

Bat White-Nose Syndrome: An Emerging Fungal Pathogen?

David S. Blehert,^{1*} Alan C. Hicks,² Melissa Behr,^{3†} Carol U. Meteyer,¹ Brenda M. Berlowski-Zier,¹ Elizabeth L. Buckles,⁴ Jeremy T. H. Coleman,⁵ Scott R. Darling,⁶ Andrea Gargas,⁷ Robyn Niver,⁵ Joseph C. Okoniewski,² Robert J. Rudd,³ Ward B. Stone²

¹USGS – National Wildlife Health Center, 6006 Schroeder Rd., Madison, WI 53711, USA.

²NY Department of Environmental Conservation, 625 Broadway, Albany, NY 12233, USA.

³NY Department of Health, P.O. Box 22002, Albany, NY 12201, USA.

⁴Cornell University, VRT T6008, Ithaca, NY 14853, USA.

⁵US Fish and Wildlife Service, 3817 Luker Rd., Cortland, NY 13045, USA.

⁶Vermont Fish and Wildlife Department, 271 North Main St., Rutland, VT 05701, USA.

⁷Symbiology LLC, Middleton, WI 53562, USA.

*To whom correspondence should be addressed. E-mail: dblehert@usgs.gov

†Present address: WI Veterinary Diagnostic Laboratory, 445 Easterday Ln., Madison, WI 53706, USA.

First evidence of bat white-nose syndrome (WNS) was documented in a photograph taken at Howes Cave, 52 km west of Albany, New York on February 16, 2006. Since emerging in the northeastern United States, WNS has been confirmed by gross and histologic examinations of bats at 33 sites in Connecticut, Massachusetts, New York, and Vermont (fig. S1). Current bat population surveys suggest a two-year population decline in excess of 75% (see supporting online material (SOM) text for further detail).

WNS has been characterized as a condition of hibernating bats and was named for the visually striking white fungal growth on muzzles, ears, and/or wing membranes of affected bats (Fig. 1A). Detailed post-mortem evaluations were completed for 97 little brown myotis (*Myotis lucifugus*; Mylu), 9 northern long-eared myotis (*Myotis septentrionalis*; Myse), 5 big brown bats (*Eptesicus fuscus*; Epfu), 3 tricolored bats (*Perimyotis subflavus*; Pesu), and 3 unidentified bats from 18 sites within the WNS-affected region. Distinct cutaneous fungal infection was observed in histologic sections from 105 of the 117 necropsied bats [91 Mylu (94%), 8 Myse (89%), 0 Epfu (0%), 3 Pesu (100%), and 3 unidentified (100%)]. Fungal hyphae replaced hair follicles and associated sebaceous and sweat glands, breaching the basement membrane and invading regional tissue. Hyphae also eroded the epidermis of ears and wings (Fig. 1B). Additionally, 69 of the 105 bats [62 Mylu (64%), 6 Myse (67%), 0 Epfu (0%), 1 Pesu (33%), and 0 unidentified (0%)] with cutaneous fungal infection had little or no identifiable fat

reserves, crucial for successful hibernation [see SOM text for additional mortality investigation details].

A fungus with a previously undescribed morphology was isolated from ten bats (table S1) with histologic evidence of WNS-associated cutaneous fungal infection. These bats were collected between February 1 and April 1, 2008 from all states within the confirmed WNS-affected region (fig. S1). The distinctive curved conidia (Fig. 1C) of the isolates were identical to conidia scraped directly from the muzzles of WNS-affected little brown myotis collected at Graphite Mine and to conidia observed histologically on the surface of infected bat skin (Fig. 1B, inset). Isolates were initially cultured at 3°C, grew optimally between 5°C and 10°C, but grew marginally above 15°C. The upper growth limit was approximately 20°C. Temperatures in WNS-affected hibernacula seasonally range between 2°C and 14°C, permitting year-round growth and reservoir maintenance of the psychrophilic fungus.

Phylogenetic analysis of the identical internal transcribed spacer region (fig. S2) and small subunit (fig. S3) rRNA gene sequences from the ten psychrophilic fungal isolates placed them within the inoperculate ascomycetes (Order Helotiales) near representatives of the anamorphic genus *Geomyces* (Teleomorph *Pseudogymnoascus*) (*I*). In contrast to the genetic data, morphology of the psychrophilic fungal isolates differed from that known for *Geomyces* species. The bat isolates produced single, curved conidia (Fig. 1C) morphologically distinct from clavate, arthroconidia

characteristic of *Geomyces* (2). Species of *Geomyces* are terrestrial saprophytes that grow at cold temperatures (3). Placement of the WNS fungal isolates within *Geomyces*, members of which colonize the skin of animals in cold climates (4), is consistent with properties predicted for a causative agent of WNS-associated cutaneous infection.

Worldwide, bats play critical ecological roles in insect control, plant pollination, and seed dissemination (5), and the decline of North American bat populations would likely have far-reaching ecological consequences. Parallels can be drawn between the threat posed by WNS and chytridiomycosis, a lethal fungal skin infection that has recently caused precipitous global amphibian population declines (6). A comprehensive understanding of the etiology, ecology, and epidemiology of WNS is essential to develop a strategy to manage this current devastating threat to bats of the northeastern United States.

References

1. A. V. Rice, R. S. Currah, *Mycologia* **98**, 307 (2006).
2. L. Sigler, J. W. Carmichael, *Mycotaxon* **4**, 349 (1976).
3. S. M. Duncan *et al.*, *Antarct. Sci.* 10.1017/S0954102008001314 (2008).
4. W. A. Marshall, *Microbiol. Ecol.* **36**, 212 (1998).
5. T. H. Kunz, M. B. Fenton, *Bat Ecology* (University of Chicago Press, Chicago, 2003).
6. L. F. Skerratt *et al.*, *EcoHealth* **4**, 125 (2007).

Supporting Online Material

www.sciencemag.org/cgi/content/full/1163874/DC1

Materials and Methods

SOM Text

Figs. S1 to S4

Table S1

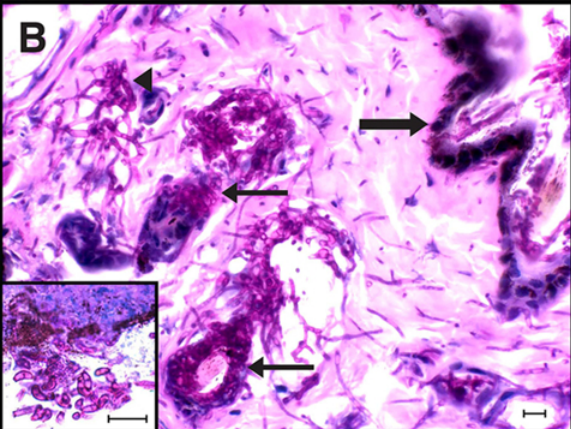
References

28 July 2008; accepted 30 September 2008

Published online 30 October 2008; 10.1126/science.1163874

Include this information when citing this paper.

Fig. 1. (A) A little brown bat, found in Howes Cave on January 6, 2008, exhibits white fungal growth on its muzzle, ears, and wings. (B) Fungal invasion of bat skin (PAS stain). Hyphae cover the epidermis (thick arrow), fill hair follicles, sebaceous glands, and sweat glands (thin arrows), breach the basement membrane and invade regional tissue (arrowhead). Inset: Curved conidia associated with the epidermis. (C) WNS-associated *Geomyces spp.* isolate stained with lactophenol cotton blue. Scale bars, 10 μ m.





Supporting Online Material for

Bat White-Nose Syndrome: An Emerging Fungal Pathogen?

David S. Blehert,* Alan C. Hicks, Melissa Behr, Carol U. Meteyer, Brenda M. Berlowski-Zier, Elizabeth L. Buckles, Jeremy T. H. Coleman, Scott R. Darling, Andrea Gargas, Robyn Niver, Joseph C. Okoniewski, Robert J. Rudd, Ward B. Stone

*To whom correspondence should be addressed. E-mail: dblehert@usgs.gov

Published 30 October 2008 on *Science Express*
DOI: 10.1126/science.1163874

This PDF file includes:

Materials and Methods
SOM Text
Figs. S1 to S4
Table S1
References and Notes

Supporting Online Material For: Bat White-Nose Syndrome: An Emerging Fungal Pathogen?

David S. Blehert,* Alan C. Hicks, Melissa Behr, Carol U. Meteyer, Brenda M. Berlowski-Zier, Elizabeth L. Buckles, Jeremy T. H. Coleman, Scott R. Darling, Andrea Gargas, Robyn Niver, Joseph C. Okoniewski, Robert J. Rudd, and Ward B. Stone

* To whom correspondence should be addressed. E-mail: dblehert@usgs.gov

Abstract: White-nose syndrome (WNS) is a condition associated with an unprecedented bat mortality event in the northeastern United States. Since the winter of 2006-2007 bat declines exceeding 75% have been observed at surveyed hibernacula. Affected bats often present with visually striking white fungal growth on their muzzles, ears, and/or wing membranes. Direct microscopy and culture analyses demonstrated that the skin of WNS-affected bats is colonized by a psychrophilic fungus that is phylogenetically related to *Geomyces spp.*, but with a conidial morphology distinct from characterized members of this genus. This report characterizes the cutaneous fungal infection associated with WNS.

Materials and Methods: Finite annual population growth rates (R) were estimated for the two caves that had at least three surveys since 2005, Hailes ($R = 0.47$) and Schoharie ($R = 0.17$). These corresponded with two-year population declines of 78% and 97%, respectively. We assumed the geometric population model $N_{t+i} = N_t R^i$, where N_t is the population at time t , and R is the finite annual growth rate. We estimated $\log(R)$ for each cave using the semilog regression model $\log(N_{t+i}) = \log(N_t) + \log(R)i$, and obtained the estimate of R as $R = \exp(\log(R))$. The estimated two-year decline was obtained as $100(1-R^2)$. Although we assumed a model of constant change, the semilog plots suggest an accelerating decline (Fig. S4).

DNA was extracted from each fungal isolate using microLYSIS-PLUS reagent (The Gel Company, San Francisco, California) following the manufacturer's instructions. rRNA gene internal transcribed spacer (ITS) region DNA (ITS1, 5.8S, and ITS2) was PCR amplified using primers ITS4 and ITS5 (S1) and ExTaq proof-reading DNA polymerase (Takara Mirus Bio, Madison, Wisconsin). Cycling parameters were an initial 2 min denaturation at 98°C followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. rRNA gene small subunit (SSU) DNA was PCR amplified using primers nu-SSU-0021-5' (S2) and nu-SSU-1750-3' (S3) as above, except the extension time was increased to 2 min. Sequencing primers were PCR primers with the addition of nu-SSU-0402-5' (S3), nu-SSU-1150-5' (S1), nu-SSU-0497-3' (S3), and nu-SSU-1184-3' (S4) for the SSU. PCR products were submitted to the University of Wisconsin – Madison Biotechnology Center DNA Sequencing Facility for direct, double-stranded sequence determination using the BigDye Terminator v3.1 (Applied Biosystems, Foster City, California) DNA sequencing system. Reaction products were analyzed using an Applied Biosystems 3730xl automated DNA sequencing instrument. Complementary strand sequencing reaction results were assembled and edited for accuracy using Lasergene 5.0 (DNASTar, Madison, Wisconsin). rRNA gene ITS (EU854569-EU854572, EU884920-EU884924, and FJ170115) and SSU (FJ231093-FJ231102) sequences are archived in GenBank. As the ITS and SSU sequences from each of the ten WNS fungal isolates were identical to each other, they were represented in phylogenetic analyses by single sequences (EU854571 for ITS and FJ231093 for SSU). Although excluded from the sequences used in analysis of the ITS region, additional genetic support comes from the presence of a putative group I intron of ca 415 nt, located at small

subunit position 1506 (S4) of each isolate, with 97% sequence similarity to insertions in *Geomyces spp.* AY345348 and AY345347. ITS and SSU sequences for comparison were selected from similar sequences archived in GenBank determined through BLAST search hits to query WNS isolate sequences, including only taxa with near complete gene sequences. Sequences were aligned visually using Se-AL (v2.0a11) (S5). The ITS alignment of 537 nt for 20 taxa and the SSU alignment of 1725 nt for 18 taxa are archived in TreeBase SN3954-18967. Parsimony phylograms were determined with PAUP* (4.0b10) (S6). Reliability of nodes was assessed with Bayesian posterior probabilities calculated using MCMC (MrBayes 3.1.2) (S7, S8) using the GTR model and running four chains with 1,000,000 generations, sampling each 1,000th tree and discarding as burn-in all pre-convergence trees; and bootstrap percentages based on 1,000 replicates in PAUP* (S4).

Supporting Text: Following the emergence of WNS during the winter of 2006-2007, the number of reports of day-flying bats recorded by the New York State Department of Health rabies laboratory for Schoharie County peaked in mid-March, 2007 at approximately 10 times the previous 25-year record high. This trend continued throughout the winter of 2007-2008 for Schoharie county and expanded to include Ulster County. All bats tested negative for rabies. Additional bacteriological and virological analyses of internal organs from WNS-suspect bats revealed no known pathogens. Disease-causing parasites were not found following examination of intestinal tracts. No consistent, significant lesions were observed upon gross or microscopic examination of internal organs from bats with the WNS-associated cutaneous fungal infection. Post-mortem evaluations were also completed for five little brown myotis from an unaffected mine in Wisconsin and eight little brown myotis from an unaffected cave in Kentucky, and no lesions were seen in their skin or internal organs.

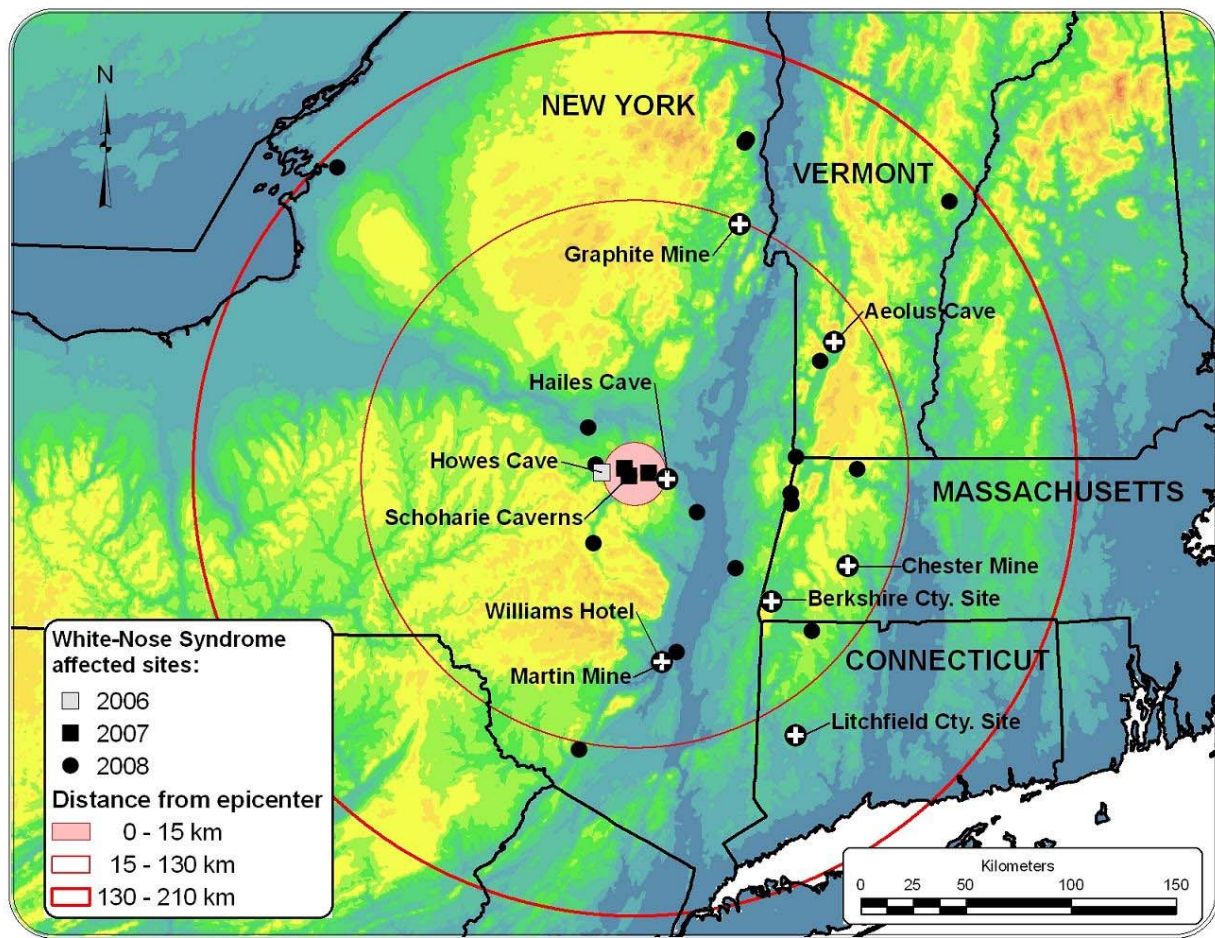


Fig. S1. Hibernacula locations, including the index site Howes Cave, confirmed by survey to be positive for WNS. Fungal isolates from which ITS and SSU sequence data were generated were cultured from bats collected at sites designated with plus signs.

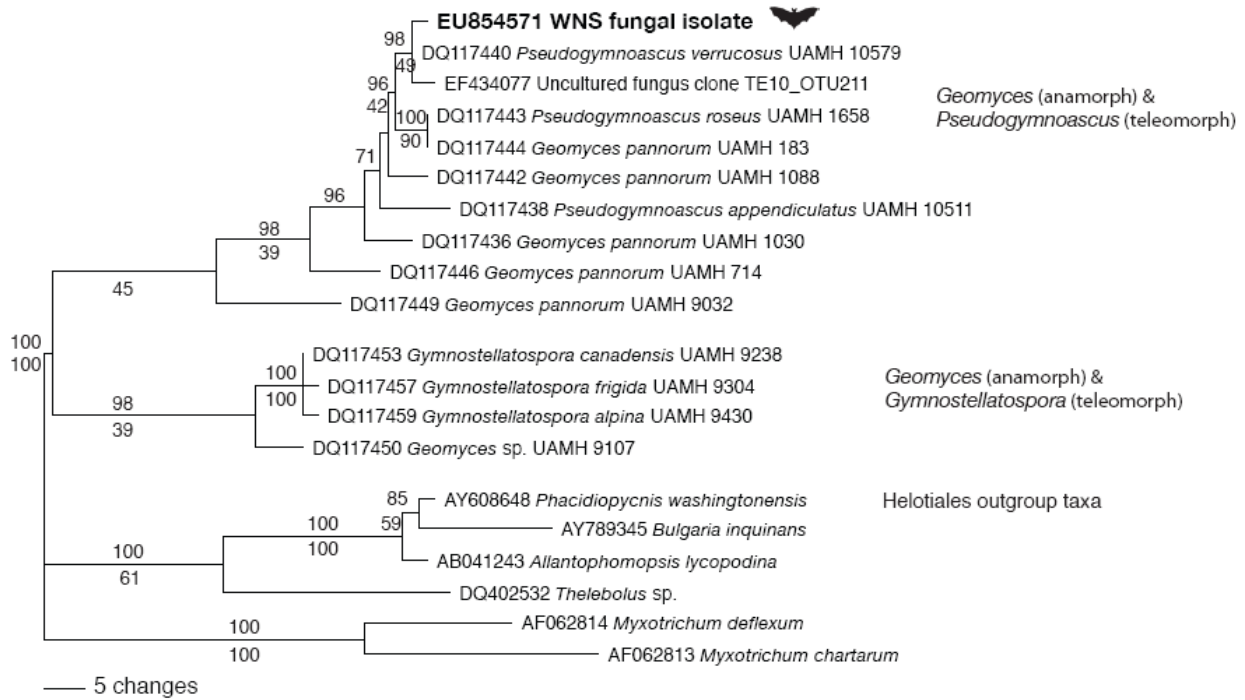


Fig. S2. One of 13 equally parsimonious trees for the ITS alignment (Length = 286, CI = 0.734, RI = 0.805). GenBank accession numbers precede taxa names, and the WNS fungal isolate sequence is indicated in bold with a bat image. Branch length is relative to the number of substitutions per site. Posterior probability values are shown above each supported node, and bootstrap percentages are shown below supported nodes.

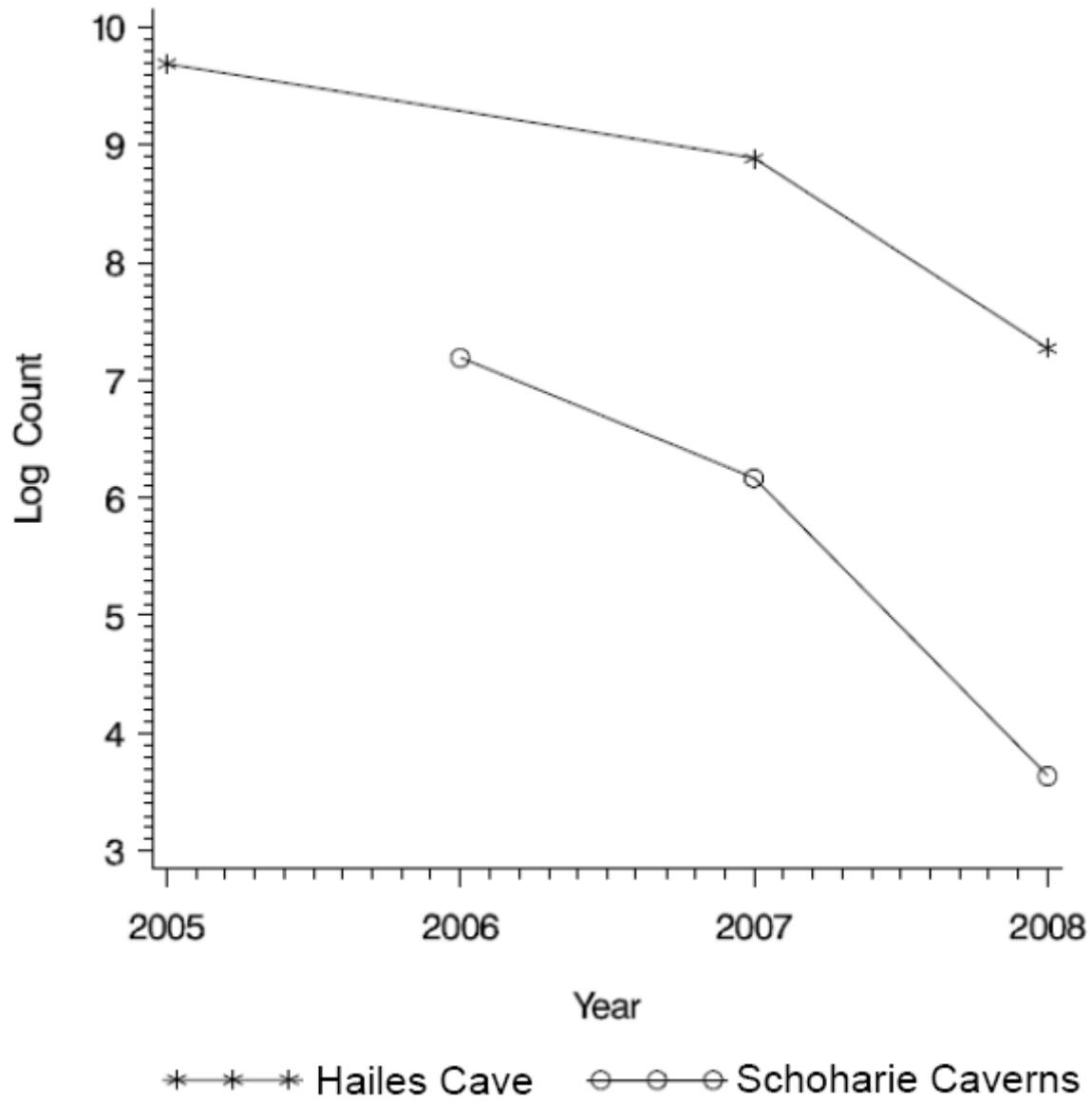


Fig. S4. Bat population trends for Hailes Cave and Schoharie Caverns.

Table S1. Summary of *Geomyces spp.* isolates.

Bat Species	Collection Date	Collection Location	State	GenBank Accessions ITS ^a	SSU ^b
<i>Myotis lucifugus</i>	1 April, 2008	Litchfield County Site	CT	EU884924	FJ231093
<i>Myotis septentrionalis</i>	21 March, 2008	Berkshire County Site	MA	EU854570	FJ231094
<i>M. septentrionalis</i>	21 March, 2008	Berkshire County Site	MA	EU854569	FJ231095
<i>M. lucifugus</i>	26 March, 2008	Chester Mine	MA	EU884923	FJ231096
<i>M. lucifugus</i>	29 January, 2008	Hailes Cave	NY	EU884920	FJ231097
<i>M. lucifugus</i>	2 February, 2008	Williams Hotel	NY	EU884921	FJ231098
<i>M. lucifugus</i>	5 March, 2008	Martin Mine	NY	EU854571	FJ231099
<i>M. lucifugus</i>	6 March, 2008	Graphite Mine	NY	EU854572	FJ231100
<i>M. septentrionalis</i>	18 March, 2008	Aeolus Cave	VT	FJ170115	FJ231101
<i>M. lucifugus</i>	18 March, 2008	Aeolus Cave	VT	EU884922	FJ231102

^a rRNA gene internal transcribed spacer

^b rRNA gene small subunit

Supplemental References:

- S1. T. J. White, T. D. Bruns, S. B. Lee, J. W. Taylor, in *PCR protocols: a guide to methods and applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, New York, 1990), pp. 315-321.
- S2. A. Gargas, P. T. DePriest, *Mycologia* **88**, 745 (1996).
- S3. A. Gargas, J. W. Taylor, *Mycologia* **84**, 589 (1992).
- S4. A. Gargas, P. T. DePriest, J. W. Taylor, *Mol. Biol. Evol.* **12**, 208 (1995).
- S5. A. Rambaut, "Se-AL: Sequence Alignment Editor Version 2.0a11," (2002); available at <http://tree.bio.ed.ac.uk/software/seal/>.
- S6. D. L. Swofford, *PAUP*: Phylogenetic analysis using parsimony (*and other methods) Version 4* (Sinauer Associates, Sunderland, Massachusetts, 2002).
- S7. J. P. Huelsenbeck, F. Ronquist, *Bioinformatics* **17**, 754 (2001).
- S8. F. Ronquist, J. P. Huelsenbeck, *Bioinformatics* **19**, 1572 (2003).
- S9. We thank T. Kunz (Boston University), M. Friend (USGS-National Wildlife Health Center), and D. Constantine for manuscript suggestions. At the USGS-National Wildlife Health Center, supporting pathology was provided by D. Green, N. Thomas, and V. Shearn-Bochsler; laboratory support was contributed by H. Ip (virology), D. Berndt (microbiology), and M. Sterner (parasitology). Population trend analysis was conducted by D. Heisey. At Cornell University, we acknowledge the anatomic pathology and wildlife medicine faculty and staff; supporting pathology was provided by K. Hulme. We also thank A. Lowell (USFWS), C. Herzog (NYSDEC), A. Davis (NYSDOH), B. Wood (NYSDOH), and the many individuals who provided survey data and specimen collection assistance for hibernacula throughout the northeastern United States. Additional field support was provided by D. Redell (WI Department of Natural Resources) and T. Hemberger (KY Department for Fish and Wildlife Resources). We acknowledge Bat Conservation International for helping to bring researchers together from across North America to share data and ideas on WNS. Work reported here was supported directly by the US Geological Survey, the US Fish and Wildlife Service, the New York State Department of Environmental Conservation, and the New York State Department of Health.