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DNA-based detection of the fungal pathogen *Geomyces destructans* in soils from bat hibernacula

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Abstract: White-nose syndrome (WNS) is an emerging disease causing unprecedented morbidity and mortality among bats in eastern North America. The disease is characterized by cutaneous infection of hibernating bats by the psychrophilic fungus *Geomyces destructans*. Detection of *G. destructans* in environments occupied by bats will be critical for WNS surveillance, management and characterization of the fungal lifecycle. We initiated an rRNA gene region-based molecular survey to characterize the distribution of *G. destructans* in soil samples collected from bat hibernacula in the eastern United States with an existing PCR test. Although this test did not specifically detect *G. destructans* in soil samples based on a presence/absence metric, it did favor amplification of DNA from putative *Geomyces* species. Cloning and sequencing of PCR products amplified from 24 soil samples revealed 74 unique sequence variants representing 12 clades. Clones with exact sequence matches to *G. destructans* were identified in three of 19 soil samples from hibernacula in states where WNS is known to occur. *Geomyces destructans* was not identified in an additional five samples collected outside the region where WNS has been documented. This study highlights the diversity of putative *Geomyces* spp. in soil from bat hibernacula and indicates that further research is needed to better define the taxonomy of this genus and to develop enhanced diagnostic tests for rapid and specific detection of *G. destructans* in environmental samples.

Key words: disease surveillance, environmental sampling, skin infection, wildlife disease

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INTRODUCTION

Since first photo-documented near Albany, New York, in 2006, white-nose syndrome (WNS) in bats and/or the associated fungus *Geomyces destructans* has been detected in 13 additional US states and two Canadian provinces (Blehert et al. 2009, Frick et al. 2010, Turner and Reeder 2009). The disease, linked to the deaths of more than 1 000 000 bats (Frick et al. 2010, Turner and Reeder 2009), is named for the often visible psychrophilic fungus *G. destructans* (Ascomycota, Helotiales [Chaturvedi et al. 2010, Gargas et al. 2009]) colonizing exposed bat muzzle, ear and/or wing skin.

Although aspects of the complex interactions among the disease, the environment and the host remain unknown, characteristic cutaneous infection of hibernating bats by *G. destructans* is the only consistently identified contributor to WNS (Blehert et al. 2009, Courtin et al. 2010, Meteyer et al. 2009). Little is known about the geographic distribution of *G. destructans* (Puechmaille et al. 2010, Wibbelt et al. 2010) or its taxonomic delimitation (Gargas et al. 2009), but as with other fungal pathogens of mammals (e.g. *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Coccidioides* spp., *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii*) (Casadevall 2005) it is likely that environmental reservoirs play a key role in the dynamics of *G. destructans* infection and resulting WNS. A comprehensive understanding of the incidence, growth dynamics and persistence of *G. destructans* in association with hibernating bats and the environments that they inhabit will be critical to inform surveillance and management strategies for WNS.

FIG. 1. White-nose syndrome (WNS) occurrence and environmental sample collection locations. States where WNS was documented when the samples were collected (winter 2008–2009) are light gray; states where the disease had not yet been identified are dark gray. Sample collection locations are designated by numbers 1–24, and sites from which a clone with a sequence diagnostic for *Geomyces destructans* was identified are indicated with white stars.

The intent of this study was to conduct an expeditious PCR-based survey to determine the distribution of *G. destructans* in soil samples collected from bat hibernacula in the eastern United States (FIG. 1) with respect to the occurrence of WNS. This initial survey is based on PCR amplification with a previously designed primer pair (Lorch et al. 2010) with demonstrated selectivity for amplifying *G. destructans* DNA from bat wing skin by targeting conserved 1506 intron and rRNA gene internal transcribed spacer (ITS) sequence elements.

MATERIALS AND METHODS

Soil samples were collected by volunteers during winter 2008–2009 from bat hibernacula both within and outside the known range of WNS (FIG. 1). To avoid cross contaminating the samples collectors wore a new pair of vinyl laboratory gloves for each sample. Soil samples were collected with 11/16-inch-wide sterile wooden splints (Fisher Scientific, Pittsburgh, Pennsylvania), placed in sterile sampling bags with flat-wire closures (Fisher Scientific, Pittsburgh, Pennsylvania) and immediately shipped on wet ice to the USGS-National Wildlife Health Center (Madison, Wisconsin) where samples were stored at −80 C until DNA was extracted.

Nineteen soil samples from hibernacula in states within the known range of WNS at the time the samples were collected (Connecticut, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Virginia, Vermont, West Virginia) and five samples from hibernacula in states where WNS had not been detected as of December 2009 (Indiana, Kentucky, Minnesota, Mississippi, Wisconsin) were analyzed (TABLE I, FIG. 1). Due to the sensitive nature of bat hibernacula, names and coordinates for collection sites are not published here.

DNA was isolated from samples with the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, California) as per the manufacturer's instructions. PCR was conducted with primers (1506)-184-5′-Gd and nu-5.8S-144-3′-Gd (Lorch et al. 2010), hereafter referred to as *Gd*-enrichment primers, or the panfungal primers ITS4 and ITS5 (White et al. 1990), with ExTaq proofreading DNA polymerase (Takara Bio Inc., Madison, Wisconsin). For the Gd-enrichment primers and the ITS4/ITS5 primer pair PCR cycling conditions were as described in Lorch et al. (2010) and Blehert et al. (2009) respectively, with extension times increased to 3 min and the number of cycles reduced to 25 to avoid chimera sequence production (Jumpponen 2007). *Gd*-enrichment primers were used to generate clone libraries for all 24 soil samples. Panfungal primers were used to generate libraries from eight soil samples—three that yielded clones with *Gd*-enrichment primers that exactly

TABLE I. Summary of clones sequenced from *Gd*-enrichment PCR product clone libraries generated from 24 soil samples. Collection site numbers correspond to the designations used in FIG. 1. Collection sites from which an exact match for *Geomyces destructans* was found are indicated with an asterisk

Collection site	State	Clones sequenced[a]	Sequence variants identified
1*	NH	3	1
2	NH	0	0
3	VT	1	1
4	NY	9	2
5	NY	102	30
6	VT	0	0
7*	MA	25	4
8*	CT	11	3
9	CT	1	1
10	NY	1	1
11	MA	2	1
12	NJ	0	0
13	PA	65	5
14	PA	6	1
15	PA	6	3
16	WV	0	0
17	WV	1	1
18	VA	6	3
19	VA	0	0
20	KY	1	1
21	IN	1	1
22	MS	3	1
23	WI	38	11
24	MN	52	16

[a] The number of clones sequenced from each sample was based on the number of clones generated.

matched *G. destructans*, as well three additional samples from within and two from outside the WNS-affected region.

PCR products were stored at 4 C after amplification and were cloned within 8–24 h as described by Lindner and Banik (2009). Regardless of whether a PCR amplification product was visible with gel electrophoresis, attempts were made to generate clone libraries from all 24 soil samples. To amplify the cloned DNA regions from bacterial colonies 15 μL PCR reactions were prepared with GoTaq DNA polymerase (Promega, Madison, Wisconsin). Primers used to amplify cloned DNA were the same as those used in the initial PCR. Each primer was used at a final concentration of 0.2 μM, and each dNTP (Promega, Madison, Wisconsin) was used at a final concentration of 200 μM. Template DNA was added by placing a small amount of a transformed bacterial colony into the reaction with a sterile 200 μL pipette tip. Thermocycler conditions and cleanup of PCR products from bacterial colonies were as described by Lindner and Banik (2009). We ran negative controls consisting of blank samples to detect background DNA contamination throughout the extraction, PCR amplification and cloning process. DNA extracted from a culture of *Laetiporus cincinnatus* was used as the positive control. All negative and positive controls performed as expected. Using a variation of the ''Taq test'' (Simon and Weiss 2008), the overall error rate for our procedures was less than one per 7000 nt PCR product.

Direct, double-stranded sequencing reactions of PCR products followed the BigDye Terminator 3.1 protocol (Applied Biosystems, Foster City, California) with the same primers as the initial amplification. Sequencing products were cleaned with CleanSeq (Agencourt, Fullerton, California) magnetic beads following the manufacturer's protocol. Cleaned sequencing products were analyzed at the University of Wisconsin at Madison Biotechnology Center with an Applied Biosystems 3730xl automated DNA sequencing instrument. Sequences initially were aligned with Sequencher 4.2 (GeneCodes Corp., Ann Arbor, Michigan).

Seven *Geomyces* spp. sequences (GenBank accession Nos. AM901700, AY345347, AY345348, DQ402527, EF434077, EU884921 and FJ362279) identified from BLAST queries (Altschul et al. 1997) with default parameters were included in the analysis to orient sequences generated in this study with the clade of *G. destructans*. Eighty-one sequences, including 74 sequence variants from this study (TABLE II), were aligned manually with SeAl 2.0a11 and archived in TreeBASE (TB2:S10696). This alignment of 639 nt (including introduced gaps) was composed of 266 nt from the SSU 1506 intron (Gargas et al. 1995), 31 nt from the SSU rRNA gene, 181 nt from ITS1, 157 nt from the 5.8S rRNA gene and 4 nt from ITS2. Maximum likelihood searches were conducted with GARLI 0.96b8 (Zwickl 2006) with default parameters. Trees were viewed with FigTree 1.3.1, and graphics were exported for final illustrations. Clades were identified based on sequence variants that formed clusters and were numbered consecutively following their relationship to clade 1, the clade containing *G. destructans*; clades 2–12 include sequences with progressively greater genetic distance from clade 1. Sequence variants without subterminal branches are indicated with dots on the phylogram, and intermediate sequence variants 9 and 53 are tentatively grouped respectively with clades 3 and 7.

RESULTS

Clones (n = 334) were successfully produced and sequenced with *Gd*-enrichment primers from 19 of 24 soil samples; five samples yielded ≥ 25 clones (TABLE I, FIG. 1; see collection sites 5, 7, 13, 23 and 24). Cloned inserts were 623–632 nt. A total of 74 sequence variants (based on 100% sequence identity within each variant group) representative of 12 clades were observed (TABLE II, FIG. 2). Twenty-seven of the 74 sequence variants (36%) were the expected size for *G. destructans* (624 nt), indicating that size alone is not sufficient to determine the identity of a PCR product when analyzing environmental samples. Thirty individual clones (9%), including 21 from Massachusetts, six from Connecticut and three from New Hampshire, exactly matched the sequence diagnostic for *G. destructans*.

TABLE II. Sequence variants identified through this study, including their collection site(s) and state(s) as indicated in FIG. 1, their phylogram designation and assigned clade as indicated in FIG. 2 and their GenBank accession numbers

Phylogram designation	Clade	Collection site(s)	US state(s) of origin	GenBank accession number
1	1	8	CT	HM848979
2	1	7	MA	HM848976
3	1	8	CT	HM848977
4	1	1, 7, 8	NH, MA, CT	HM848972
5	1	7	MA	HM848975
6	1	7	MA	HM848978
7	2	24	MN	HM848985
8	3	20	KY	HM848992
9	3	17, 18	WV, VA	HM848965
10	4	5	NY	HM848963
11	4	5	NY	HM848958
12	4	5	NY	HM848966
13	4	5	NY	HM848948
14	4	5	NY	HM848935
15	4	5	NY	HM848947
16	4	5	NY	HM848929
17	4	5	NY	HM848941
18	4	5	NY	HM848960
19	4	5	NY	HM848950
20	4	5	NY	HM848927
21	4	5	NY	HM848931
22	4	5	NY	HM848937
23	4	5	NY	HM848959
24	4	5	NY	HM848932
25	4	5	NY	HM848946
26	4	5	NY	HM848934
27	4	5	NY	HM848945
28	4	5	NY	HM848940
29	4	3	VT	HM848996
30	4	5	NY	HM848961
31	4	5	NY	HM848949
32	4	5	NY	HM848930
33	4	5	NY	HM848939
34	4	5	NY	HM848943
35	4	5	NY	HM848964
36	4	5	NY	HM848928
37	4	5	NY	HM848933
38	4	5	NY	HM848942
39	4	5	NY	HM848962
40	4	5	NY	HM848944
41	5	15	PA	HM848994
42	6	24	MN	HM848997
43	6	24	MN	HM848984
44	6	24	MN	HM848987
45	6	24	MN	HM848968
46	6	24	MN	HM848983
47	6	24	MN	HM848982
48	6	5, 15, 24	NY, PA, MN	HM848971
49	6	24	MN	HM848991
50	6	24	MN	HM848988
51	6	24	MN	HM848981
52	7	24	MN	HM848990
53	7	24	MN	HM848980
54	7	24	MN	HM848989
55	7	21, 24	IN, MN	HM848970
56	7	24	MN	HM848986
57	8	14	PA	HM848993
58	9	13, 18	PA, VA	HM848969
59	9	4, 9, 11, 13, 22	NY, CT, MA, PA, MS	HM848973
60	9	13, 18	PA, VA	HM848974
61	9	4, 13	NY, PA	HM848967
62	9	13	PA	HM848936
63	10	23	WI	HM848938
64	10	23	WI	HM848924
65	10	23	WI	HM848925
66	10	23	WI	HM848926
67	10	23	WI	HM848951
68	10	23	WI	HM848952
69	10	23	WI	HM848954
70	10	23	WI	HM848955
71	10	23	WI	HM848956
72	10	23	WI	HM848957
73	11	23	WI	HM848953
74	12	15	PA	HM848995

Five sequence variants from soil samples collected from hibernacula in Connecticut (five clones) and Massachusetts (four clones) grouped within the *G. destructans* clade (FIG. 2, clade 1) but did not match *G. destructans* exactly, each exhibiting 1–3 single nucleotide polymorphisms (SNPs). The samples that yielded these sequence variants also yielded clones with sequence that exactly matched *G. destructans*. To determine the significance of these SNPs a small ITS clone library was generated from a pure culture of *G. destructans* type strain 20631-21 with a high fidelity polymerase. Sequence analyses of 16 clones from this library revealed five unique A/G transitions, suggesting that SNPs occur among the multicopy rRNA gene tandem arrays within individual isolates of *G. destructans*. In contrast direct sequence analysis (without cloning) of ITS region PCR products from more than 50 fungal isolates with microscopic and gross morphologies consistent with *G. destructans* showed no variations from the type strain sequence (GenBank accession number EU884921).

Sequences of 272 clones from eight soil samples generated with panfungal primers ITS4 and ITS5

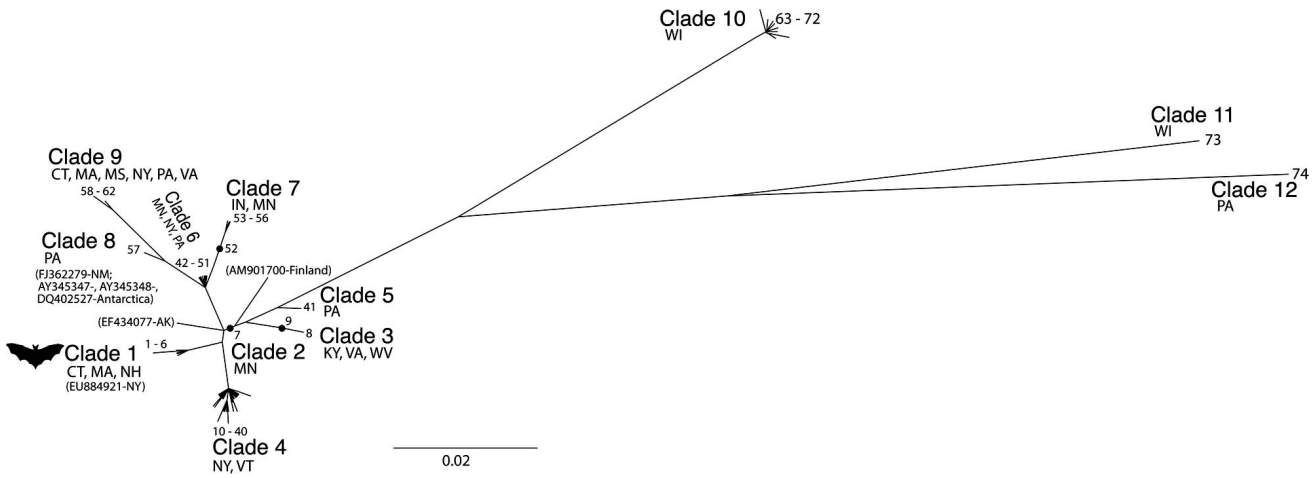

FIG. 2. Maximum likelihood phylogram based on GARLI (0.96b8) analysis of aligned *Gd*-enrichment primer PCR product (1506 intron and ITS) sequences from soil sample clone libraries and from sequences published in GenBank. Clades of *Geomyces destructans* (designated with a bat icon) and allied *Geomyces* spp. based on analysis of 74 unique sequence variants from 334 clones (duplicate variants not shown) are represented. Tip labels include phylogram designation numbers from TABLE II or GenBank accession numbers. Branch lengths are proportional to the number of substitutions per site (scale bar on figure). State, country or continent of origin for sequence variants is shown adjacent to each clade. Sequence variants not from this study (GenBank accession Nos. AM901700, AY345347, AY345348, DQ402527, EF434077, EU884921 and FJ362279) and their origin are shown within parentheses.

comprised 97 unique sequences which varied in length from 520–944 nt (data not shown). Clones with exact sequence matches to *G. destructans* were not identified in ITS4/ITS5-generated clone libraries, even in the three samples from which a sequence diagnostic for this species was detected previously with the *Gd*-enrichment primers.

DISCUSSION

This study yielded two key results: (i) Intron/ITS sequence variants of presumptive *Geomyces* spp. closely related to *G. destructans* are common in soil from underground environments where bats hibernate, and their presence confounds the ability to specifically detect *G. destructans* with the only existing PCR test (Lorch et al. 2010) as a presence/absence metric; and (ii) a taxon-specific sequence indicative of *G. destructans* was identified in soil samples collected from three of 19 bat hibernacula in three states where WNS occurs, establishing the environment as a potential reservoir for the fungus. This report provides the first analysis of the environmental occurrence of *G. destructans* within the context of related fungi and underscores a critical need for more specific diagnostic tests to better characterize the prevalence of this fungus and the role of the environment in WNS epidemiology.

The numerous sequence variants closely related but not identical to *G. destructans* identified through this investigation highlight a need for systematic research to classify these new variants within genus *Geomyces*. The most closely related clone identified in this study belonging to a clade different from *G. destructans* was generated from a soil sample collected in Minnesota (TABLE II, GenBank accession number HM848985, FIG. 2, clade 2). The intron/ITS sequence from this clone was greater than 99% identical to *G. destructans* (four SNPs within the Type I intron and a single insertion in the ITS1 region). The small genetic distance between the *G. destructans* sequences in clade 1 and the related sequence variant in clade 2 indicates that minor changes within the rRNA gene region might distinguish pathogenic variants from previously undescribed clades. A priority of future research will be to determine how these clades based on intron/ITS sequence correspond to species boundaries and specifically whether members of particular clades are able to exchange genetic information through sexual or parasexual processes. Exploration of additional loci likely will be necessary to differentiate *G. destructans* from closely related clades in environmental samples.

Although PCR amplification with *Gd*-enrichment primers detected a sequence diagnostic for *G. destructans* in soils collected from bat hibernacula in three WNS-positive states, the panfungal ITS primers, ITS4 and ITS5, did not. This suggests that DNA from *G. destructans* does not dominate the overall population of fungal DNA present in soil samples. Nonetheless this study provides evidence suggesting that *G. destructans* occurs in soil from underground environ-

ments where bats hibernate and indicates that if the fungus is viable it could be translocated by humans or other animals that enter infested sites. More research is needed to determine the role of soils as a reservoir in the transmission cycle of *G. destructans*.

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