
Mucor	2.0 (3)
Ambomucor	0.68 (1)
Eurotiomycetes	13 (19)
Eurotiales	13 (19)
Penicillum	13 (19)
Tremellomycetes	13 (19)
Tremellales	8.2 (12)
Trichosporon	8.1 (12)
Cystofilobasidiales	4.8 (7)
Guehomyces	4.8 (7)
Agaricomycetes	1.4 (2)
Polyporales	1.4 (2)
Bjerkandera	1.4 (2)
Mitosporic Ascomycota	0.68 (1)
Incertae sedis	0.68 (1)
Trichocladium	0.68 (1)
Saccharomycetes	0.68 (1)
Saccharomycetales	0.68 (1)
Galactomyces	0.68 (1)

Among the Ascomycota, the most frequent classes isolated were Sordariomycetes (23%), Dothideomycetes (20%), Leotiomycetes (16%), and Eurotiomycetes (13%) (Table 3). Other isolated classes of Ascomycota with ≤1% were Saccharomycetes and an unclassified mitosporic species. Only one class of Basidiomycota was frequently isolated, the Tremellomycetes (13%), although two additional isolates were obtained from Agaricomycetes within the Basidiomycota.

Common fungal orders isolated within Ascomycota included Helotiales (14%), Eurotiales (13%), Capnodiales (12%), and Hypocreales (11%) (Table 3). Other common isolated fungal orders included Cystofilobasidiales, Tremellales, Mortierellales, Mucorales, Pleosporales, Sordariales, and Xylariales (Table 3). *Cladosporium*, *Geomyces*, *Mortierella*, *Penicillium*, and *Trichosporon* were the most commonly isolated genera (Table 3; see Table S1 in the supplemental material).

Basidiomycota represented 14% (21 sequences) of sequenced isolates, including fungi within Cystofilobasidiales and Polyporales (Tables 2 and 3). Cystofilobasidiales were dominated by *Trichosporon*. Fungi in the Polyporales were closely related to a fungus in the genus *Bjerkandera*.

The phylum Zygomycota represented 13% (20 sequences) and contained the common orders Mortierellales and Mucorales, which include the genera *Mortierella* and *Mucor* (Table 3; see Table S1 in the supplemental material). *Mortierella* species were frequently isolated (8.8% of total isolates sequenced; 13 isolates). Genera closely related to *Helicostylum* did not amplify using ITS nrDNA primers, so they were sequenced with LSU primers.

A total of 11 *Geomyces* cultures (not including *G. destructans* from WNS-positive bats) were isolated (7.4%). *Geomyces* was isolated from all bat species surveyed: eight from *M. septentrionalis*, two from *M. sodalis*, and one from *P. subflavus* (Fig. 1; see Table S1 in the supplemental material). The majority of isolates came from Siloam Springs State Park (six isolates) (see Table S1 in the supplemental material). *Geomyces* isolates were obtained from all bat hibernacula, showing 97 to 99% similarity with respect to *G. destructans* (Pennsylvania isolate) for the ITS region. Culture morphology (see Fig. S2 in the supplemental material) as well as light and electron microscopy (data not shown) revealed that the *Geomyces* isolates were morphologically different from *G. destructans* based on the appearance of conidia. Temperature experiments showed that all 11 *Geomyces* isolates were able to grow at 25°C and 6°C (psychrotolerant), with higher growth rates at room temperature. Excluding *G. destructans*, the growth rates at 25°C ranged from 4.8 to 11 mm/week. Growth rates at 6°C ranged from 1.5 to 6 mm/week. *Geomyces destructans* isolates from Indiana and Pennsylvania did not exhibit growth at 25°C and had lower growth rates (compared to those of other *Geomyces* isolates) of 0.7 to 1.4 mm/week at 6°C (see Fig. S3 in the supplemental material).

A total of 199 sequences from GenBank along with our 13 ITS sequences were included in ML phylogenetic analyses of *Geomyces*. These results showed very low resolution, but the tree topology is consistent with previously published phylogenies for this genus (15, 24). The 11 *Geomyces* isolates from Illinois cluster into seven distinct clades, three of which are highly supported by BS and BPP. *Geomyces* strains isolated from wings of healthy bats in Illinois are diverse, representing at least seven different clades, and are closely related to *Geomyces* strains isolated or sequenced from soils in Alberta (Canada), Indiana, Latvia (from *Alnus incana*, gray alder), Massachusetts, Minnesota, New Hampshire, New Jersey,

New York, Pennsylvania, Virginia, and West Virginia. The *G. destructans* isolates from Indiana clustered with the European sequence (GU350442) and the type *G. destructans* sequence (EU884921) (Fig. 1).

LSU sequences showed low variability ($x = 0.5\%$) for all *Geomyces* isolates and were not included in the analysis. *MCM7* showed much higher variability ($x = 10.6\%$) for this genus, suggesting that it could be used as an additional marker to distinguish among *Geomyces* species (data not shown). However, limited *MCM7* sequences were available for species of *Geomyces*, preventing further phylogenetic analyses.

DISCUSSION

This study focused on the psychrophilic fungal components of the microbial community on bats. Taxa found in this study are consistent with previously documented fungal taxa in caves and mines (25, 30, 33, 68). The study of fungal diversity is crucial to determine the true impact of a potential invasive species such as *G. destructans* in the fungal community structure among bats and hibernacula. Samples collected from a WNS-positive cave (Mesmore Cave, IN) in this study showed that after WNS arrival, the possibility to study other common fungi on bats decreases by almost 100% (see Fig. S1 in the supplemental material). For the majority of Indiana samples, we obtained almost pure cultures of *G. destructans* in the swab plates. These monoculture *G. destructans* samples from Indiana were different from those from hibernating bats in Illinois (a WNS-free state at the time of sampling), which had an average of seven isolates per bat (Table 1). Previous studies show that WNS-positive bats have an overabundance of *G. destructans* conidia (18), but very little is known about how *G. destructans* interacts with other species and the impact this fungus may have on the different components of cave ecosystems.

The phylum Ascomycota dominated the fungal community. *Cladosporium*, *Fusarium*, *Mortierella*, and *Penicillium* strains were frequently isolated from bat wings. Vanderwolf et al. (25, 33) also reported that these fungal taxa are abundant in caves and mines. In contrast, possibly due to a bias toward mesophilic fungi and different culture methods, small overlap was seen compared to results by Voyron et al. (69) and Larcher et al. (68). The psychrotolerant fungi documented by Lorch et al. (15) in soils show major overlap with this study.

Based on this and other studies, *Geomyces* spp. seem to be common inhabitants of bat wings (33, 56). Our study shows that culture-based techniques are effective for the isolation of *Geomyces*. Lindner et al. (24), Lorch et al. (15), and Puechmaille et al. (70) documented bat hibernacula as potential reservoirs for *Geomyces*. It is likely that fungi found on bat wings represent a snapshot of the spore bank found in caves and mines instead of actively growing fungi. Some of these fungi could also act as minor pathogens, since many of the samples in this study were collected from wings that showed some minor damage. Samples collected from Siloam Springs State Park showed the highest damage, with WDI scores of 2, and we obtained six *Geomyces* isolates from these samples. These psychrotolerant *Geomyces* strains seem to represent part of a native mycobiota on bats. Some species of *Geomyces* have been isolated as keratinophilic fungi (28, 29), suggesting that some species isolated from bats could represent mild pathogens that are actively growing on bat wings. Wings with damage caused by some of these *Geomyces* spp. could represent active points of entry for a more aggressive pathogen such as *G. destructans*. Future research
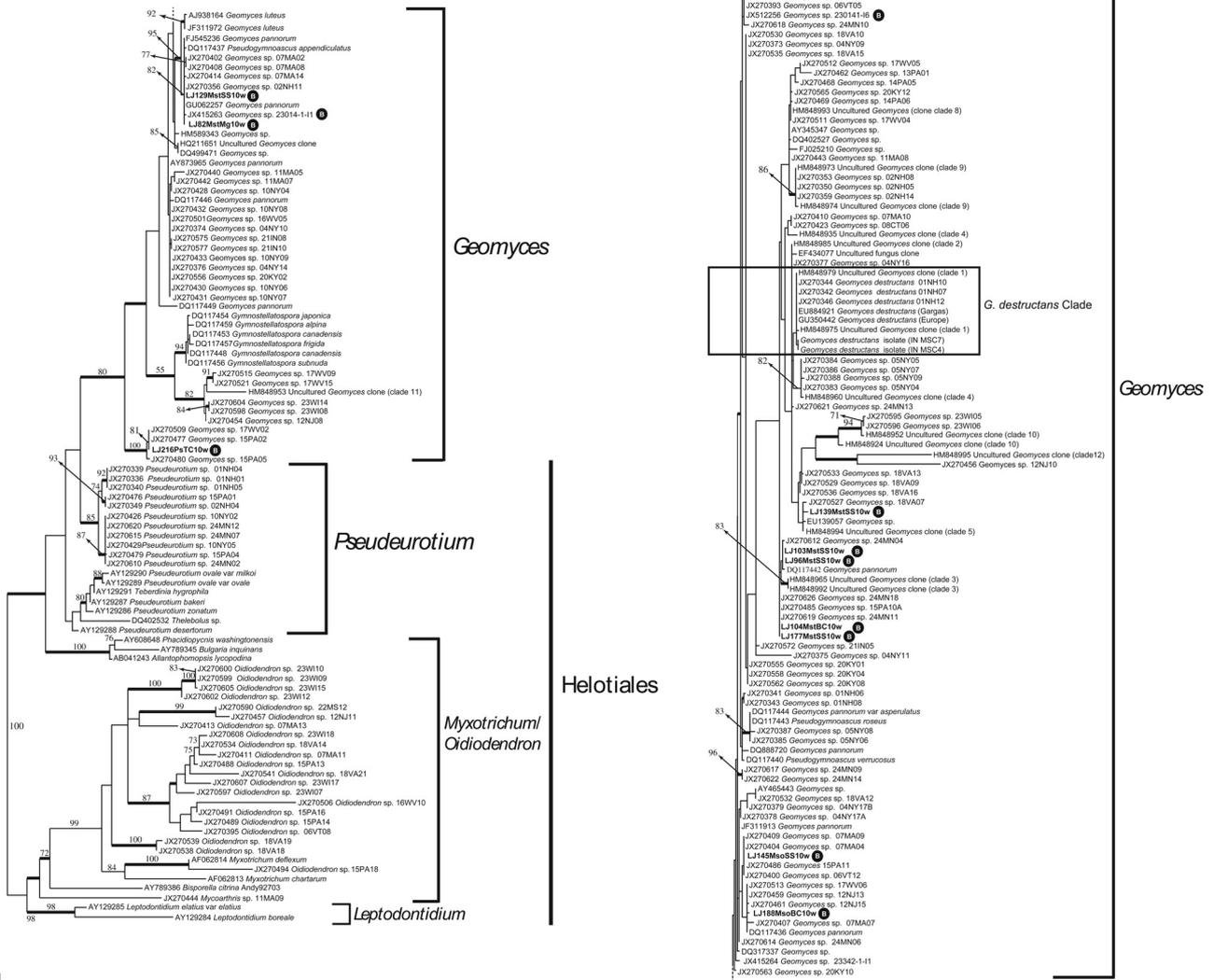
FIG 1 Maximum-likelihood phylogeny of *Geomyces* based on an ITS nrDNA data set of 89 taxa using PhyML [(−ln) L score, 14,971]. Thickened branches indicate significant Bayesian posterior probabilities of ≥95%; numbers refer to PhyML/RAxML bootstrap support values of ≥70% based on 1,000 replicates. *Bisporella citrina* was used as an outgroup taxon. Classification as described by Lorch et al. (15) is shown on the right. Sequences from this study are shown in bold.

on more *Geomyces* isolates from areas free of WNS could elucidate important information about *Geomyces* as a genus and result in a better understanding of the mechanisms of infection, pathogenicity, and management of the disease.

Sequences with 100% similarity to *G. destructans* were recovered only from Indiana, where mortality has been observed. Phylogenetic analysis confirmed that isolates from Indiana do cluster with European and other northeastern U.S. *G. destructans* strains (Fig. 1). Our results support the evidence that *G. destructans* is an invasive pathogen, because *G. destructans* was not recovered from any of the Illinois caves that were free of WNS at the time of sampling (32, 71).

All 11 *Geomyces* isolates from Illinois grouped into seven clades previously reported in other phylogenetic studies (15, 24). *Geomyces* sequences grouped with several soil clones from the study

by Lindner et al. (24), suggesting that the genus *Geomyces* may be more diverse than the literature has previously represented. A *Geomyces* strain isolated from a *Lasionycteris noctivagans* bat (silver-haired bat) from Tennessee, 23014-1-I1 (GenBank accession number, JX415263), grouped with isolates LJ129MstSS10w and LJ82MstMg10w, which suggests a wide distribution of these *Geomyces* strains in North America. The overlap between fungi found in different bat species in this study also suggests that bat-to-bat transmittance of fungi (other than *G. destructans*) may be a common occurrence. Most of the sequences in this study grouped with samples studied by Lorch et al. (15) from states of the U.S. East Coast (Massachusetts, New Jersey, and New York) and Midwest (Indiana and Minnesota), showing that bats may harbor many similar taxa from bat hibernaculum soils across the United States.

Our phylogenetic analyses confirm that the ITS region pro-

vides poor resolution in resolving different taxa of *Geomyces* (15, 24, 56). *Geomyces* cultures isolated from bats show great variation in colony morphology (see Fig. S2 in the supplemental material) and temperature requirements (see Fig. S3 in the supplemental material) with respect to *G. destructans* but cannot be distinguished at the species level using a common barcode marker such as the ITS. *MCM7* sequences show greater potential as a phylogenetic marker than ITS and LSU sequences, but *MCM7* can be difficult to amplify because it is a single-copy protein-coding gene (72, 73).

Temperature experiments showed that the *Geomyces* cultures isolated in this study are psychrotolerant and can be distinguished from *G. destructans* based on their growth rate at specific temperatures (see Fig. S3 in the supplemental material). This temperature restriction of *G. destructans* with respect to the other *Geomyces* isolates may be an important factor determining the pathogenicity of the species and requires further exploration.

The culturing techniques used in this study proved to be effective for the isolation of *Geomyces* strains and other psychrophilic fungi. Most *Geomyces* strains were isolated from damaged wings, indicating that other *Geomyces* strains could be acting as pathogens in bats. The impact of these fungi on bat health and the effects of the arrival of *G. destructans* in cave microbial communities require further exploration.

ACKNOWLEDGMENTS

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