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A culture-based survey of fungi in soil from bat hibernacula in the eastern United States and its implications for detection of *Geomyces destructans*, the causal agent of bat white-nose syndrome

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Abstract: The recent emergence of white-nose syndrome (WNS), a fungal disease causing unprecedented mortality among hibernating bats of eastern North America, has revealed a knowledge gap regarding fungal communities associated with bats and their hibernacula. We used culture-based techniques to investigate the diversity of fungi in soil samples collected from 24 bat hibernacula in the eastern United States. Ribosomal RNA regions (internal transcribed spacer and partial intergenic spacer) were sequenced to preliminarily characterize isolates. *Geomyces* species were one of the most abundant and diverse groups cultured, representing approximately 33% of all isolates. *Geomyces destructans* was isolated from soil samples from three hibernacula in states where WNS is known to occur, and many of the other cultured *Geomyces* isolates likely represent undescribed taxa. Further characterization of the diversity of fungi that occur in hibernacula both will facilitate an improved understanding of the ecology of *G. destructans* within this complex fungal community and provide an opportunity to identify characteristics that differentiate *G. destructans* from non-pathogenic relatives.

Key words: bat, *Geomyces*, skin infection, white-nose syndrome, wildlife disease

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Introduction

Since first photo documented in New York in February 2006, bat white-nose syndrome (WNS) has spread to 19 U.S. states and four Canadian provinces (see http://www.whitenosesyndrome.org/resources/map). The disease has been linked to the deaths of over 5 000 000 bats (see http://www.whitenosesyndrome.org/news/north-american-bat-death-toll-exceeds-55-million-white-nose-syndrome), and the high mortality caused by WNS has prompted concern over the future of North American bat populations (Blehert et al. 2009, Frick et al. 2010). White-nose syndrome is named for characteristic white growth caused by the recently described psychrophilic fungus, *Geomyces destructans* (Ascomycota, Helotiales) (Gargas et al. 2009, Chaturvedi et al. 2010), as it colonizes and invades exposed muzzle, ear and/or wing skin of infected bats (Meteyer et al. 2009). While laboratory studies have confirmed that *G. destructans* causes WNS in healthy bats (Lorch et al. 2011, Warnecke et al. 2012), little is known about the ecology or life cycle of this fungal pathogen.

Because active infection by *G. destructans* only occurs while bats hibernate and the fungus cannot grow at temperatures in excess of approximately 20 C (Gargas et al. 2009), it is possible that the cool caves and mines inhabited by bats during hibernation serve as environmental reservoirs for the fungus. Members of the genus *Geomyces* frequently are isolated from soil (States and Christensen 2001, Domsch et al. 2007, Izzo and Mazzola 2009, Arenz and Blanchette 2011), and many pathogenic fungi are known for their ability to persist in the environment even in the absence of hosts (Fisher et al. 2012). Thus, the environment might play an important role in the lifecycle of *G. destructans* and consequent manifestation of WNS in bats. A better understanding of the ecology of *G. destructans* is necessary to develop effective management strategies to control this disease.

Current efforts to study *G. destructans* in bat hibernacula are complicated by a limited knowledge

of fungal communities in these sites. Although previous researchers have investigated the fungal communities of particular cave systems (see Orpurt 1964, Koilraj et al. 1999, Nováková 2009, Shapiro and Pringle 2010, Vaughan et al. 2011), no broad-scale geographic sampling of cave mycota has been conducted in eastern North America. In addition, DNA-based identification of fungal isolates has become common only in the past decade, so relatively little work using modern species concepts has been conducted on the fungal communities that inhabit bat hibernacula where WNS has become prevalent.

A recent PCR-based study revealed that soil from bat hibernacula in the eastern United States contained a diversity of undescribed *Geomyces* species genetically similar to *G. destructans* (Lindner et al. 2011); DNA from these *Geomyces* species cross-reacted with primers previously designed to amplify DNA from *G. destructans* (Lorch et al. 2010), complicating identification of *G. destructans* in environmental samples. DNA from *G. destructans* was confirmed in soil samples collected from three hibernacula that housed infected bats, although *G. destructans* DNA appeared to be at low abundance relative to DNA from other fungi (Lindner et al. 2011). To expand upon this work, we conducted a culture-based survey to characterize psychrophilic and psychrotolerant fungi in soil samples collected from 24 bat hibernacula in the eastern United States. Greater knowledge of these fungal communities, particularly with respect to species of *Geomyces*, will be important to understand the ecology, evolution and pathogenicity of *G. destructans*.

MATERIALS AND METHODS

The same 24 soil samples used for molecular analyses by Lindner et al. (2011) were used for the culture analyses described in this study. Soil samples were collected from bat hibernacula both within and outside the known range of WNS during the winter of 2008–2009 as described by Lindner et al. (2011). Following collection, soil samples were shipped to the USGS, National Wildlife Health Center (Madison, Wisconsin) on wet ice and stored at −80 C. The 24 samples analyzed for this study were collected from 19 hibernacula in states within the known range of WNS (Connecticut, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Virginia, Vermont, West Virginia) and five hibernacula in states where WNS had not been observed at the time the samples were collected (Indiana, Kentucky, Minnesota, Mississippi, Wisconsin) (TABLE I). The names and coordinates of the collection sites have been withheld due to the sensitive nature of bat hibernacula.

Approximately 200 mg of each frozen soil sample was suspended in 0.5 mL sterile, deionized water, and serially diluted to 10^{-4}. An aliquot (150 µL) of each of the 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions was transferred onto duplicate Sabouraud dextrose agar plates containing chloramphenicol and gentamycin (BD Diagnostic Systems, Sparks, Maryland) and distributed with a sterile glass spreader until the liquid was absorbed by the medium. Plates were sealed with laboratory film (Bemis Flexible Packaging, Neenah, Wisconsin), incubated in the dark at 7 C for 90 d and examined for fungal growth once weekly. During each inspection, colonies of filamentous fungi appearing to be morphologically distinct from others within the same soil sample were isolated by transferring to fresh medium. Fungal tape impressions (St-Germain and Summerbell 2011) were examined for each isolate by light microscopy with a 100× objective.

DNA was extracted from fungal isolates with the protocol of Lindner and Banik (2009) with reagent volumes modified for use with 0.2 mL PCR strip tubes. Mycelium was scraped from cultures with a flattened transfer needle and placed in 200 µL cell lysis solution in PCR strip tubes. Tubes were then frozen at −80 C for a minimum of 30 min and subsequently placed in a 65 C water bath for 2 h. The samples were centrifuged at 10 000 rcf for 5 min, after which 100 µL supernatant was removed and transferred to a new strip tube. Next, 150 µL 0 C 2-propanol was added to each supernatant, tubes were inverted to mix, incubated at −80 C for 15 min and centrifuged at 10 000 rcf for 20 min at 0 C. Supernatants were discarded, 175 µL 75% ethanol (v/v) was added, and tubes were centrifuged at 16 000 rcf for 5 min at room temperature. Supernatants were removed and pellets were air-dried at room temperature for 10 min and resuspended in 45 µL molecular biology-grade water. DNA in aqueous solution was cleaned with the GeneClean III kit (MP Biomedicals, Solon, Ohio) following the manufacturer's protocol with the following modifications. First, 45 µL aqueous DNA solution was combined with 135 µL NaI solution and 5 µL glassmilk. Tubes then were agitated continuously for 5 min followed by centrifugation at 16 000 rcf for 8 s. The supernatant was discarded, and the pellet was washed once with 175 µL New Wash solution provided with the kit. After removal of solution, pellets were air-dried 15 min and template DNA was eluted in 50 µL molecular-grade water.

PCR was performed with 5× Green GoTaq reaction buffer and GoTaq DNA polymerase (Promega, Madison, Wisconsin). GoTaq reaction buffer was diluted to a 1× working concentration, and 0.25 units GoTaq DNA polymerase were used per reaction. Primers ITS1-F and ITS4 (Gardes and Bruns 1993) were used to amplify the ITS region. The intergenic spacer region (IGS) was amplified with primers CNL12 and CNS1 (White et al. 1990, Anderson and Stasovski 1992, Mbofung et al. 2007). All primer pairs were used at a final concentration of 0.4 µM and each dNTP (Promega, Madison, Wisconsin) had a final concentration of 200 µM. Thermo-cycler conditions for amplification of ITS were: initial denaturation at 94 C for 2 min followed by 30 cycles of denaturing at 94 C for 30 s, annealing at 55 C for 45 s, and extension at 72 C for 1 min and a final extension of 72 C for 10 min. Thermo-cycler conditions for IGS followed Mbofung et al. (2007).

Before sequencing, PCR products were analyzed with 1% agarose gels stained with ethidium bromide to confirm

TABLE I. Three hundred thirty-two fungal isolates cultured from 24 soil samples collected in bat hibernacula in eastern North America and tentative taxonomic placement based on morphology and/or BLAST queries of GenBank

Fungal isolate	Tentative taxonomic placement				GenBank accession nos.	
	Phylum	Class	Order	Genus	ITS	IGS
22MS03	Ascomycota	Dothideomycetes	Capnodiales	Incertae sedis	JX270582	JX270306
09CT02	Ascomycota	Dothideomycetes	Capnodiales	*Pseudocercosporella*	JX270425	—
07MA20	Ascomycota	Dothideomycetes	Incertae sedis	*Arthrographis*	JX270420	JX270227
22MS01	Ascomycota	Dothideomycetes	Incertae sedis	*Epicoccum*	JX270580	JX270305
19VA07	Ascomycota	Dothideomycetes	Pleosporales	*Dictyosporium*	JX270548	—
13PA04	Ascomycota	Dothideomycetes	Pleosporales[c]	Incertae sedis	JX270465	—
15PA17A	Ascomycota	Eurotiomycetes	Eurotiales	*Aspergillus*	JX270492	JX270264
15PA17B	Ascomycota	Eurotiomycetes	Eurotiales	*Aspergillus*	JX270493	JX270265
17WV10	Ascomycota	Eurotiomycetes	Eurotiales	*Aspergillus*	JX270516	JX270273
17WV11	Ascomycota	Eurotiomycetes	Eurotiales	*Aspergillus*	JX270517	JX270274
17WV13	Ascomycota	Eurotiomycetes	Eurotiales	*Aspergillus*	JX270519	JX270275
17WV14	Ascomycota	Eurotiomycetes	Eurotiales	*Aspergillus*	JX270520	JX270276
02NH09	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270354	—
02NH16	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270361	—
03VT06	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	—	—
03VT09	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270368	—
04NY07	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	—	—
04NY08	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	—	—
06VT04	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270392	—
06VT06	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270394	JX270207
06VT09	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270396	—
07MA05	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270405	JX270214
07MA17	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270417	JX270224
08CT04	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270422	JX270229
08CT05	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*[c]	—	—
11MA10	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270445	JX270238
11MA11	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270446	JX270239
12NJ04	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270450	—
12NJ06	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270452	—
14PA08	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270471	—
16WV01	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270496	—
16WV04	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270500	—
18VA01	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	—	—
19VA03	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270544	—
20KY03	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270557	—
20KY09	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	—	—
21IN04	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270571	JX270299
21IN09	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270576	—
22MS02	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270581	—
22MS04	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	—	—
22MS05	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270583	JX270307
22MS06	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270584	JX270308
22MS10	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270588	JX270309
22MS11	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270589	JX270310
24MN01	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270609	—
24MN05	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270613	—
24MN15	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270623	—
24MN16	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	JX270624	—
24MN22	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	—	—
07MA15	Ascomycota	Eurotiomycetes	Onygenales	*Arthroderma*	JX270415	—
20KY13	Ascomycota	Eurotiomycetes	Onygenales	*Arthroderma*	JX270566	JX270297
07MA16	Ascomycota	Eurotiomycetes	Onygenales	*Auxarthron*	JX270416	JX270223
14PA12	Ascomycota	Eurotiomycetes	Onygenales	*Chrysosporium*	JX270474	—

TABLE I. Continued

Fungal isolate	Tentative taxonomic placement				GenBank accession nos.	
	Phylum	Class	Order	Genus	ITS	IGS
18VA20	Ascomycota	Eurotiomycetes	Onygenales	*Chrysosporium*	JX270540	JX270282
21IN11	Ascomycota	Eurotiomycetes	Onygenales	*Chrysosporium*	JX270578	—
21IN12	Ascomycota	Eurotiomycetes	Onygenales	*Chrysosporium*	JX270579	JX270304
24MN30	Ascomycota	Eurotiomycetes	Onygenales	*Gymnascella*	JX270629	JX270334
14PA14	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascoideus*	JX270475	—
24MN34	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascoideus*	JX270631	—
02NH07	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270352	—
12NJ07	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270453	JX270242
12NJ14	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270460	JX270247
15PA08	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270483	—
15PA15	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270490	JX270262
18VA04	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270524	—
20KY15	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270567	JX270298
22MS07	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270585	—
22MS08	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270586	—
22MS09	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270587	—
23WI02	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270593	—
23WI13	Ascomycota	Eurotiomycetes	Onygenales	*Gymnoascus*	JX270603	JX270317
02NH15	Ascomycota	Eurotiomycetes	Onygenales	Incertae sedis	JX270360	—
14PA11	Ascomycota	Eurotiomycetes	Onygenales	*Neogymnomyces*	JX270473	—
19VA05	Ascomycota	Eurotiomycetes	Onygenales	*Trichophyton*	JX270546	—
19VA06	Ascomycota	Eurotiomycetes	Onygenales	*Trichophyton*	JX270547	JX270287
19VA08	Ascomycota	Eurotiomycetes	Onygenales	*Trichophyton*	JX270549	—
19VA09	Ascomycota	Eurotiomycetes	Onygenales	*Trichophyton*	JX270550	JX270288
19VA10	Ascomycota	Eurotiomycetes	Onygenales	*Trichophyton*	JX270551	JX270289
19VA12	Ascomycota	Eurotiomycetes	Onygenales	*Trichophyton*	JX270553	JX270291
19VA13	Ascomycota	Eurotiomycetes	Onygenales	*Trichophyton*	JX270554	—
20KY07	Ascomycota	Eurotiomycetes	Onygenales	*Trichophyton*	JX270561	—
23WI03	Ascomycota	Incertae sedis	Incertae sedis	Incertae sedis	—	JX270314
01NH06	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270341	—
01NH07	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[a]	JX270342	JX270192
01NH08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270343	—
01NH10	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[a]	JX270344	JX270193
01NH12	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[a]	JX270346	JX270195
02NH05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270350	—
02NH08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270353	JX270196
02NH11	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270356	—
02NH14	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270359	JX270198
03VT05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[c]	—	—
04NY09	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270373	—
04NY10	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270374	—
04NY11	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270375	—
04NY14	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270376	—
04NY16	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270377	—
04NY17A	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270378	JX270201
04NY17B	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270379	JX270202
05NY04	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270383	—
05NY05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270384	—
05NY06	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270385	—
05NY07	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270386	JX270203
05NY08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270387	—
05NY09	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270388	JX270204
06VT05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270393	JX270206
06VT12	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270400	JX270210

TABLE I. Continued

Fungal isolate	Tentative taxonomic placement				GenBank accession nos.	
	Phylum	Class	Order	Genus	ITS	IGS
07MA02	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270402	JX270212
07MA04	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270404	JX270213
07MA07	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270407	JX270215
07MA08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270408	JX270216
07MA09	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270409	JX270217
07MA10	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[a]	JX270410	JX270218
07MA14	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270414	JX270222
08CT06	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[a]	JX270423	JX270230
10NY04	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270428	JX270231
10NY06	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270430	—
10NY07	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270431	—
10NY08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270432	JX270232
10NY09	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270433	—
10NY10	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270434	—
11MA03	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270438	JX270233
11MA05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270440	—
11MA07	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270442	JX270236
11MA08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270443	JX270237
12NJ08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270454	JX270243
12NJ10	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270456	—
12NJ13	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270459	JX270246
12NJ15	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270461	JX270248
13PA01	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270462	—
14PA05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270468	—
14PA06	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270469	JX270249
14PA09	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	—	—
14PA13	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	—	JX270251
15PA02	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270477	JX270252
15PA05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270480	—
15PA10A	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270485	JX270256
15PA10B	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	—	JX270257
15PA11	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270486	JX270258
16WV05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270501	JX270267
17WV02	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270509	—
17WV03	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270510	JX270269
17WV04	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270511	JX270270
17WV05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270512	JX270271
17WV06	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270513	JX270272
17WV09	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270515	—
17WV15	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270521	—
18VA07	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270527	—
18VA08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270528	—
18VA09	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270529	—
18VA10	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270530	—
18VA12	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270532	—
18VA13	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270533	JX270279
18VA15	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270535	—
18VA16	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270536	JX270281
18VA17	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270537	—
20KY01	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270555	—
20KY02	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270556	—
20KY04	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270558	—
20KY08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270562	JX270293
20KY10	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270563	JX270294

TABLE I. Continued

Fungal isolate	Tentative taxonomic placement				GenBank accession nos.	
	Phylum	Class	Order	Genus	ITS	IGS
20KY12	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270565	JX270296
21IN01	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270568	—
21IN05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270572	JX270300
21IN06	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270573	JX270301
21IN08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270575	JX270302
21IN10	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270577	JX270303
23WI04	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270594	—
23WI05	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270595	—
23WI06	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270596	JX270315
23WI08	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270598	JX270316
23WI14	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270604	JX270318
24MN03	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270611	—
24MN04	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270612	JX270322
24MN06	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270614	JX270323
24MN09	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270617	JX270325
24MN10	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270618	JX270326
24MN11	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270619	JX270327
24MN13	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270621	JX270328
24MN14	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270622	JX270329
24MN17	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270625	JX270330
24MN18	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270626	JX270331
24MN21	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	—	—
24MN24	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	—	—
24MN25	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[c]	—	—
24MN26	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[c]	—	—
24MN27	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[c]	—	—
24MN28	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270628	JX270333
24MN29	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[c]	—	—
24MN31	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*[c]	—	—
24MN32	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	—	—
24MN33	Ascomycota	Leotiomycetes	Helotiales	*Geomyces*	JX270630	JX270335
11MA09	Ascomycota	Leotiomycetes	Helotiales	*Mycoarthris*	JX270444	—
06VT08	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270395	—
07MA11	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270411	JX270219
07MA13	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270413	JX270221
07MA18	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270418	JX270225
12NJ11	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270457	JX270244
15PA07	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270482	JX270254
15PA13	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270488	JX270260
15PA14	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270489	JX270261
15PA16	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270491	JX270263
15PA18	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270494	—
16WV10	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270506	—
16WV11	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*[b]	JX270507	—
18VA14	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270534	JX270280
18VA18	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270538	—
18VA19	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270539	—
18VA21	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270541	JX270283
22MS12	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270590	JX270311
23WI01A	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270591	JX270312
23WI01B	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270592	JX270313
23WI07	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270597	—
23WI09	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270599	—
23WI10	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270600	—

TABLE I. Continued

Fungal isolate	Tentative taxonomic placement				GenBank accession nos.	
	Phylum	Class	Order	Genus	ITS	IGS
23WI11	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270601	—
23WI12	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270602	—
23WI15	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270605	—
23WI16	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270606	JX270319
23WI17	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270607	JX270320
23WI18	Ascomycota	Leotiomycetes	Helotiales	*Oidiodendron*	JX270608	JX270321
01NH01	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270336	—
01NH04	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270339	—
01NH05	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270340	—
02NH04	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270349	—
10NY02	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270426	—
10NY05	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270429	—
15PA01	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270476	—
15PA04	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270479	—
24MN02	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270610	—
24MN07	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270615	—
24MN12	Ascomycota	Leotiomycetes	Helotiales	*Pseudeurotium*	JX270620	—
12NJ03	Ascomycota	Saccharomycetes	Saccharomycetales	*Candida*	JX270449	—
16WV03B	Ascomycota	Saccharomycetes	Saccharomycetales	*Candida*	JX270499	—
10NY11	Ascomycota	Saccharomycetes	Saccharomycetales	*Debaryomyces*	JX270435	—
21IN03	Ascomycota	Saccharomycetes	Saccharomycetales	*Debaryomyces*	JX270570	—
15PA19	Ascomycota	Sordariomycetes	Hypocreales	*Hypomyces*	JX270495	JX270266
07MA12	Ascomycota	Sordariomycetes	Hypocreales	*Isaria*	JX270412	JX270220
07MA19	Ascomycota	Sordariomycetes	Hypocreales	*Isaria*	JX270419	JX270226
13PA02	Ascomycota	Sordariomycetes	Hypocreales	*Isaria*	JX270463	—
13PA03	Ascomycota	Sordariomycetes	Hypocreales	*Isaria*	JX270464	—
06VT10	Ascomycota	Sordariomycetes	Hypocreales	*Nectria*	JX270397	—
06VT11A	Ascomycota	Sordariomycetes	Hypocreales	*Nectria*	JX270398	JX270208
06VT11B	Ascomycota	Sordariomycetes	Hypocreales	*Nectria*	JX270399	JX270209
11MA06	Ascomycota	Sordariomycetes	Hypocreales	*Neonectria*	JX270441	JX270235
01NH11	Ascomycota	Sordariomycetes	Hypocreales	*Tolypocladium*	JX270345	JX270194
03VT07	Ascomycota	Sordariomycetes	Hypocreales	*Verticillium*	JX270366	JX270199
03VT08	Ascomycota	Sordariomycetes	Hypocreales	*Verticillium*	JX270367	JX270200
14PA10	Ascomycota	Sordariomycetes	Hypocreales	*Verticillium*	JX270472	JX270250
17WV12	Ascomycota	Sordariomycetes	Hypocreales	*Verticillium*	JX270518	—
24MN19	Ascomycota	Sordariomycetes	Hypocreales	*Verticillium*	JX270627	JX270332
02NH06	Ascomycota	Sordariomycetes	Microascales	*Doratomyces*	JX270351	—
02NH10	Ascomycota	Sordariomycetes	Microascales	*Doratomyces*	JX270355	JX270197
02NH13	Ascomycota	Sordariomycetes	Microascales	*Doratomyces*	JX270358	—
07MA03	Ascomycota	Sordariomycetes	Microascales	*Doratomyces*	JX270403	—
15PA12	Ascomycota	Sordariomycetes	Microascales	*Doratomyces*	JX270487	JX270259
18VA03	Ascomycota	Sordariomycetes	Microascales	*Doratomyces*	JX270523	—
18VA05	Ascomycota	Sordariomycetes	Microascales	*Doratomyces*	JX270525	—
18VA06	Ascomycota	Sordariomycetes	Microascales	*Doratomyces*	JX270526	JX270277
18VA11	Ascomycota	Sordariomycetes	Microascales	*Doratomyces*	JX270531	JX270278
02NH12	Ascomycota	Sordariomycetes	Microascales	*Kernia*[b]	JX270357	—
04NY18	Ascomycota	Sordariomycetes	Microascales	*Kernia*	JX270380	—
12NJ12	Ascomycota	Sordariomycetes	Microascales	*Kernia*	JX270458	JX270245
14PA07	Ascomycota	Sordariomycetes	Microascales	*Kernia*	JX270470	—
15PA06	Ascomycota	Sordariomycetes	Microascales	*Kernia*	JX270481	JX270253
15PA09	Ascomycota	Sordariomycetes	Microascales	*Kernia*	JX270484	JX270255
16WV08	Ascomycota	Sordariomycetes	Microascales	*Kernia*	JX270504	—
16WV09	Ascomycota	Sordariomycetes	Microascales	*Kernia*	JX270505	—
21IN07	Ascomycota	Sordariomycetes	Microascales	*Kernia*	JX270574	—

TABLE I. Continued

Fungal isolate	Tentative taxonomic placement				GenBank accession nos.	
	Phylum	Class	Order	Genus	ITS	IGS
12NJ05	Ascomycota	Sordariomycetes	Sordariales	*Trichocladium*	JX270451	JX270241
19VA01	Ascomycota	Sordariomycetes	Sordariales	*Trichocladium*	JX270542	JX270284
19VA02	Ascomycota	Sordariomycetes	Sordariales	*Trichocladium*	JX270543	JX270285
19VA04	Ascomycota	Sordariomycetes	Sordariales	*Trichocladium*	JX270545	JX270286
19VA11	Ascomycota	Sordariomycetes	Sordariales	*Trichocladium*	JX270552	JX270290
20KY11	Basidiomycota	Agaricomycetes	Agaricales	*Coprinellus*	JX270564	JX270295
16WV07	Basidiomycota	Agaricomycetes	Agaricales	Incertae sedis	JX270503	—
20KY06	Basidiomycota	Agaricomycetes	Cantharellales	*Burgoa/Sistotrema*	JX270560	—
01NH03	Basidiomycota	Tremellomycetes	Tremellales	*Trichosporon*	JX270338	JX270191
02NH01	Basidiomycota	Tremellomycetes	Tremellales	*Trichosporon*	JX270347	—
03VT02	Basidiomycota	Tremellomycetes	Tremellales	*Trichosporon*	JX270363	—
04NY03	Basidiomycota	Tremellomycetes	Tremellales	*Trichosporon*	JX270371	—
05NY01	Basidiomycota	Tremellomycetes	Tremellales	*Trichosporon*	JX270381	—
06VT03	Basidiomycota	Tremellomycetes	Tremellales	*Trichosporon*	JX270391	JX270205
16WV06	Basidiomycota	Tremellomycetes	Tremellales	*Trichosporon*	JX270502	JX270268
20KY05	Basidiomycota	Tremellomycetes	Tremellales	*Trichosporon*	JX270559	JX270292
24MN08	Basidiomycota	Tremellomycetes	Tremellales	*Trichosporon*[b]	JX270616	JX270324
02NH03	Incertae sedis	Incertae sedis	Incertae sedis	Incertae sedis	—	—
04NY12	Incertae sedis	Incertae sedis	Incertae sedis	Incertae sedis	—	—
04NY13	Incertae sedis	Incertae sedis	Incertae sedis	Incertae sedis	—	—
04NY15	Incertae sedis	Incertae sedis	Incertae sedis	Incertae sedis	—	—
06VT07	Incertae sedis	Incertae sedis	Incertae sedis	Incertae sedis	—	—
17WV08	Incertae sedis	Incertae sedis	Incertae sedis	Incertae sedis	—	—
24MN20	Incertae sedis	Incertae sedis	Incertae sedis	Incertae sedis	—	—
24MN23	Incertae sedis	Incertae sedis	Incertae sedis	Incertae sedis	—	—
03VT04	Zygomycota	Incertae sedis	Mortierellales	Incertae sedis	JX270365	—
01NH02	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270337	—
02NH02	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270348	—
03VT01	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*[b]	JX270362	—
03VT03	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270364	—
04NY01	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270369	—
04NY02	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270370	—
04NY05	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270372	—
05NY02	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270382	—
06VT02	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270390	—
07MA01	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270401	JX270211
07MA06	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270406	—
08CT03	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270421	JX270228
09CT01	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270424	—
10NY03	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270427	—
11MA01	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*[b]	JX270436	—
11MA04	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270439	JX270234
12NJ01	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270447	JX270240
12NJ02	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270448	—
14PA01	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270466	—
15PA03	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270478	—
16WV02	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270497	—
16WV03A	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270498	—
17WV01	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270508	—
17WV07	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270514	—
18VA02	Zygomycota	Incertae sedis	Mortierellales	*Mortierella*	JX270522	—
06VT01	Zygomycota	Incertae sedis	Mucorales	*Helicostylum*[b]	JX270389	—
12NJ09	Zygomycota	Incertae sedis	Mucorales	*Helicostylum*[b]	JX270455	—
14PA04	Zygomycota	Incertae sedis	Mucorales	*Helicostylum*[b]	JX270467	—

TABLE I. Continued

Fungal isolate	Tentative taxonomic placement				GenBank accession nos.	
	Phylum	Class	Order	Genus	ITS	IGS
04NY04	Zygomycota	Incertae sedis	Mucorales	*Mucor*	—	—
04NY06	Zygomycota	Incertae sedis	Mucorales	*Mucor*	—	—
08CT01A	Zygomycota	Incertae sedis	Mucorales	*Mucor*	—	—
10NY01	Zygomycota	Incertae sedis	Mucorales	*Mucor*	—	—
11MA02	Zygomycota	Incertae sedis	Mucorales	*Mucor*[b]	JX270437	—
14PA02	Zygomycota	Incertae sedis	Mucorales	*Mucor*	—	—
21IN02	Zygomycota	Incertae sedis	Mucorales	*Mucor*[b]	JX270569	—
01NH09	Zygomycota[c]	Incertae sedis	Incertae sedis	Incertae sedis	—	—
05NY03	Zygomycota[c]	Incertae sedis	Incertae sedis	Incertae sedis	—	—

[a] Isolate with a 100% ITS match to *Geomyces destructans*.
[b] ITS sequence excluded from the alignment used to construct the tree in FIG. 1.
[c] Likely taxonomic placement, but taxonomy is not certain.

presence of a single amplicon. ITS PCR products were sequenced in both directions with the BigDye Sequencing Kit 3.1 and an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, California) at the University of Wisconsin at Madison Biotechnology Center using 1 : 10 diluted PCR product and the same primers from the initial amplification. IGS PCR products were sequenced as described above but in only one direction using the CNL12 primer. Sequencing products were cleaned with CleanSeq (Agencourt, Fullerton, California) magnetic beads following the manufacturer's protocol.

Sequence chromatograms were visually inspected with Sequencher 4.2 (GeneCodes Corp., Ann Arbor, Michigan), and all alignments were conducted with MAFFT 6.864 with the Q-INS-i algorithm (Katoh 2008). The 296 ITS sequences generated for this study were combined with eight reference sequences from GenBank (*Candida albicans* JN882321, *Davidiella tassiana* EF679363, *Geomyces destructans* EU884921, *Geomyces pannorum* var. *asperulatus* DQ117444, *Oidiodendron tenuissimum* AF062807, *Penicillium expansum* DQ339562, *Trichophyton rubrum* AF170472, *Trichosporon chiarellii* HQ999971), and a global alignment of all ITS sequences was completed. All unknown sequences were compared to the NCBI GenBank database with BLAST (Altschul et al. 1997). A small number of poor quality sequences and sequences with BLAST matches to Zygomycota s.l. (Mucorales) were excluded from the final global alignment (such sequences are marked with a superscript b in TABLE I) because they could not be confidently aligned with other sequences.

Based on an initial analysis of all sequences, a subset of sequences composed of *Geomyces* species and closely related taxa were selected and a second alignment was created with representative sequences of *Geomyces*, *Pseudeurotium* and other helotialean outgroup taxa obtained from GenBank; selection of sequences from GenBank was based on the work of Sogonov et al. (2005), Rice and Currah (2006), Wang et al. (2006a, b) and Gargas et al. (2009). ITS sequences from three *Geomyces* isolates cultured from bats also were incorporated into this second alignment to determine their relationship to *Geomyces* spp. occurring in the soil of bat hibernacula. These bat isolates included two described by Muller et al. (2012) (230141-I2 and 233421-I1; GenBank accession numbers JX415263 and JX415264 respectively) and an additional isolate cultured from a silver-haired bat (*Lasionycteris noctivagans*) from Tennessee (230141-I6; GenBank accession number JX512256).

Phylogenetic analyses were conducted with MEGA5 (Tamura et al. 2011) and all alignments and trees are archived in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S13306). The global alignment of all ITS sequences was analyzed with the neighbor joining (NJ) method. The NJ tree (FIG. 1) was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree; the absolute value was used for any branch with negative length. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. All positions containing gaps and missing data were eliminated.

The alignment of *Geomyces* and related species was analyzed with the maximum likelihood (ML) method based on the Jukes-Cantor model and 1000 bootstrap replicates. The tree (FIG. 2) with the highest log likelihood was selected as the final tree, while initial trees for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one-fourth the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories). Trees were drawn to scale with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Trees were viewed with FigTree 1.3.1, and graphics were exported for final illustrations.

RESULTS

Following incubation of soil samples at 7 C, a total of 332 fungal isolates were cultured from the 24 hibernacula. Complete ITS and partial IGS region sequences were successfully generated for 296 and

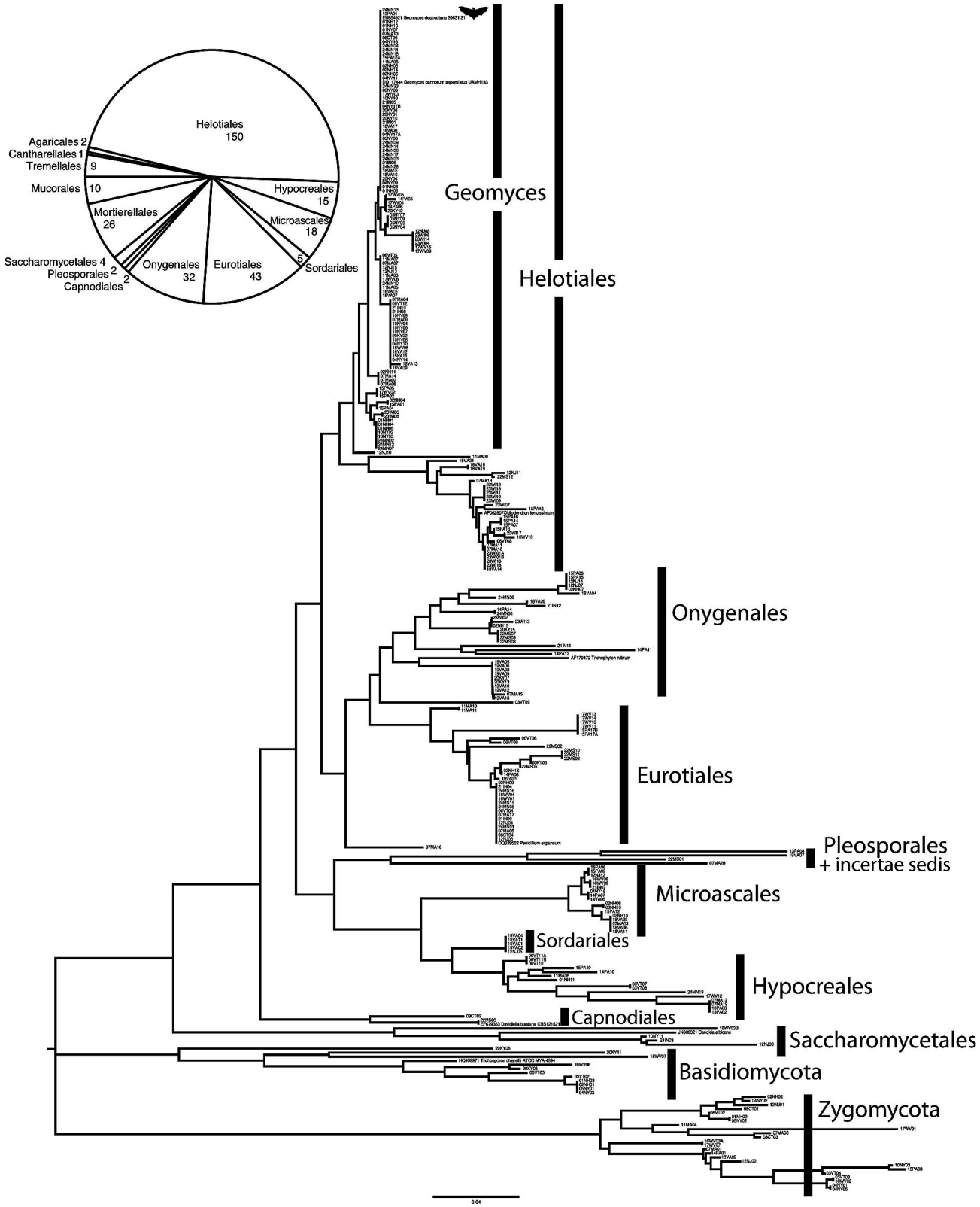

FIG. 1. Taxonomic diversity and ITS neighbor joining phylogeny of fungi recovered from soil samples collected in 24 bat hibernacula in eastern North America. The Geomyces clade includes 11 isolates identified as *Pseudeurotium* (FIG. 2, TABLE I).

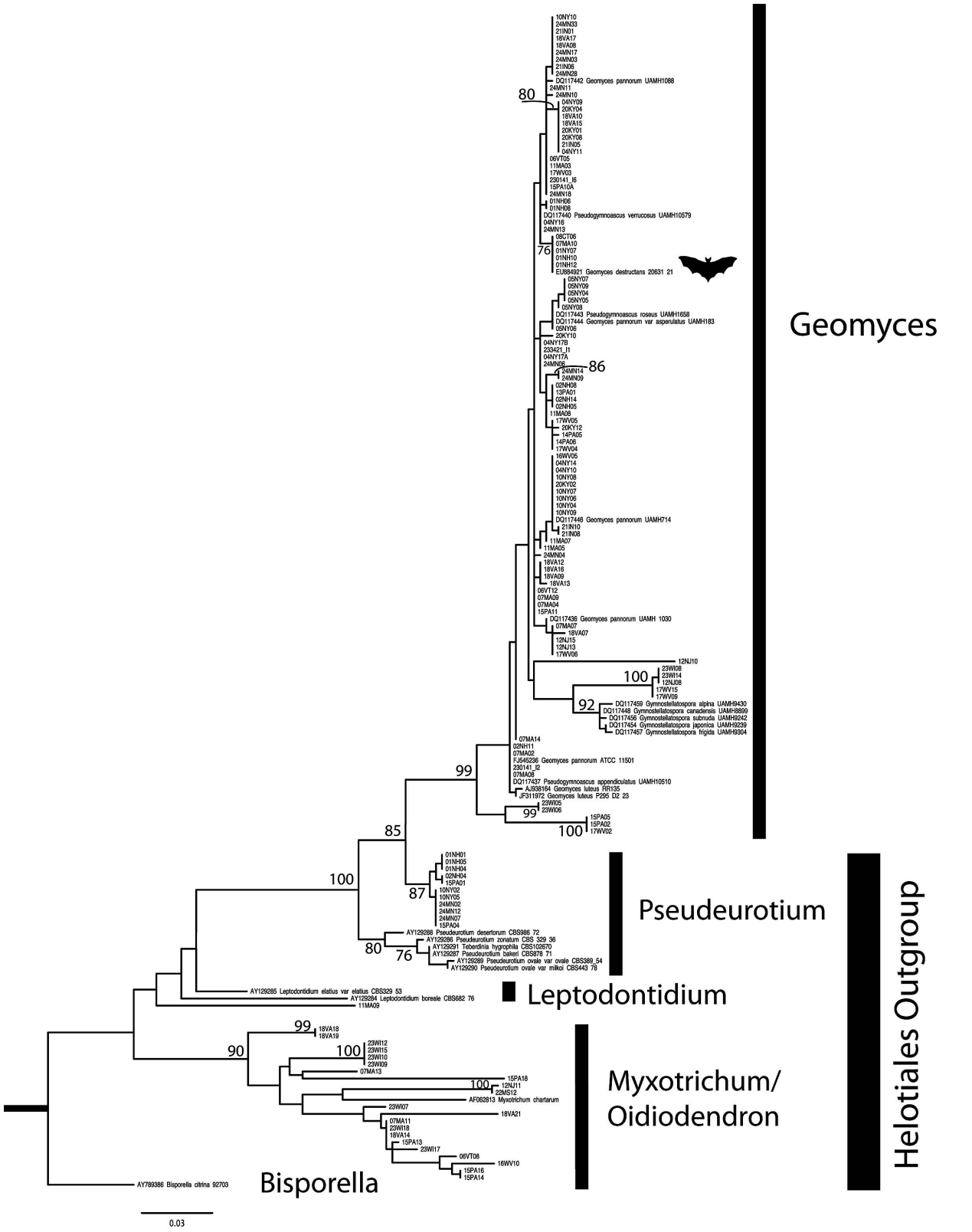

FIG. 2. ITS maximum likelihood phylogeny of *Geomyces* and related species isolated from soil samples collected in 24 bat hibernacula in eastern North America. All isolates in the Geomyces clade produced anamorphs typical of *Geomyces*. Bootstrap values ≥ 75% are presented.

172 of the 332 isolates respectively (TABLE I). Cultured fungal isolates were diverse, including many within the phylum Ascomycota and a smaller number of Zygomycota and Basidiomycota (FIG. 1, TABLE I). A total of 274 isolates (83%) were Ascomycota, 38 isolates (11%) were Zygomycota, 12 isolates (4%) were Basidiomycota, and eight isolates (2%) could not be confidently placed in a phylum. Ascomycota isolates were recovered from all 24 sites (100%), while Zygomycota were recovered from 18 of 24 sites (75%) and Basidiomycota from nine of 24 sites (38%).

Among the Ascomycota, the most represented orders were Helotiales, Eurotiales and Onygenales (225 of the 332 isolates; 68%). Helotiales was the single most dominant order, representing approximately 45% of cultured isolates (150 of 332); isolates belonging to this order were found in 22 of the 24 sites (92%; FIG. 1; TABLE I). In particular, *Geomyces* spp. comprised a large and diverse group of the cultured isolates (110 of 332; 33%; FIG. 2; TABLE I) and were recovered from 21 of the 24 sites (88%). Three *Geomyces* isolates from bats included in the analysis did not cluster together based on ITS sequences but were interspersed with the *Geomyces* isolates from soil (FIG. 2). Although ITS sequence provided poor species-rank resolution within *Geomyces*, the genus was well supported by ITS sequence characters (99% bootstrap support, FIG. 2). All basal taxa within *Geomyces* were confirmed by light microscopy to produce anamorphs typical of genus *Geomyces*, while isolates in the next most closely related clade (genus *Pseudeurotium*) produced *Teberdinia*-like anamorphs typical of species with or without known *Pseudeurotium* teleomorphs (Sogonov et al. 2005). However, *Pseudeurotium* consists of two clades as recognized herein (FIG. 2) and might represent two distinct genera. We prefer to identify both clades as *Pseudeurotium* due to morphological and ITS sequence similarity until further taxonomic work is performed.

High representation among the Eurotiales (43 of 332 isolates; 13%) stemmed from the numerous isolates within the genus *Penicillium* (37 of 332 isolates; 11%), which were found at 16 of 24 sites (67%). *Aspergillus* was poorly represented (six of 332 isolates; 2%) and was isolated only from two sites (8%), perhaps due to the cool incubation temperatures used in this study (Domsch et al. 2007). Other orders within Ascomycota (Capnodiales, Hypocreales, Microascales, Pleosporales, Sordariales, Saccharomycetales) were represented to a lesser degree, along with two orders of Zygomycota (Mortierellales, Mucorales) and three orders of Basidiomycota (Agaricales, Cantharellales, Tremellales) (TABLE I).

Geomyces destructans was isolated from soil samples from three sites (TABLE I). These three sites corresponded to the sites where DNA from *G. destructans* was detected (Lindner et al. 2011). The ITS sequences of cultured isolates of *G. destructans* were identical to that of the type of *G. destructans* (GenBank accession number EU884921), and morphology of the isolates was consistent with the description of *G. destructans* (Gargas et al. 2009). With the exception of the isolates with a 100% ITS match to *G. destructans*, none of the isolates in *Geomyces* produced conidia that were morphologically similar to those of *G. destructans*.

DISCUSSION

This study highlights the range of cold-tolerant fungal species that can be cultured from bat hibernacula. Based upon the culture conditions used for this study, Ascomycota was the dominant phylum in cave and mine soils and this was due in part to the large number of isolates within the order Helotiales, most of which belonged to the genus *Geomyces*. *Geomyces* species are commonly found in soil and are noted for their adaptation to cool environments (Domsch et al. 2007), which may explain their abundance in hibernacula. Eurotiales, which includes the ubiquitous genus *Penicillium*, was the second most common order of fungi isolated, and *Penicillium* species also are known for their cold tolerance (Domsch et al. 2007). Onygenales, which includes a saprotrophic, geophilic group of dermatophytes (Domsch et al. 2007), accounted for the third largest order of fungi isolated. Compared to Ascomycota, the phylum Zygomycota was represented by fewer isolates and Basidiomycota were cultured only rarely.

The mycota isolated from caves and mines in this study may reflect the types of energy sources available in the soil of hibernacula. For example, the ability of Onygenales to degrade keratin (Domsch et al. 2007), a substance that tends to accumulate in mammalian dwellings (Battelli et al. 1978) and that may be common in the soil of caves occupied (or previously occupied) by large numbers of hibernating bats, could explain the abundance of these fungi. The recovery of *Kernia* and *Doratomyces* (Microascales) isolates is also noteworthy because both genera are found on animal dung (Malloch and Cain 1971, Domsch et al. 2007) and also may exist in association with bat guano. In addition, numerous members of the order Hypocreales (15 isolates from 10 sites) and the genus *Mortierella* (Mortierellales; 25 isolates from 17 sites) were isolated, and many of these are known metabolizers of chitin (Domsch et al. 2007), a component of waste produced by insectivorous bats (Emerson and Roark 2007). Together these findings suggest that cave and mine soils contain a diversity of

fungi supported by diverse sources of nutrients, including keratin and chitin. Further work with defined media will be needed to characterize the substrates upon which *G. destructans* can grow, thus providing a more complete understanding of the ability of the fungus to persist and/or proliferate using the resources available in soil from bat hibernacula.

As the single-most commonly cultured fungal genus in this study (110 of 332 isolates), this work emphasizes the abundance and diversity of *Geomyces* species in soil from bat hibernacula. Through this effort we recovered in culture the majority of the *Geomyces* clades (nine of 12 clades; matches to clades 3, 11 and 12 were not recovered in culture) described previously based upon partial sequence analysis of the ITS region (Lindner et al. 2011), providing the opportunity to determine full-length ITS sequences for these clades. However, even full-length ITS sequences provided poor taxonomic resolution among the clades (FIG. 2). To overcome the lack of taxonomic resolution provided by ITS sequences, we successfully obtained partial sequences of the IGS region for 172 of the 332 isolates. Partial IGS sequences offered utility for differentiating species of *Geomyces* and served as the basis for development of a sensitive and specific real-time PCR test for detecting *G. destructans* (Muller et al. 2012). However, IGS sequences were of limited phylogenetic value due to our inability to produce alignments spanning the genus and a lack of reference data in GenBank. Multilocus sequencing, including single-copy protein coding genes, will be necessary to phylogenetically characterize the numerous *Geomyces* species isolated for this study and to determine whether there are phylogeographic trends in the occurrence of these isolates across eastern North America. In particular, species-rank characterization will make it possible to determine whether there is evidence of endemism among fungal species within particular hibernacula or regions and to determine whether certain bat species are associated with individual *Geomyces* species. Because only 24 sites were surveyed for this work, it is likely the diversity observed in this study represents only a fraction of the fungal species present in these environments.

Based on our assessment of key taxonomic literature on *Geomyces* that includes teleomorphic names classified in *Gymnostellatospora* and *Pseudogymnoascus* (Carmichael 1962, van Oorschot 1980, Sigler et al. 2000, Rice and Currah 2006) as well as lists of species in Index Fungorum (http://indexfungorum.org/) and MycoBank (http://www.mycobank.org/), approximately 17 discrete species would be accepted in this group under a one name system of classification (Norvell 2011). However, numerous heterotypic synonyms listed by van Oorschot (1980) and Domsch et al. (2007) are widely recognized to represent distinct species (Domsch et al. 2007). Although a critical reassessment of named *Geomyces* species, especially under a one name system of classification, is not available at this time, the diversity of *Geomyces* taxa observed in this sampling effort indicates the actual number of *Geomyces* taxa is far greater than previously recognized based on traditional taxonomic methods. Specifically, currently named taxa classified among *Geomyces*, *Gymnostellatospora* and *Pseudogymnoascus* are differentiated by as few as 4–7 SNPs across the ITS region. By that measure, we may have isolated as many as 35 species of *Geomyces*. Similarly low genetic variation in the ITS region, as was observed in the examined isolates of *Geomyces*, also has been observed among species in many other genera of Ascomycota including *Aspergillus*, *Beauveria*, *Colletotrichum*, *Fusarium*, *Penicillium* and *Trichoderma*; this apparent lack of genetic variation often obscures a remarkable diversity of distinct species (Aoki et al. 2003, Chaverri et al. 2003, Rehner and Buckley 2005, Balajee et al. 2007, Houbraken et al. 2010, Rojas et al. 2010). This work on *Geomyces* cultured from hibernacula suggests that much diversity within the genus remains to be discovered.

Our culture-based studies favored growth of fungi that grew readily on artificial medium in darkness at the relatively low temperature of 7 C. Under these conditions designed to approximate those found in bat hibernacula, *Geomyces* species were a dominant and diverse part of the culturable fungal community. Although others have identified *Geomyces* species in caves (e.g. Domsch et al. 2007, Nováková 2009), the diversity of this group has been underestimated. If *Geomyces* species are similarly diverse in hibernacula of temperate regions in Eurasia where *G. destructans* is hypothesized to have originated (Puechmaille et al. 2011, Warnecke et al. 2012), genetic interactions among this assemblage of closely related fungi may have provided the capacity for an evolutionary transition from soil dweller to bat pathogen. With an array of *Geomyces* spp. isolated from caves and mines now in culture, in-depth genetic, morphological and physiological analyses can be conducted to determine which unique traits *G. destructans* possesses that mediate its pathogenicity to hibernating bats.

The ability to culture viable *G. destructans* from soil (this study) and other abiotic surfaces in bat hibernacula (Puechmaille et al. 2011) demonstrates the existence of environmental reservoirs of this pathogenic fungus in locations occupied by hibernating bats, thus presenting distinct challenges for managing WNS. However, researchers were unable to cultivate *G. destructans* from 26 soil and debris

samples collected from bat hibernacula in New York (Chaturvedi et al. 2010); and through application of dilution technique, we succeeded only in culturing the fungus from soil samples from three of 19 sites within the range of WNS at the time the samples were collected. Consequently, the role of the environment, both as a long-term reservoir for *G. destructans* and as a potential source for fungal spread, remains a topic of active investigation. Nonetheless, the ability of *G. destructans* to persist, proliferate and perhaps sexually recombine in soils of hibernacula has the potential to fundamentally change our understanding of the transmission dynamics of WNS. Thus, the ability to effectively manage WNS will be dependent on gaining a solid understanding of the lifecycle of *G. destructans* as it colonizes and spreads among bats and their environments.

Bat hibernacula are cool, humid, dark environments that, as exemplified by this study, are conducive to the growth of a diverse assemblage of fungi. However, we are unaware of any reports of epizootic fungal disease among hibernating bats in North America before the emergence of WNS. This suggests that, compared to other fungi that occur in bat hibernacula, *G. destructans* possesses unique characteristics that let it proliferate on the skin of hibernating bats and cause severe disease. Further genetic and physiological characterization of the presumably non-pathogenic fungi that we have cultivated from soil from bat hibernacula, including other species of *Geomyces*, may provide important clues regarding the unique ecology and pathogenicity of *G. destructans*.

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